Boraginaceae Varronia rupicola (Urb.) Brtton : biogeography, systematic placement and conservation genetics of a threatened species endemic to the Caribbean

Boraginaceae
Varronia rupicola (Urb.) Britton

Biogeography, systematic placement and conservation genetics of a threatened species endemic to the Caribbean

Thesis submitted for the degree of

Doctor of Philosophy

by

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Declaration

I hereby confirm that this thesis is my own work and that any materials from other sources used in this work have been fully and appropriately acknowledged.

Martin Allen Hamilton

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Abstract

In the Caribbean region, *Varronia rupicola* (Boraginaceae) is a medium to large, woody shrub endemic to the Puerto Rican Bank where it is threatened with extinction due to its limited area of occupancy, small populations and on-going threats. The greatest of these is currently loss of suitable habitat through development and degradation. These are caused by human activities that are expected to continue and possibly worsen. The species is also threatened by sea level rise and drought as well as natural disasters, particularly hurricanes and tsunamis. Combined, the effects of anthropogenic and climate change induced threats could push the species to extinction over the coming century.

Through interrogation of the findings of cyto-, phylo- and population genetic as well as biogeographical research, it is clear that *V. rupicola* is a distinct species that is endemic to the islands of Puerto Rico, Vieques and Anegada where five populations were detected. The species has lost genetic diversity in the wild through a reduction in population size with allelic diversity proportional to the size of the population. The five populations were found to have lower than expected levels of heterozygosity as well as significant genetic differentiation and inbreeding. *Varronia rupicola* plants were found in an extremely limited area of intact habitat (<90 km^2) overlying substrates that cover <200 km^2 across the three islands. Protected areas contain less than a third (<30 km^2) of the remaining intact habitat that supports the species and established *ex-situ* collections capture less than half of the private alleles found in the wild. An integrated approach to the species conservation is needed to maximise genetic diversity and potentiality allow adaptation of *V. rupicola* to environmental change and new threats.

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"If we range through the whole territory of nature, and endeavour to extract from each department the rich stores of knowledge and pleasure they respectively contain, we shall not find a more refined or purer source of amusement, or a more interesting and unfailing subject for recreation, than that which the observation and examination of the structure, affinities, and habits of plants and vegetables, afford." Sir Joseph Paxton (1838).

This work is dedicated to my wife, Michele. Your love gives me purpose and your support gives me strength. Thank you for always being there to provide assistance and encouragement.

Chapter 1. Introduction

Varronia rupicola (Urb.) Britton is a woody shrub in the family Boraginaceae Jussieu endemic to Puerto Rico and the Virgin Islands (Acevedo-Rodríguez and Strong, 2012) in the Caribbean (Figure 1). The species is threatened with extinction and was assigned the category of 'Critically Endangered' on the IUCN Red List of Threatened Species (Clubbe *et al.*, 2003). Due to limited distribution and on-going threats to its habitat in Puerto Rico, *V. rupicola* has been listed as 'Threatened' by the US Fish and Wildlife Service (2014b). There are no known uses of the species by humans (Wenger *et al.*, 2010) and little is known about the species in the wild. The few investigations undertaken for *V. rupicola* prior to this research have only focused on part of the species distribution and most have relied solely on previously published findings, few herbarium vouchers and relatively limited survey data as is evidenced by the lack of detailed information available (Woodbury *et al.*, 1975; Proctor, 1991; Clubbe *et al.*, 2003; Pollard and Clubbe, 2003; U.S. Fish and Wildlife Service, 2010).

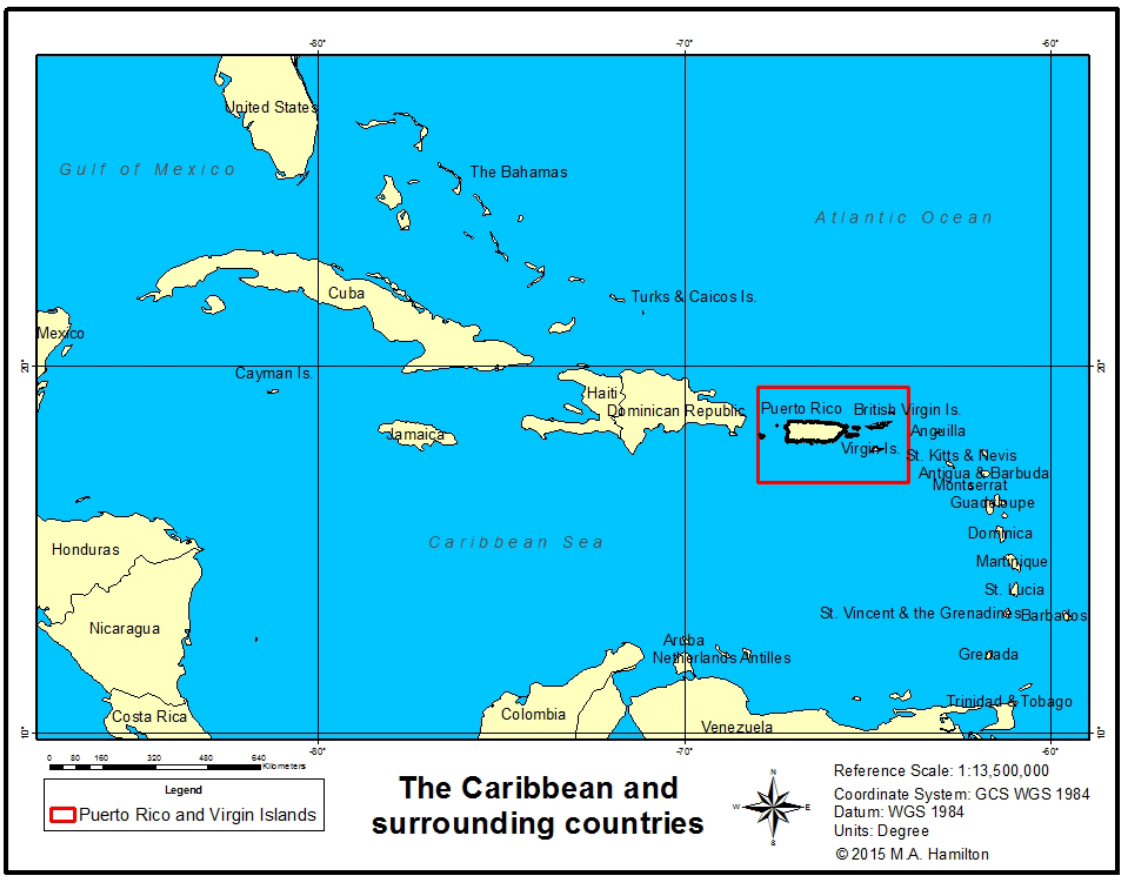

Figure 1: The Caribbean islands and surrounding countries showing location of Puerto Rico and the Virgin Islands.

1.1 The Puerto Rican Bank

The Puerto Rican Bank (PRB) (Figure 2) comprises the chain of islands that stretches from Puerto Rico in the west to Anegada in the east encompassing the countries of Puerto Rico (excluding Monito, Mona and Desecheo), the US Virgin Islands (excluding St Croix) and the British Virgin Islands (Acevedo-Rodríguez, 1996; McGowan *et al.*, 2006b; Gore, 2013).

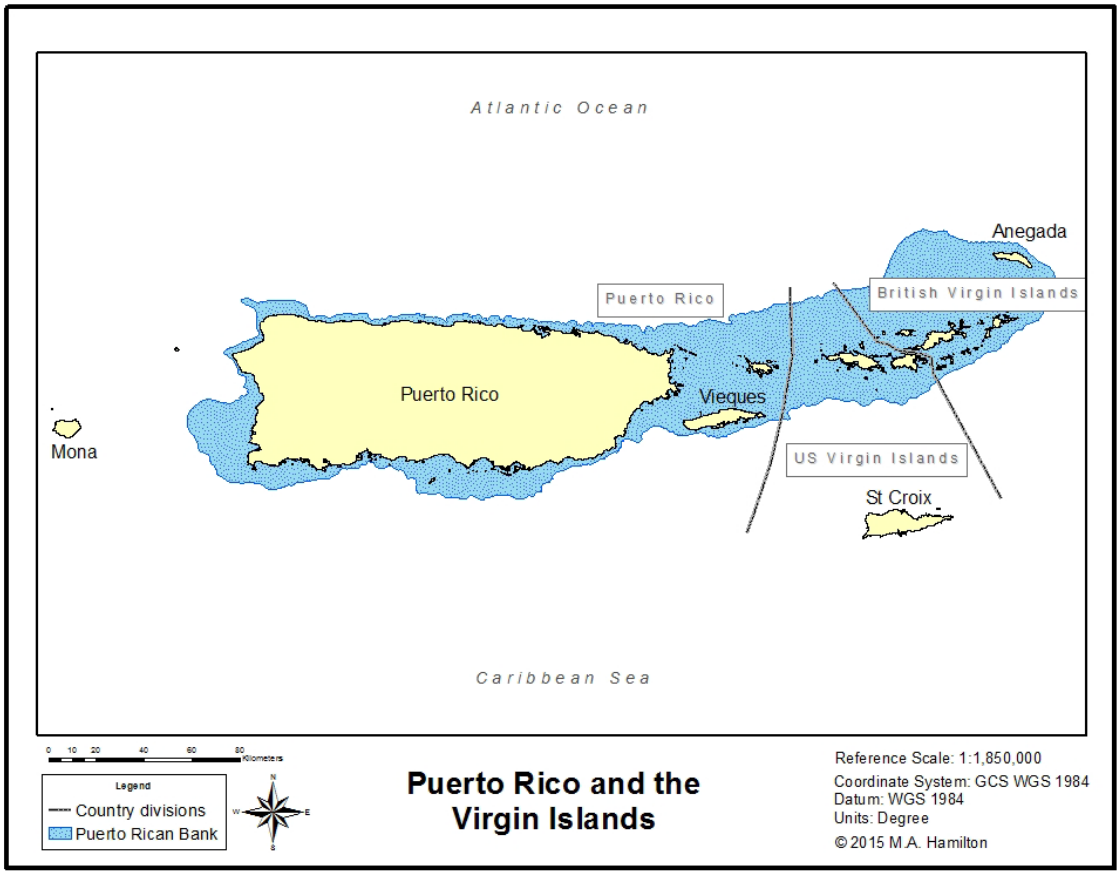

Figure 2: Map showing the countries of Puerto Rico, the US Virgin Islands and the British Virgin Islands with the current islands shaded yellow and the area of the Puerto Rican Bank during the Last Glacial Maximum when sea level was ca. -120 m lower than today shaded blue.

The PRB forms part of the Greater Antilles (Howard, 1970) along with the Cayman Islands, Jamaica, Cuba, Haiti and the Dominican Republic (see Figure 1); the last two countries located on the island of Hispaniola (Acevedo-Rodríguez and Strong, 2012). Except for the island of St Croix which has always been separated, the Puerto Rico and Virgin Islands archipelago is a tectonic microplate with an insular shelf that is 50 km broad (north to south) and 300 km wide (east to west) (Renken *et al.*, 2002) along the seismically active boundary between the Caribbean and North American plates (Mann, 2005). The islands making up the microplate were a single land mass (except Desecheo, Monito and Mona) at several times in the past with the most recent period during the Last Glacial Maximum (Lambeck *et al.*, 2002a) when sea levels reached a maximum of 120 m below the levels of today (Siddall *et al.*, 2003). Sea level rise is further discussed in 2.1.3. Sea level rise.

In 1493, Christopher Columbus discovered Puerto Rico and the Virgin Islands during his second voyage to the New World. Puerto Rico and its neighbouring islands were settled by the Spanish soon after their discovery by Europeans (Britton, 1918; Meyerhoff, 1926; Miller and Lugo, 2009). In 1508, Ponce de Leon was named governor of Puerto Rico, then called San Juan Bautista, and began exploiting the island of its natural resources. The islands remained under Spanish control until the Spanish-American War in 1898 which lasted only a few months and resulted in the transfer of the islands to the United States (Miller and Lugo, 2009). The Virgin Islands were not successfully colonised by Europeans until, in 1666, the British took control of the eastern islands which included Tortola, Virgin Gorda and Anegada. St. Thomas and St. John were settled in 1672 by the Danes. St. Croix changed hands many times between 1643 and 1733 when the French sold the island to Denmark. The United States, in 1917, purchased what would become known as the US Virgin Islands from Denmark (Britton, 1918; Meyerhoff, 1926). The complex political boundaries and the distribution of *V. rupicola* across international and state borders complicate the species conservation.

1.1.1. Physical environment

The land area of the Commonwealth of Puerto Rico, including Vieques, Mona, Culebra and several other smaller islands, is ca. (circa) 8,900 km^2, with ca. 8740 km^2 on the main island and ca. 125 km^2 on Vieques. Puerto Rico's highest point, Cerro de Punta, in the Cordillera Central on the main island, reaches 1,388 m asl (Daly *et al.*, 2003) and is the highest point in the Puerto Rican Bank. The highest point on Vieques is Monte Pirata at ca. 300 m (Renken *et al.*, 2002). The mountain ranges on Puerto Rico create precipitation gradients on the island from the northeast where predominantly moist habitats are found to the south-west where predominantly dry habitats prevail in the rain shadow of the Cordillera Central (Keel, 2005).

The Virgin Islands group with an area of ca. 505 km^2, including the US Virgin Islands with ca. 352 km^2 and the British Virgin Islands with ca. 153 km^2, is made up of ca. 100 rocks, keys and islands with a nearly even split between the two political entities (Meyerhoff, 1926; Acevedo-Rodríguez and Strong, 2008). The highest point in the group is found on Tortola where Sage Mountain reaches ca. 500 m (Kennaway *et al.*, 2008) and several other islands have ca. 300 m elevations at their highest points (D'Arcy, 1971). Meaning "drowned", Anegada was so named by the Spanish due to the low topography and many wetlands on the island. Anegada is 38 km^2 and the most northerly and easterly of the Virgin Islands group (Gore, 2013). With a maximum height of ca. 8 m asl (BVI Department of Town and Country Planning, 1993; Gore, 2013), it has the lowest elevation and least topography of any island in the archipelago (Howard, 1970). It is unique in the Virgin Islands as it is the only island completely composed of limestone (Howard, 1970).

Soils vary greatly across the archipelago and within individual islands with Puerto Rico having nine out of the eleven possible soil orders present (Miller and Lugo, 2009). This variation is due to its complex climate, topography and geology. The last two of these are further discussed in Chapter 2: Biogeography of *Varronia rupicola.* The first is covered below.

1.1.2. Climate

The Puerto Rican Bank has a predominantly maritime, tropical climate (Daly *et al.*, 2003). There is little variation in temperature throughout the year with most locations experiencing somewhere between 25 °C and 35 °C daily with high humidity. The dry forest habitats experience the highest temperatures and the mountains the coolest across the archipelago (Miller and Lugo, 2009). Daly *et al.* (2003) calculated mean annual temperatures for Puerto Rico, Culebra and Vieques to be 29.7 °C maximum and 19.4 °C minimum. The islands receive rainfall throughout the year with November to April being the drier period and January to February the driest months. Rain produced in this period is the result of cold fronts coming from eastern North America (Daly *et al.*, 2003; Miller and Lugo, 2009). The wettest months are on average September and October (Meyerhoff, 1926) during disturbances in the trade winds that occur from May to November (Daly *et al.*, 2003). During this period and mainly between the months of June and October, the islands are subjected to hurricanes (Miller and Lugo, 2009).

The rain shadow created by mountain ranges on Puerto Rico cause the south-west of the island to be considerably drier (Keel, 2005). These dry forest areas include the Guánica State Forest where *V. rupicola* is known to occur and that receives less than 1000 mm rainfall annually (Murphy and Lugo, 1986; Monsegur, 2009). At the eastern end of the archipelago, Anegada receives between 889 mm and 1016 mm rainfall annually (BVI Department of Town and Country Planning, 1993).

According to the International Panel on Climate Change (IPCC, 2012), a 2 °C to 5 °C rise in the daily maximum of global temperature by the end of 2100 will likely result in more intense droughts, higher frequency of heavy precipitation and higher wind speeds in tropical cyclones. Elsner and Jagger (2010) found that hurricanes are already increasing in intensity, especially in the Caribbean Sea and Gulf of Mexico which has the highest ocean heat capacity. The upward trend in intensity is related to an increase in sea-surface temperature. Hurricanes Gabrielle and Hugo in 1989 caused major changes to the coastline of Anegada. On its north shore, 15 horizontal meters of beach was lost at Loblolly Bay and 30 meters was gained at Cow Wreck Bay. Sites on the south shore at the Anegada Reef Hotel and Nutmeg Point lost over 15 m and 32 m of beach, respectively (UNESCO, 1989). Gore (2013) reported that in the period between

1861 and 2009 the beaches on the far western side of Anegada within the eroded 285 m while those in the South accrued up to 135 m, suggesting a counter clockwise beach movement adjusted to changes in prevailing conditions. Beaches along the north-western coast within the showed no more than 30 m of erosion or accretion between 1953 and 2002 due to the stabilising effect of exposed rock along the shoreline.

1.1.3. Population and development

The most heavily developed areas with the majority of human settlements in the Caribbean are located along the coasts (Burkett *et al.*, 2008) in dry forest habitats that receive between 500 and 1000 mm of rainfall annually (Robbins *et al.*, 2010). According to Lugo *et al.* (2001), the limestone regions of coastal Puerto Rico are threatened by human activities such as groundwater contamination, vegetation removal and development. These threats may be exacerbated by an increase in human population and unchanged development practices.

Puerto Rico is divided into 78 municipalities which are sub-divided into 875 wards that make up the political and legal divisions. Within the municipalities, zoning laws are defined and regulated, often poorly. The majority of the land in Puerto Rico is in private ownership and, therefore, individuals and developers often make decisions that shape the future of the land's use and forest cover within each ward (Yackulic *et al.*, 2011). Martinuzzi *et al.* (2007) found that between 1977 and 1994 in Puerto Rico, urban areas grew to nearly 125,000 ha, a 27% increase. This expansion was mainly in the lower elevations near existing infrastructure with 8,000 ha of side slopes adjacent to the plains being developed. This area of lower elevation and side slopes contains portions of suitable habitat for *V. rupicola* in Puerto Rico. According to Yackulic *et al.* (2011), reforestation in Puerto Rico following the transition from agrarian to service industries was mainly in western areas of Puerto Rico formerly used for coffee production. Human modification of the forests is further discussed in Chapter 2: Biogeography of *Varronia rupicola*.

Nicholls *et al.* (2011) suggested that a 4 °C rise in temperature is possible by 2100 and could result in sea level rise (SLR) between 0.5 and 2.0 m. This would mean displacement of between 1.2 and 2.2 million people over the next century in the Caribbean, Pacific Ocean and Indian Ocean. In this scenario, abandonment of areas affected by SLR may be the outcome due to the costs and challenges associated with implementing SLR protection (Nicholls *et al.*, 2011). This could have profound consequences for inland areas that would require new developments to house the displaced. Future sea level rise is further discussed in 2.1.3. Sea level rise.

1.1.4. Vegetation and flora

Collectively, the archipelago of islands from Anegada in the east to Mona in the west is known as the Puerto Rican Bank floristic province and forms part of the Antillean phytogeographical subregion of the Caribbean vegetation classification system (Lugo *et al.*, 2006). The Caribbean biodiversity hotspot is of global importance floristically containing 2.3% of the world's endemic plants (Myers *et al.*, 2000). The complex history of the region, both environmental and geological, and its physical location between North and South America have driven the complex vegetation assemblages and unique flora in limited land area (Fritsch and McDowell, 2003).

The Virgin Islands are covered by dry forest, except for the highest areas that receive more rainfall, and often have extremely dry eastern areas covered by xerophytic vegetation due to the salt-laden winds (Ewel and Whitmore, 1973). Kennaway *et al.* (2008) identified 26 vegetation formations and land cover types for the Virgin Islands group of which 16 were found on Anegada. The varied geology and topography of Puerto Rico give rise to 28 geo-climatic zones harbouring diverse taxa and habitats (Keel, 2005). Dinerstein *et al.* (1995) identified two broad ecoregions, subtropical moist and dry, for Puerto Rico which Helmer *et al.* (2002) divided into ten ecoregions that contain 26 vegetation formations and land cover types. Puerto Rico has six subtropical life zones according to the Holdridge system (Lugo *et al.*, 1999) with four occurring at higher elevation and two at lower elevation (Miller and Lugo, 2009). Further discussion about mapping and observed change of vegetation across the PRB is provided in 2.1.2. Land cover.

Serious studies of Puerto Rican flora started after the loss of 90% of the forests when only ca. 1% of the mature vegetation was still untouched (Figueroa-Colón, 1996). Several of the islands have had published accounts in the form of checklists or complete floras. One of the earliest was an account from Anegada made by Schomburgk (1832) in which he described the noticeable differences between Anegada and the other Virgin Islands particularly with regard to the flora, listing several peculiar species for which no specimens seem to exist (Britton, 1916). Of particular note is Schomburgk's description of the vegetation being cut for transport and sale in St Thomas, presumably for firewood and fence posts. Nathaniel Lord Britton (1916) of the New York Botanical Garden was the next worker to undertake botanical survey of Anegada with W. C. Fishlock, a former staff member of the Royal Botanic Gardens, Kew sent to Tortola to run the agriculture station. Their work resulted in several new records including the endemic species *Metastelma anegadense* Britton (Apocynaceae) and *Acacia anegadensis* Britton (Leguminosae). Both species are still extant on Anegada but their ranges have been extended such that the former has also been recorded on Virgin Gorda and Tortola (Goyder *et*

al., 2014) and the latter found on Fallen Jerusalem (Bárrios, 2015). Britton and many collaborators also undertook studies for the entire Puerto Rican Bank in a series of publications that set the stage for all modern works (Britton, 1918; Britton and Wilson, 1924, 1925a, 1925b; Britton, 1927). D'Arcy provided accounts of the flora of Tortola (D'Arcy, 1967) and Anegada (D'Arcy, 1971, 1975). In the former, he listed only 60 species for the Virgin Islands that are absent from Puerto Rico. Little *et al.* (1976) produced the only thorough account to date for Virgin Gorda. Acevedo-Rodríguez (1996) listed 642 native vascular plants on St. John, of which two are endemic. Axelrod (2011) provided the most recent and thorough treatment of the Puerto Rican flora, listing 2,337 native vascular plants species, of which 243 are endemic. The most comprehensive work to date for the Caribbean region was undertaken by Acevedo-Rodríguez and Strong (2012), who listed 183 families, 1,474 genera and 10,401 species of native seed plants. Of these 182 genera (12.4%) and 7,383 species (71%) are thought to be endemic (Acevedo-Rodríguez and Strong, 2012).

1.2. Caribbean dry forest

The dry forest habitat makes up ca. 47% of the approximately 11,424,544 ha of forested lands of the Caribbean (Robbins *et al.*, 2010). According to Ewel and Whitmore (1973) the subtropical dry forest zone found in the Puerto Rican Bank receives between 600 mm and 1100 mm rainfall annually with the soil moisture levels being highest from August to December. The structure of the vegetation is generally single layered and flattened with a maximum canopy height of 15 m (Lugo *et al.*, 2006). Many of the woody species are naturally coppiced and this is due, in part, to hurricane winds and their influence on canopy removal through uprooting larger and poorly anchored stems as well as inducing sprouts, even on undamaged stems (Van Bloem *et al.*, 2006). The resulting forests are often dense stands of small stems, up to 12,000 per ha, growing close together to the point of becoming entangled (Miller and Lugo, 2009). Many of the dry forest habitats in the Puerto Rican Bank occur over limestone and species found in these areas exhibit xerophytic adaptations such as small leaves that are coriaceous or succulent and deciduous during the driest periods (Ewel and Whitmore, 1973).

1.2.1. Dry forest composition and protection

Due to the previously mentioned socio-economic factors, the Caribbean dry forest is a threatened habitat (Janzen, 1988). This, in turn, means that many species in the dry forest are also threatened. An assessment by Figueroa-Colón and Woodbury (1996) found that roughly a third of all rare and threatened Puerto Rican and Virgin Island species are found in the dry forests and 42% are found on calcareous substrate which is usually associated with dry habitats at lower elevations. Among the list of rare and threatened taxa produced by Figueroa-

Colón and Woodbury (1996) were four species in the Boraginaceae, including *V. rupicola* which only grows in the dry forests on calcareous substrate.

Lugo *et al.* (2001) reported that the southern limestone region of Puerto Rico supports 14,764 ha of dry forest on dry limestone. Keel (2005) showed that ca. 3,545 ha, or 24%, of the total dry forest area in Puerto Rico falls within the protected area system. Within the southern limestone region, Figueroa-Colón (1996) listed 13 species-rich sites and found that the Guánica dry forest ranked third in Puerto Rico for tree species richness, with 657 species, behind Luquillo Mountains and Maricao which had 830 species and 845 species, respectively. The last two areas are both strictly moist forest habitats as are all the other sites listed by Figueroa-Colón (1996) except Guánica and Sierra Bermeja, which are strictly dry forest habitats, and Susua that has both dry and moist forest habitats. Guánica is the only dry forest site on a calcareous substrate as the other two dry forest sites are on ultramafic substrates. The important point to draw from these figures is that Guánica is a unique dry forest site due to the topography, geology and historic land use. Guánica is different because the area was mainly used for charcoal production; therefore, the substrate was not heavily disturbed and trees were allowed to regrow after coppicing. Figueroa-Colón (1996) pointed out that all of the sites played a major role in keeping species richness across the island by acting as refugia during the centuries of intensive forest exploitation and habitat conversion that occurred over most of the island.

1.2.2. Threats to the dry forest

Dry forests face a range threats from natural events through to human-induced impacts. Miller and Lugo (2009) suggested that 60% of the savannah and scrub forests of today were once dry forests that were impacted by humans to the extent that they altered irreversibly. Today dry forests are impacted by development for human settlements and infrastructure including roads, wind farms, gas and power lines and ports; tourism and recreation including off road driving and biking; illegal dumping; human-induced fire; agriculture and mining for sand and bedrock (Huggins *et al.*, 2007).

Many of the rare dry forest species are only found in isolated localities and in association with each other (U.S. Fish and Wildlife Service, 2010). Figueroa-Colón (1996) stated that just under half of the Puerto Rican endemic plants occur in less than three localities. This extremely restricted area of occupancy makes these species much more susceptible to extinction from localised, threatening events and stochastic events. The impact of these events can be magnified on the smaller Virgin Islands leading to species extirpation or even extinction.

1.2.2.1. Natural events

Climate change and sea level rise

According to the IPCC (2012), the global climate is likely to change over the next century leading to more severe droughts and higher wind speeds in tropical cyclones (see 1.1.2. Climate). Dry forests are already known to suffer a water deficit for ten months of the year (Miller and Lugo, 2009). The species found in dry forests are adapted to these conditions; however, more intense droughts brought on by climate change in a relatively short period may prove to be too much for some species to cope with in already altered landscapes. Angeles *et al.* (2006) modelled future climate scenarios and found that three of the four areas on Puerto Rico that would experience greater surface temperatures occur in south-western Puerto Rico. The IPCC (2013b) reported that the global sea level is expected to rise between 0.28 and 0.98 m by 2100; however, many feel that sea level may rise as much as 3 m by 2100 and 6 m by 2300 (Bellard *et al.*, 2014; Gregory, 2014; Horton *et al.*, 2014). See also 2.1.3. Sea level rise.

Hurricanes

The worst recorded hurricanes to strike Anegada were Hurricane Donna in 1960 and Hurricane Hugo in 1989, the latter of which caused flooding in The Settlement (BVI Department of Town and Country Planning, 1993) and extensive damage across the Virgin Islands and Puerto Rico (Committee on Natural Disasters, 1994). Human infrastructure can impede storm surge drainage and thus saltwater can remain on the land in depressions causing damage and mortality to the surrounding vegetation as it evaporates or penetrates the fresh water lens. Natural seepage into the freshwater lens following storm surges is possible and studies in the Florida Keys have shown increased mortality of larger stem diameter trees due to increased salinity and water stress following hurricane induced storm surge events (Sah *et al.*, 2010). Increased salinity of the fresh water lens, particularly on small islands, may also be caused by sea level rise.

Van Bloem *et al.* (2006) studied the impacts on Guánica State Forest of the Category 3 Hurricane Georges that hit Puerto Rico in 1998. The storm dumped 151 mm of rain and had sustained winds of ca. 180 km/hr and gusts of ca. 240 km/hr. Above-ground stem damage on woody plants was low with only 13% showing significant damage that was biased to larger diameter stems. Mortality was also low with less than 2% showing no regeneration after nine months. The forests of the Caribbean are adapted to withstand the effects of hurricanes; however, increasing hurricane intensity coupled with habitat degradation and fragmentation may have dire consequences for nature and humans alike.

Earthquakes and tsunamis

The Puerto Rican Trench is an area of high seismic activity and has been the source of several earthquakes that can also spawn tsunamis. There are ca. ten documented tsunamis to cause significant impact to the northern Caribbean since 1498, with many of these affecting the Greater Antilles, especially Puerto Rico and the Virgin Islands (Parsons and Geist, 2009). Recent studies by Atwater *et al.* (2012) have suggested that Anegada was struck in 1755 by a transatlantic tsunami that formed after the Lisbon earthquake. W. C. Fishlock (1912) and BVI Department of Town and Country Planning (1993) reported an earthquake-induced tsunami in 1867. This tsunami impacted most of the Caribbean with maximum water heights of 1.5 m in Road Town in Tortola in the BVI and 6.1 m on Vieques and eastern Puerto Rico (Parsons and Geist, 2009). The most recent reports of tremors in the BVI were in 1992 (BVI Department of Town and Country Planning, 1993). Probability modelling by Parsons and Geist (2009) found a higher hazard for a >0.5 m tsunami run-up for the Virgin Islands and Puerto Rico due to the proximity of the islands to the Caribbean and North American plates subduction zone.

1.2.2.2. Human-induced threats

Agriculture

Most of the Virgin Islands and the majority of Puerto Rico were cleared for plantations of crops such as sugarcane (*Saccharum officinarum* L.), coffee (*Coffea arabica* L.) and bananas (*Musa* x *paradisiaca* L.) following European colonisation and the introduction of slave labour (D'Arcy, 1967; Miller and Lugo, 2009). This had devastating impacts on the species and habitats native to the islands, both directly through changes in land cover and indirectly through secondary impacts like erosion. Since there were few written accounts of the original vegetation, only speculation exists for the true extent of loss during the plantation era (Britton, 1918; Miller and Lugo, 2009). Acevedo-Rodríguez (1996) reported that 60% of St John was used for sugarcane production and the majority of the rest of the island was used for cultivation of secondary crops between 1765 and 1830. In the mid-19th century the sugar industry collapsed in the Caribbean and some plantations like those on St. John were allowed to revert to forest. Crop production today in the dry forest is not common due to the water resources naturally available; however, some farming of crops takes place, especially where the soils are better developed.

In areas with poorer soils, forestry and livestock grazing are practised. Forestry plantations in Guánica State Forest of non-native (e.g. *Swietenia mahagoni* (L.) Jacq. (Meliaceae)) and native (e.g. *Guaiacum officinale* L. (Zygophyllaceae)) species were common in 20th century (Ewel and Whitmore, 1973; Miller and Lugo, 2009). Livestock, often allowed to roam freely or even go

feral, can have detrimental effects on the dry forest through suppression of the native vegetation, spread of non-native species and breakage of the soil surface which allows erosion. Acevedo-Rodríguez (1996) reported the most immediate threat to the regeneration of native forests on St. John to be grazing by feral cows, donkeys and goats and uprooting of plants by pigs. D'Arcy (1971, 1975) commented on the impact of grazing animals on Anegada, particularly around The Settlement. Recent vegetation surveys found that Anegada is dominated by unpalatable and spiny species of plants (Clubbe *et al.*, 2004). D'Arcy (1971, 1975) described the cutting of the vegetation for human use (e.g. charcoal and fence posts) which was also mentioned by Schomburgk (1832). The Puerto Rican forests were also exploited for centuries and these extractive practices still occur, although to a lesser extent (Ewel and Whitmore, 1973; Ventosa-Febles *et al.*, 2005). These practices have no doubt left altered and depauperate vegetation in many areas.

Invasive species and diseases

The international exchange of goods, both living and processed, has opened many new routes for invasive plants, insect pests and pathogens to enter the PRB. Of particular concern is the horticultural trade in living plants produced abroad and imported with soil on the roots (Serra *et al.*, 2003; Clubbe *et al.*, 2010). Once established, these invaders are difficult to control and nearly impossible to eradicate.

Kairo *et al.* (2003) found 179 plant species in the Caribbean that are invasive or encroaching on native habitats. In the Greater Antilles, invasive species from a range of life forms from trees, e.g. *Azadirachta indica* A.Juss. (Meliaceae), to herbaceous perennials, e.g. *Catharanthus roseus* (L.) G.Don (Apocynaceae), and vines, e.g. *Antigonon leptopus* Hook. & Arn. (Polygonaceae), have been recorded in the dry forests (Serra *et al.*, 2003). There are several exotic and invasive plant species known to occur in the dry forest habitat on Puerto Rico such as the herbaceous grass species *Cenchrus ciliaris* L. (Poaceae) and the woody species of Leguminosae: *Haematoxylum campechianum* L., *Leucaena leucocephala* (Lam.) de Wit (Monsegur, 2009), *Parkinsonia aculeata* L. and *Prosopis juliflora* (Sw.) DC. (Ewel and Whitmore, 1973). Monsegur (2009) listed 761 taxa for Guánica State Forest and found 63 to be non-native. Vieques has many heavily disturbed landscapes and invasive plants such as *Leucaena leucocephala* and the tree *Melicoccus bijugatus* Jacq. (Sapindaceae) (Monsegur, 2009). D'Arcy (1971) provided a short list of exotic species for Anegada which did not include *Casuarina equisetifolia* L., *Scaevola taccada* (Gaertn.) Roxb. or *Cryptostegia madagascariensis* Bojer ex Decne. that are all now serious invasive problems on the island (McGowan *et al.*, 2006a).

The dry forests are also suffering attack from insect pests with many examples of exotic pests causing extensive damage to island ecosystems and individual species within those ecosystems (Liebhold, 1995; Serra *et al.*, 2003; Malumphy *et al.*, 2012). There are already several serious pests established in Puerto Rico attacking threatened species. For example, *Leptocereus quadricostatus* (Bello) Britton & Rose, a Critically Endangered species in the Cactaceae, is under attack in the Guánica State Forest and surrounding areas by the Harrisia cactus mealybug, *Hypogeococcus pungens*, (Segarra Carmona and Ramírez-Lluch, 2007). The pest effectively stops reproduction of plants by attacking the apical meristem which haults the formation of flowers. Many cactus plants are affected by the pest and mortality rates are high (Segarra-Carmona *et al.*, 2010).

Fire

Robbins *et al* (2010) found that the majority of forest fires in the Caribbean occur in the dry forest habitat which is the location of most human settlements. Fire is not a natural process in the Puerto Rican Bank (Miller and Lugo, 2009) and is increasing in frequency (Robbins *et al.*, 2010); therefore, many of the species lack the necessary adaptions to survive wildfires. Local wildfires are mainly caused by humans through escaped fires near settlements, land use disputes, protest, and land clearance for hunting or agriculture (Robbins, 2006). Non-natural, human-induced fires are known to occur and cause damage to habitats in Guánica State Forest, particularly along the roads (Ventosa-Febles *et al.*, 2005; Wolfe, 2009). The author has also seen damage to large areas of dry forest in the proximity of known habitat for *V. rupicola* adjacent to Highway 2 in Canas ward of Ponce municipality and within Guánica State Forest. Wildfires can also assist the establishment and spread of exotic and invasive plant species through clearance of the native vegetation and increasing soil fertility (Wolfe, 2009; Robbins *et al.*, 2010). Continued development in the dry forest habitat expands the interface of urban areas with natural areas and increases the risk for wildfires. Changes in the Caribbean climate may also exacerbate the effects of wildfires through warming and drying (Robbins *et al.*, 2010).

1.3. Legislation and protected areas

As the Puerto Rican Bank includes three political divisions, the establishment of protected areas, legal frameworks for species and habitat protection and enforcement of laws and protected area regulations vary considerably. The following sections are provided to give an overview of the current situation in the Puerto Rican Bank with a specific focus on *V. rupicola*; therefore, only the countries of Puerto Rico and BVI will be addressed here.

1.3.1. Legislation

The United States Congress (1973) passed the Endangered Species Act (ESA) to protect plants and animals threatened with extinction and their habitat. The U.S. Fish and Wildlife Service (USFWS) is tasked with administration of freshwater and terrestrial organisms listed under the ESA (U.S. Fish and Wildlife Service, 2013b). A species listed for protection is 'Endangered' when there is the risk of extinction across all or a significant portion of its known range. If in the foreseeable future a species is likely to become 'Endangered', it is listed as 'Threatened' (U.S. Fish and Wildlife Service, 2013b). Prior to formal listing, a species may be held on a candidate list for review as was the case for *V. rupicola* when this research began (U.S. Fish and Wildlife Service, 2010). During the course of this research, the species was listed under the ESA as a 'Threatened' species (U.S. Fish and Wildlife Service, 2014b) and critical habitat was designated for the species in Puerto Rico (U.S. Fish and Wildlife Service, 2014a). These designations have raised the profile of *V. rupicola*, both regionally and globally, and require that a species recovery plan is developed for the species in U.S. Territory; however, plants listed under the ESA are not explicitly protected from collection outside of Federal lands unless state laws exist that restrict collection of listed species.

In Puerto Rico, *V. rupicola* is currently listed as a 'Critical Element' (Department of Natural and Environmental Resources, 2007). Although this designation does not provide legal protection outside state lands, any proposed actions that might impact the species may result in the local government requiring measures that would avoid or limit such impacts (U.S. Fish and Wildlife Service, 2010). The Commonwealth of Puerto Rico enacted Law 241, the New Wildlife Law of Puerto Rico, that declares all wildlife under Puerto Rican jurisdiction the property of the Commonwealth and enables regulation through collection and hunting permits (Department of Natural and Environmental Resources, 1999). The Puerto Rico Forest Law, Law 133, prohibits the collection or damage of flora within public forests, including Guánica State Forest. This research was undertaken through the acquisition of a collecting permit (#2012-EPE-012) valid throughout Puerto Rico which was issued by the Department of Natural and Environmental Resources (DRNA) with reference to relevant laws and regulations.

Existing legislation in the BVI [*i.e.* Physical Planning Act, 2004, and National Parks Act, 2006 (Government of the Virgin Islands, 2004, 2005)] provides some level of protection for over 13% of the country's terrestrial environment (Gardner *et al.*, 2008). Unfortunately, *V. rupicola* occurs outside this area and no formal legal protection is currently afforded to the species or its habitat even though laws exist that could be used to protect the species, *i.e.* Protection of endangered animals, plants and articles (removal and possession), CAP. 95 (Government of the Virgin Islands, 1981). According to Deputy Premiere, the Honourable Dr Pickering (2015), a

new ‘Natural Resources and Climate Change Bill’ is being drafted by the BVI Government that will address environmental management, conservation of biodiversity and protection of natural resources. It is unclear if the proposed legislation will include provisions for threatened species to be formally listed under an article or schedule by name, similar to the legislation enacted in Puerto Rico.

In both countries, the availability of adequate legal protection is overshadowed by the lack of enforcement of existing laws and wanting implementation of regulations. This is generally a result of limited resources, both funding and capacity, that leads to the continued threat of extinction for *V. rupicola* and many other species. This can be either direct impacts, e.g. plants killed due to road works, or indirect impacts like habitat loss and degradation. These issues will be discussed further in Chapter 2: Biogeography of *Varronia rupicola*.

1.3.2. Protected areas in Puerto Rico

Federal and privately managed lands combined result in 5.2% of the land area under protection in the Commonwealth of Puerto Rico (Helmer *et al.*, 2002). There are two existing protected areas, Guánica State Forest and Vieques National Wildlife Refuge (NWR), within the known historical range of *V. rupicola*. The species is also now present in the Cabo Rojo NWR as discussed below. Protected areas that include limestone deposits of similar age to that known to support extant *V. rupicola* are further discussed in 2.4. Discussion and conclusions.

Cabo Rojo NWR

First established in 1974 with 238 ha of degraded land, the Cabo Rojo NWR (Figure 3) has expanded into a 750 ha protected area managed by the USFWS focused on providing bird habitat and undertaking restoration/reforestation (U.S. Fish and Wildlife Service, 2013a). The refuge in the extreme south-west of Puerto Rico is outside the known historical range of *V. rupicola*. As part of the general restoration efforts on the refuge and as a specific conservation action for *V. rupicola*, a small planting (n = 30) of the species was undertaken in November 2012 using nursery grown plants originating from wild source seed collected in Yauco and Guánica (Morales and Martinez, 2013).

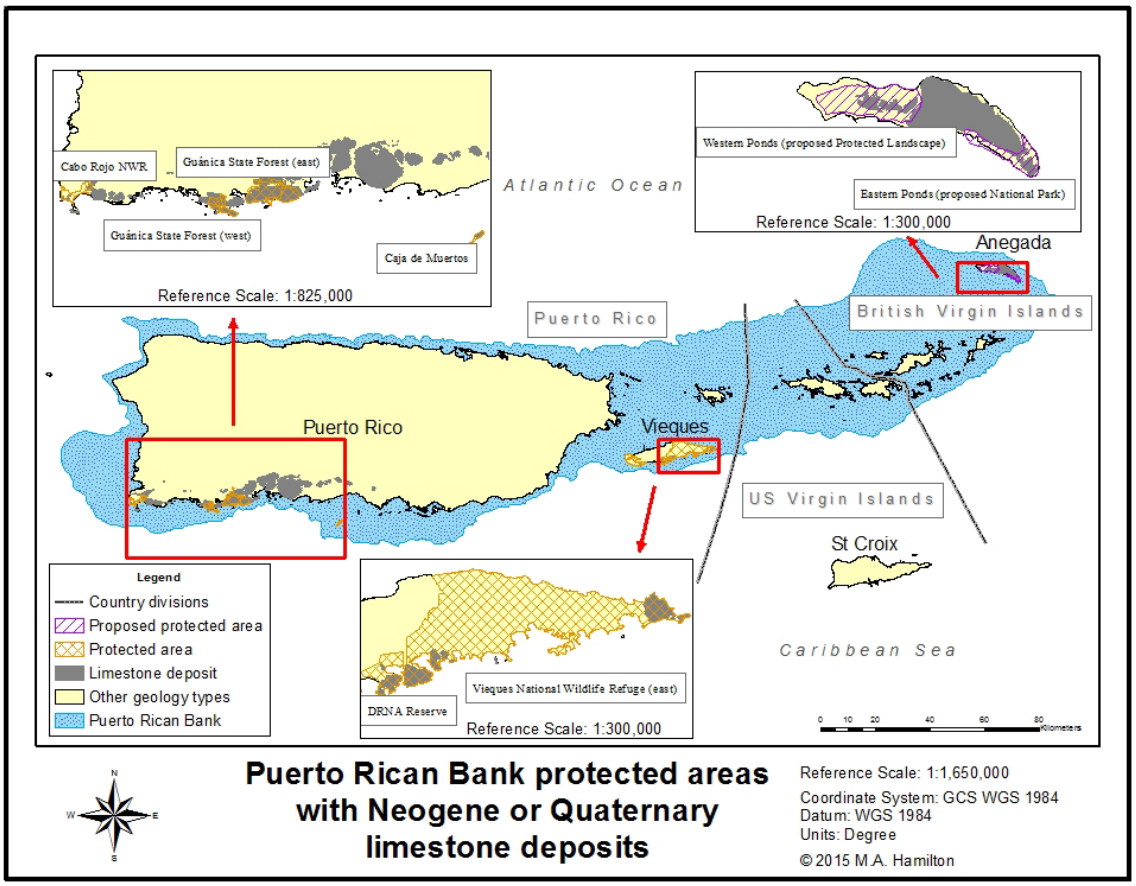

Figure 3: Protected areas (established and proposed) across the Puerto Rican Bank that include deposits of limestone of similar age to that known to support extant *Varronia rupicola*.

Guánica State Forest

Designated in 1917 and formally managed from 1930 by the Commonwealth of Puerto Rico, the ca. 4,400 ha of primarily semi-deciduous tropical dry forest known as Guánica State Forest (Murphy *et al.*, 1995; Van Bloem *et al.*, 2006; Monsegur, 2009) is located in south-western Puerto Rico (see Figure 3). The forest is divided into two main tracts of land that are separated by Guánica Bay and several urban areas. The eastern tract of the forest has the highest point which reaches 228 m asl (Ventosa-Febles *et al.*, 2005) and is part of three municipalities, Guánica, Yauco and Guayanilla.

One of the premier examples of dry forest habitat in the Neotropics, the forest was recognised as an International Biosphere Reserve by the United Nations in 1981 (Miller and Lugo, 2009). Monsegur (2009) found three endemic species to the forest, seven federally listed species (not including *V. rupicola*), 19 Puerto Rican endemics, 16 Puerto Rican Bank endemics (including *V. rupicola*) and a further 47 species designated as critical elements by the Commonwealth of Puerto Rico among 761 taxa of which 460 are confirmed to exist in the forest boundaries.

The eastern tract of the forest is almost certainly the collection location for one of the syntypes (Sintenis, P.E.E. 3731) coming from the Guánica municipality. The other syntype

(Sintenis, P.E.E. 4879) is from Los Indios in nearby Guayanilla municipality. The USFWS (2010) reported that the preference of the species for growing along trails and power line clearings has meant difficulties in ensuring the species is not impacted by maintenance activities inside the forest.

Vieques NWR

The Vieques NWR is divided into two main tracts on the island municipality of Vieques, east of mainland Puerto Rico (see Figure 3). To establish the refuge, the U.S. Congress transferred ca. 7191 ha between 2001 and 2003 from the U.S. Navy to the USFWS (2007). The two tracts which together cover over half of the island are separated by urban areas. The eastern tract where *V. rupicola* has been recorded is the largest portion of the refuge with ca. 5937 ha. The majority of the habitats in the refuge are altered due to historic land use in the form of agriculture and military activities. The latter has left many areas with contaminants and unexploded ordinance (UXO) that are currently being removed (U.S. Fish and Wildlife Service, 2007). The on-going clean-up process has resulted in the destruction of remnant native vegetation and habitat degradation in some areas.

1.3.3. Protected areas in the British Virgin Islands

Unfortunately, *V. rupicola* does not naturally occur within the existing protected area network of the BVI. Part of the recorded habitat for the species occurs within the Western Salt Ponds of Anegada Ramsar site (The Ramsar Convention On Wetlands, 2013) but this designation does not constitute a formally protected area or provide legal protection. According to Fenty (2015), the Government of the Virgin Islands has undertaken public consultations for a series of new protected areas (see Figure 3), two of which would encompass terrestrial environments on Anegada (Gardner *et al.*, 2008) and will be discussed further in Chapter 2: Biogeography of *Varronia rupicola*. Outside its known historic range, the species is in cultivation on the island of Tortola in the J.R. O'Neal Botanic Garden, one of the protected areas managed by the National Parks Trust of the Virgin Islands (NPTVI) (Gardner *et al.*, 2008).

1.4. Boraginaceae systematics, biogeography and morphology

1.4.1. Family divisions

The α-taxonomy of Boraginaceae has been debated for many years without a satisfactory resolution in the scientific community (Al-Shehbaz, 1991; Estrada Sánchez, 1995; Gottschling *et al.*, 2005; Cole, 2015). Around 2700 species of Boraginaceae *s.l.* are found throughout the temperate, subtropics and tropics of the world (Gottschling, 2003). Major centres of distribution are found in South and Central America and in the Mediterranean region (Heubl *et al.*, 1990). Many life forms are found in Boraginaceae from herbaceous perennials through to

woody trees and shrubs. The components of Boraginaceae are unclear as phylogenetic relationships are still being resolved. The Angiosperm Phylogeny Group (APG, 2009) placed Boraginaceae in the lamiid clade of the asterids; however, the family is not placed to a specific order. Although Stevens (2012) recognised Boraginales Juss. *ex* Bercht. & J.Presl. with eight subordinate groups (Boraginaceae *s.s.*; Codonaceae; Cordiaceae; Ehretiaceae; Heliotropiaceae; Hydrophyllaceae; Nama, etc.; and Wellstediaceae), the high level taxonomy proposed by the Angiosperm Phylogeny Group (APG, 2009) is followed here.

Many authors have proposed often rather diverse taxonomic treatments for the family based on a range of morphological features. Gürke (1893) undertook a comprehensive investigation of Boraginaceae and included four subfamilies, Boraginoideae, Cordioideae Beilschmied, Ehretioideae Arnott and Heliotropioideae Arnott. The works of Ivan M Johnston on Boraginaceae began in 1923. His final work went to press following his death in 1960 (Correll, 1961). Most modern researchers use a combination of the works of Johnston as the basis for their taxonomic treatment of the family. Several reviews of the taxonomic literature are available and provide an insight into the often confusing taxonomy that has been presented for Boraginaceae (Sahay, 1979; Al-Shehbaz, 1991; Estrada Sánchez, 1995; Gottschling, 2003). Many modern authors have suggested using a much divided taxonomy for a broadly defined Boraginaceae (Luebert and Wen, 2008; Miller and Porter Morgan, 2011). Gottschling *et al.* (2005) suggested a classification of six subordinate families within the order Boraginales. This classification would elevate the subfamilies, Cordiaceae Dumortier, Ehretiaceae Martius and Heliotropiaceae of the formerly defined Boraginaceae *s.l.*, along with the families Hydrophyllaceae Schrader and Lennoaceae Solms-Laubach. Due to the on-going α-taxonomy debate in Boraginaceae and unresolved relationships using molecular techniques and morphological characters, the taxonomy for Boraginaceae composed of six subfamilies, Boraginoideae, Cordioideae, Ehretioideae, Heliotropioideae, Hydrophylloideae Burnett and Lennooideae Craven (APG, 2009), is followed here. Cordioideae, Ehretioideae and Heliotropioideae constitute the primarily woody Boraginales (Gottschling, 2003). Conflicting dates are given for the diversification of the primarily woody taxa with Gottschling *et al* (2004) stating it occurred in the mid-Cretaceous, 90 mya (million years ago), and Moore and Jansen (2006) suggesting the end of the Cretaceous, 67-63 mya. Heubl *et al* (1990) stated that it is likely that the evolutionary radiation of woody Boraginaceae took rise from a diploid ancester with a basic chromosome number of $x = 8$ which is still present in the group, especially in *Ehretia* P.Browne and *Cordia* L.

1.4.2. Subfamily Cordioideae

An almost exclusively tropical subfamily (Al-Shehbaz, 1991), Cordioideae is dominated by woody trees, shrubs or lianas with storied cambium in the secondary meristem, a terminal style which is divided, drupaceous fruit with one to four seeds that lack endosperm and plicate cotyledons. Cordiaceae, Hoplestigmataceae Gilg and Sebestenaceae Ventenat are synonyms of Cordioideae (Stevens, 2012; USDA ARS National Genetic Resources Program, 2015). Generic limitations in this text follow that of Miller & Gottschling (2007). Species concepts and distributions follow that of Acevedo-Rodríguez & Strong (2012). Some authors include *Varronia* P.Browne in the genus *Cordia*; therefore, references to *Cordia rupicola* and closely related taxa recognised as *Cordia s.l.* section *Varronia* (P.Browne) G.Don by other authors should be recognised as part of the genus *Varronia* and considered as interchangeable unless otherwise noted. The subfamily, as recognised here, includes three genera, *Cordia* including *Patagonula* L., *Saccellium* Humb. & Bonpl. and *Auxemma* Miers. (Gottschling and Miller, 2006); *Hoplestigma* Pierre (APG, 2009) and *Varronia*.

1.4.3. The genus *Varronia*

Confusion over the distinction between *Varronia* and *Cordia* started at the beginning of the Linnaean system (Taroda and Gibbs, 1986) and is due to the morphological diversity of *Cordia s.l.* Browne (1756) first used the name *Varronia* when describing two shrubs found in Jamaica. The generic name is in honour of the ancient Roman scholar and writer Marcus Terentius Varro who lived from 116 to 27 BC (Britton and Wilson, 1925a). Linnaeus (1753) had already published *Cordia* and included three species in the genus, with *Lantana bullata* L. which would be considered congeneric by other authors. The second edition of Species Plantarum, further confused the issue as Linnaeus (1762) published ten species that would be included in *Cordia s.l.*, five in *Varronia* and five other species in *Cordia s.s.* Desvaux (1808) presented a monograph for *Varronia* and published ten new species. A detailed treatment of *Cordia s.l.* was provided by De Candolle (1845); however, he applied the name *Varronia* considerably differently than subsequent authors with the exception of Gürke (1893), according to Miller & Gottschling (2007).

Most authors since De Candolle (1845) have treated *Cordia* in a broad sense and included *Varronia.* A few notable exceptions must be recognised who segregated *Varronia* based on pollen (Nowicke and Ridgway, 1973; Nowicke and Miller, 1990) and wood anatomy (Mez, 1890). Friesen (1933) split *Varronia* and published three new genera, *Montjolya*, *Varroniopsis* and *Ulmarronia* (≡ *Varronia*), based on morphological characters. Johnston (1949) re-established *Varronia* as a section of *Cordia*. After Johnston, the next authors to recognise *Varronia* and effect taxonomic change were Borhidi *et al.* (1988); however, their new

combinations, with no consideration of synonymy, assigned to all names in the section received criticism in subsequent publications (Miller and Gottschling, 2007). A concise overview of the taxonomic treatments for *Cordia s.l.* was provided by Taroda & Gibbs (1986).

Based on genetic studies using ITS1 and the previous work of several authors (Johnston, 1949; Taroda and Gibbs, 1986; Borhidi *et al.*, 1988; Nowicke and Miller, 1990), Miller & Gottschling (2007) recognised *Varronia* as a distinct genus from the rest of *Cordia* and highlighted the distinct distribution and ecological and morphological differences. The generic limitation of Miller & Gottschling (2007) is followed in this research with *Varronia* including *Catonia* Raf., *Montjolya, Piloisia* Raf. and *Varroniopsis*. Chapter 3: Phylogenetic placement of *Varronia rupicola* explores the relationships of several Caribbean species of *Cordia* and *Varronia*.

There are ca. 100 species of *Varronia* (Gottschling *et al.*, 2005; Miller and Wood, 2008) occurring from Arizona, part of the Northern Mexico and South-western North America Ecoregion of the Nearctic Ecozone, throughout the Neotropic Ecozone to Argentina in the Southern South America Ecoregion (Udvardy, 1975; Dinerstein *et al.*, 1995; Miller and Gottschling, 2007). This distribution covers a range of vegetation types from dry savannah through to tropical rain forest. Heubl *et al.* (1990) suggested that the radiation from wet conditions of the rain forest to the xerophytic conditions of the savannah may have advanced the dominance of the shrubby life form seen in the genus. They went on to postulate that modification of flower and inflorescence structure to attract new pollinators made the transition into new, often hostile, habitats possible.

According to Acevedo-Rodríguez and Strong (2012) there are 67 *Varronia* species native to the Caribbean. Nine species are native to the Puerto Rican Bank, three of which, *V. rupicola, V. bellonis* (Urb.) Britton and *V. wagnerorum* (R.A. Howard) Borhidi, are endemic with the last two only being found on Puerto Rico.

Karyological data for *Cordia s.l.* is sparse due to the tendency of the chromosomes to stick together (Britton, 1951; Heubl *et al.*, 1990). Britton (1951) stated that extant *Cordia s.l.*, including the $x = 7$ series, would have a woody ancestor with $x = 8$. This view was shared by Opler *et al.* (1975) who went on to say that the ancestor was heterostylous and probably adapted to pollination by Lepidoptera. Several authors have reported evidence of aneuploidy in their studies of the genus in the broad sense that would support a series of base numbers (Bhattacharya, 1968; Opler *et al.*, 1975). Most notably, Heubl *et al.* (1990) found three main evolutionary lines from the cytological data available for *Cordia s.l.*, $x = 7$, 8 and 9, and a derived complex, $x = 15$. Polyploidy has been recorded several times for *Cordia s.l.* (Britton, 1951; Bhattacharya, 1968; Opler *et al.*, 1975; Heubl *et al.*, 1990) and these authors stated that

with aneuploidy several polyploidisation events occurred for species exhibiting heterostyly. Heubl *et al.* (1990) found all *Varronia* studied to be diploid or tetraploid derived from *x* = 9 with 2*n* = 18 or 2*n* = 36 being the somatic chromosome number. Heubl *et al.* (1990) suggest that colonisation of disturbed ecosystems was not always coupled with an increase in ploidy level, but was dependent on morphological adaptations to the envirnoment. Such changes are seen through the development of homostyly, dioecy and flower morphology that allows visitation by small insects (Opler *et al.*, 1975; Bawa, 1980). Although diploid taxa are present in the group, Heubl *et al.* (1990) considered *Varronia* a relatively young and advanced group.

Studies have shown distylous type heterostyly to be present in *Cordia s.l.* and across Boraginaceae. Self-incompatibility is generally associated with distyly in the family; however, exceptions like self-compatibility in *Amsinckia* Lehm. are known (Al-Shehbaz, 1991). Distyly is associated with two flower types (Figure 4) commonly called pin, long-styled/short-anthered morphs, and thrum, short-styled/long-anthered morphs. Pin are often homozygous recessive, and thrum are often heterozygous, with the latter showing little or no seed set (Bawa, 1980; Bawa and Beach, 1981, 1983; Stutzman *et al.*, 2012); however, complex multi-allelic systems are known. Generally, pin and thrum flowers are the same overall size, but some species of *Cordia s.l.* are known to have slightly larger thrum flowers (Al-Shehbaz, 1991). Studies by Stutzman *et al.*(2012) of four *Varronia* species from the Galapagos Islands suggest that they are self-incompatible and thus obligate outcrossers.

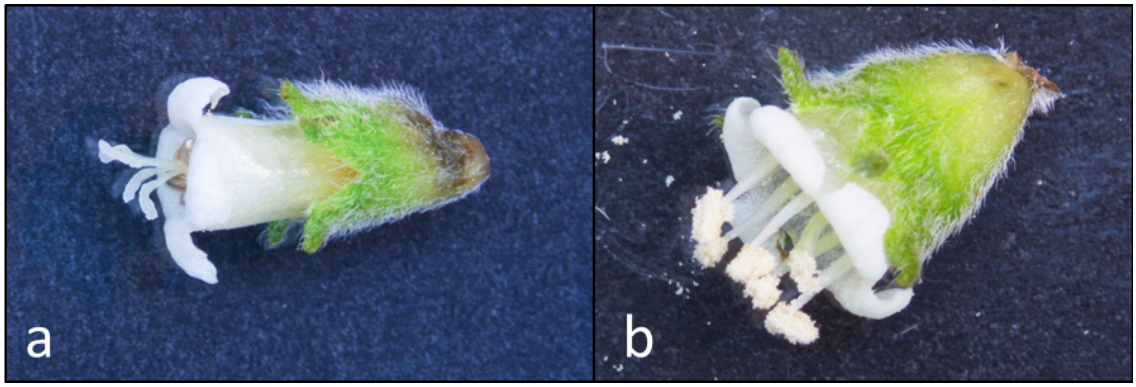

Figure 4: *Varronia rupicola* flowers demonstrating distylous type heterostyly of a) long-styled/short-anthered flower morph "pin" and b) short-styled/long-anthered flower morph "thrum". ©M.A. Hamilton.

1.4.3.1. General description

Varronia species are usually multi-stemmed, woody shrubs with crenate to serrate leaf margins, condensed inflorescences/infructescences (Gaviria, 1987; de Stapf, 2010), craspedodromous venation (Miller and Gottschling, 2007) and a fleshy, red mesocarp with ornithochorous dispersal (Heubl *et al.*, 1990). The exceptions to the dominant life-form are a few lianescent species and several suffrutescent species from Brazil with facultatively annual above-ground structures. Two Brazilian taxa, *V. longifolia* (A.DC.) Borhidi and *V. poliophylla*

(Fres.) Borhidi, exhibit brochidodromous venation which is unlike any other *Varronia* but universal in *Cordia s.s.* (Miller and Gottschling, 2007). *Cordia s.s.* are all trees with paniculate or cymose inflorescences and entire leaves (Miller and Wood, 2008).

1.4.3.2. Vegetative morphology

Varronia species are typically characterised by their shrubby habit, pubescent or scabrous leaves with alternate arrangement and white flowers in condensed inflorescences (Britton and Wilson, 1925a). A well-developed, primary root is persistent and many species develop efficient horizontal root systems to collect sparse moisture in arid environments (Gottschling, 2003). Most species are evergreen, but some species in dry or temperate regions are deciduous. Leaf blades, at least the upper half, are dentate, crenate or most commonly serrate (Miller and Gottschling, 2007) (see Figure 5 (a)). The flat, bifacial, undivided leaves usually have a single-layer of palisade tissue and anomocytic stomata (Gottschling, 2003).

Figure 5: General characters of *Varronia* species: (a) *V. rupicola* leaf showing crenate margin and craspedodromous venation; (b) *V. bahamensis* showing condensed infructescences and a fleshy, red mesocarp. ©M.A. Hamilton.

The genus *Varronia* usually has abundant indumentum, only rarely absent, which can consist of branched multicellular trichomes often with the base being slightly bulbous, unicellular hairs sometimes with cystoliths that cause the leaf surface to feel rough and uniseriate hairs (Mez, 1890; Estrada Sánchez, 1995; Gottschling, 2003). Gaviria (1987) found variation in the composition and density of the indumentum within and among species based on ecological conditions.

Throughout Cordioideae, calcium oxalate crystals are present in the wood as prismatic crystals or crystal sand which is informative systematically for the subfamily as certain crystal types are restricted to different sections of *Cordia s.l*. Crystal sand has been found in all *Varronia* species observed and is common across *Cordia s.l.* The function of crystal arrangments observed is unclear (Heubl *et al.*, 1990).

1.4.3.3. Inflorescence and flower structure

Varronia inflorescences, in most species, are composed of flowers in condensed spicate or capitate inflorescences (Miller and Wood, 2008). Many of the species with the later type of inflorescence have calyx lobes with prolonged filiform tips (visible in Figure 5 (b)) which are unique to *Varronia* within *Cordia s.l.* (Miller and Gottschling, 2007). *Varronia bifurcata* (Ruiz & Pav.) Borhidi and relatives are the only species that differ and have small cymose inflorescences a few centimeters wide and differ from those found in *Cordia s.s.* that are much broader (Miller and Gottschling, 2007). Syndesmia in *Varronia* (Hagemann, 1975; Uhlarz and Weberling, 1977; Estrada Sánchez, 1995) separate it from the rest of *Cordia s.s.* and result in the apex of the inflorescence doubling back (Gottschling *et al.*, 2005). This creates the situation where open flowers and closed buds are interspersed. Gaviria (1987) proposed spicate and cephaloid syndesmia could be distinguished in early ontogeny. The former can be derived from the latter (Uhlarz and Weberling, 1977) and can be seen as apomorphic for *Varronia* which is supported by molecular data (Gottschling *et al.*, 2005).

Flowers are distylous, rarely unisexual and subdioecious, with a urceolate to campanulate, five-lobed, rarely four-lobed, calyx (Miller and Gottschling, 2007). Synsepalous calyx has equal lobes and is persistent, sometimes enclosing, or nearly so, the mature fruit (see Figure 5 (b)). The corolla is white, sympetalous and tubular with reflexed or spreading limbs (see Figure 4). The superior gynoecium is sessile and syncarpous with four ovules and a terminal style with four stigmatic lobes. Usually the androecium is antesepalous, epipetalous and haplostemonous with linear filaments and trichomes in a patch at the attachment point. Dorsifixed anthers can be included or exserted and are tetrasporangiate opening via longitudinal slits (Gottschling, 2003). Stamens are usually pubescent at the point of insertion and number five (Miller and Gottschling, 2007), rarely four. Gottschling (2003) reports immature anthers to have stalked, glandular trichomes with a single head cell. A nectary disc is developed at the base of the ovary (Gottschling, 2003; Milet-Pinheiro and Schlindwein, 2010).

1.4.3.4. Embryology

Little is known about *Varronia* embryology. Gottschling (2003) reports that the internal architecture of the bicarpellate ovary is the result of the development of basal, apical and false septa giving rise to four locules. Four stigmatic heads result from the twice bifid, filiform, terminal style (Figure 6 (b)) which is persistent (Figure 6 (c)) in some species. The anthers have fibrous thickenings in the endothecium and one to five middle layers. The microspore is tetrahedral and microsporogenesis is thought to be simultaneous. The two theca release their pollen simultaneously (Figure 6 (a)).

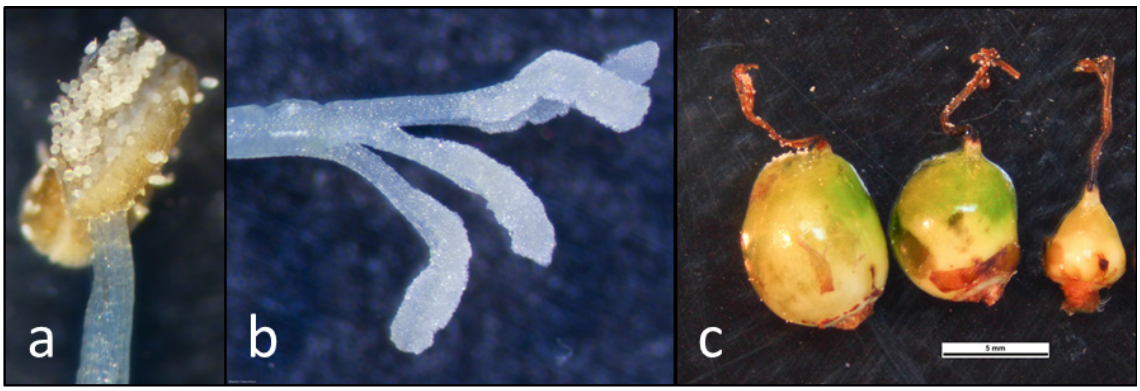

Figure 6: Embryology characters of *Varronia* species: (a) *V. rupicola* stamen showing anther with two theca releasing pollen; (b) *V. rupicola* terminal style that is twice bifid with four stigmatic heads; (c) persistent styles on *V. rupicola* developing fruits. ©M.A. Hamilton.

1.4.3.5. Palynology

Nowicke & Miller (1990) reported seven pollen types for *Cordia s.l.* Reticulate (network-like pattern) and 3-porate (circular) aperture pollen found for *Varronia* (Figure 7) is distinct from *Cordia s.s.* (Nowicke and Ridgway, 1973; Moncada and Herrera-Oliver, 1988; Miller and Nowicke, 1989; Heubl *et al.*, 1990; Nowicke and Miller, 1990) which all have colporate (compound) apertures (Miller and Gottschling, 2007).

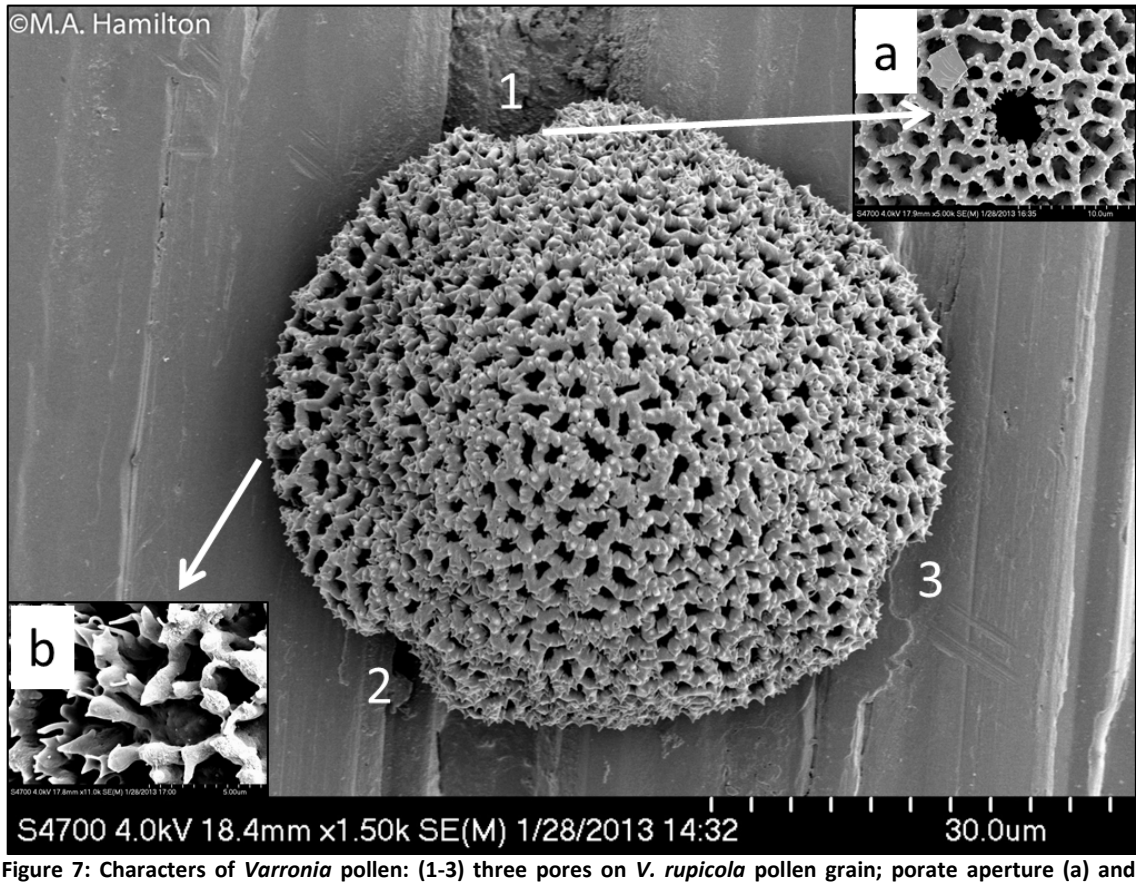

Figure 7: Characters of *Varronia* pollen: (1-3) three pores on *V. rupicola* pollen grain; porate aperture (a) and reticulum with spinulose muri (b) of *V. lima* pollen grain. ©M.A. Hamilton.

Cordia lutea Lam. (*Cordia s.s.*) reportedly has porate pollen but it is considerably larger than *Varronia* pollen (Gottschling, 2003) and the species has a basic number of *x* = 7 (Miller, 1985) not *x* = 9 as in *Varronia* (Heubl *et al.*, 1990). Pollen size dimorphism has been found for distylous species of *Cordia s.s.* (Al-Shehbaz, 1991; McMullen, 2012) and *Varronia* (Milet-Pinheiro and Schlindwein, 2010). According to Miller and Gottschling (2007), pollen grains of *Varronia* are oblate-spheroidal to spheroidal with a reticulate tectum (roof-like structure) and sparse but uniform spinulose muri (surface ornamentation).

1.4.3.6. Spermology

Varronia fruits develop inside the persistent calyx as a one-seeded (other three ovules aborted) drupe. The bony endocarp (Figure 8) is undivided and encloses the seed and three sterile chambers (Desvaux, 1808) with a mesocarp that is sometimes inflated (Miller and Gottschling, 2007). Transfer cells are found in the testa (Diane *et al.*, 2002). Embryos may be curved to straight with plicate cotyledons, a defining feature of the Cordioideae (Heubl *et al.*, 1990). Endosperm is completely absent inside the seed (Miller and Gottschling, 2007).

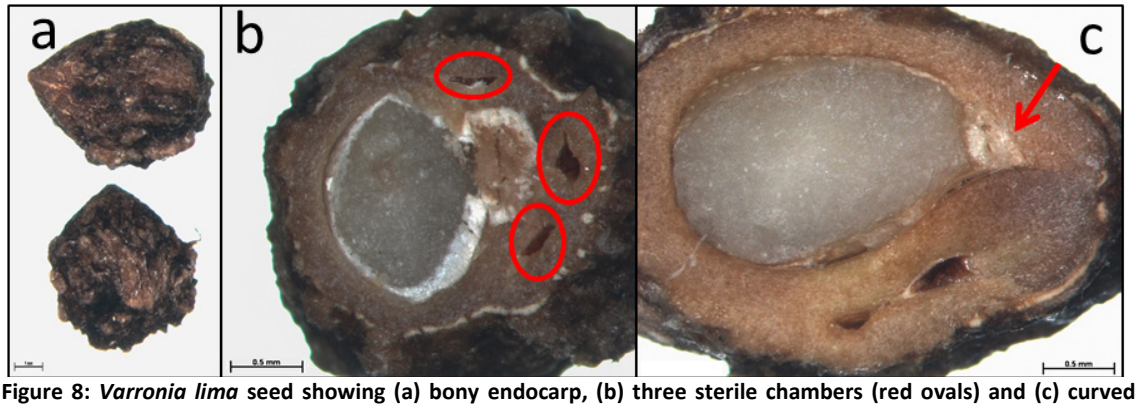

Figure 8: *Varronia lima* seed showing (a) bony endocarp, (b) three sterile chambers (red ovals) and (c) curved embryo (red arrow). ©M.A. Hamilton.

1.4.4. *Varronia rupicola*

Varronia rupicola (Urb.) Britton, Scientific Survey of Porto Rico and the Virgin Islands 6(1): 128. 1925. Syntypes: Sintenis, P.E.E. 3731 (1886); Sintenis, P.E.E. 4879 (1886)[3]

Synonyms

Cordia rupicola Urb., Symbolae Antillanae (Urban). 1: 392. 1899.

Varronia bahamensis sensu Britton & P. Wilson, Scientific Survey of Porto Rico and the Virgin Islands 6(1): 127. 1925, *non* (Urb.) Millsp.

[3] A full list of specimens consulted is available in Appendix 1: *Varronia rupicola* records

Distribution

This species is endemic to the Puerto Rican Bank and has been recorded in the countries of Puerto Rico, on the islands of Puerto Rico (south-western coastal municipalities) and Vieques (southern coastal areas), and the British Virgin Islands, on the island of Anegada (Urban, 1899; Britton and Wilson, 1925a; Pollard and Clubbe, 2003; Clubbe *et al.*, 2004; Hamilton, 2005; U.S. Fish and Wildlife Service, 2010).

Description

Varronia rupicola is a medium to large shrub reaching 5 m in height with one to many branches from the base. Stems, particularly young branches, are covered in setulose indumentum. Chartaceous leaves are highly variable, ovate to elliptic or oblong-elliptic, 2-10 cm long by 1-5 cm wide and held on short, strigose petioles, 2-10 mm long. Leaf base is obtuse or narrowed and apex is acute, obtuse or rounded. Leaf margin varies from denticulate or low-crenate to sub-entire with few teeth. Adaxial surface is scabrous and rigid while the abaxial surface is puberulous, especially the veins. Appressed, pilose hairs cover the four or five triangular lobed, 4-5 mm long, calyx which is obovoid in bud (Figure 9, Figure 10 (a)). White flowers in globose, solitary inflorescences (Figure 9), up to 2.5 cm across, are held on peduncles up to 4 cm long. Corolla is 7 mm across with four or five lobes, 4-5 mm long (see Figure 9, Figure 10 (b)). Red fruit is ovoid, obtuse and 4-5 mm long (see Figure 9, Figure 10 (c)) (Urban, 1899; Britton and Wilson, 1925a; Proctor, 1991).

Figure 9: Flowers, fruits and foliage of *Varronia rupicola* on Anegada, British Virgin Islands. ©M.A. Hamilton.

Figure 10: Development of solitary *Varronia rupicola* inflorescence showing (a) triangular lobed obovoid calyx in bud, (b) white flowers in globose inflorescences with five lobed corolla and (c) ovoid and obtuse fruit developing from green to red when mature. ©M.A. Hamilton.

According to Proctor (1991), *V. rupicola* can be distinguished from the other native *Varronia* species in the Puerto Rican Bank by morphological characters. *Cordia globosa* (= *Varronia bullata* L. subsp. *humilis* (Jacq.) Feuillet) has coarsely serrate leaves and the corolla tip is filiform. The leaves of *V. lima* are bullate, its flowers have shorter peduncles and it forms 3mm fruit which is smaller than *V. rupicola*. All other *Varronia* species lack globose, solitary, peduncled inflorescences.

1.4.4.1. Taxonomy

Urban (1899) first described *Cordia rupicola* selecting the specific name epithet meaning "growing on rocks or cliffs" based on the type location habitat from which Paul Sintenis collected the original material in south-western Puerto Rico. Britton made the combination *Varronia rupicola* as part of the revision of the flora of the Puerto Rican Bank (Britton and Wilson, 1925a). Unfortunately, Britton considered *V. rupicola* endemic to Puerto Rico and plants found on Anegada to represent *V. bahamensis*. D'Arcy (1971) originally followed Britton, but based on morphological differences of specimens collected on Anegada referred to *Varronia* plants as *Cordia rupicola* in his second publication about the island (D'Arcy, 1975). In the latter publication, D'Arcy states that further work is needed on the taxa as there are many similarities between plants in the Bahamas, Hispaniola and the Puerto Rican Bank referred to as *C. rupicola, C. lima* and *C. bahamensis*. These morphological similarities must have been obvious to Urban (1899) when he described *Cordia rupicola* and *C. bahamensis* because he refers to *C. lima* in the text as well as a fourth species from Cuba, *C. erythrococca* Wr., that he felt warranted note due to their similar characters.

George Proctor collected specimens of *Varronia* on Anegada in 1987 and also determined them to be *Cordia rupicola* (Proctor, 1991). Most collectors since have recognised plants on Anegada to represent *V. rupicola*; however, curation of specimens in the major herbaria of

Europe and North America varies considerably. Chapter 4: Conservation genetics of *Varronia rupicola* explores the genetic variation and structure of *Varronia rupicola*.

1.4.4.2. Threats to the species

Varronia rupicola is a rare species with a limited area of occupancy (AOO) that is often found as solitary individuals or in small numbers in isolated patches, especially in Puerto Rico. Human activities have impacted *V. rupicola* for decades as was documented by Woodbury *et al.* (1975) and has probably been happening since before the species was discovered by Sintenis in 1886 (Urban, 1899) given the documented levels of deforestation and land-use change across the Puerto Rican Bank (Schomburgk, 1832; Eggers, 1879; D'Arcy, 1971, 1975; Murphy and Lugo, 1990; Lugo *et al.*, 1996; Molina Colón and Lugo, 2006; Ramjohn *et al.*, 2012). The greatest current threat to the species is habitat loss through development and on-going degradation and fragmentation of existing habitat are of major concern. Due to the limited numbers of individuals and locations, the species is also threatened by human-induced fire, particularly on Puerto Rico, and natural disasters, particularly hurricanes and tsunamis (Parsons and Geist, 2009; Elsner and Jagger, 2010; U.S. Fish and Wildlife Service, 2010). On the island of Anegada the species faces threats from climate change, most specifically sea-level rise, as the highest point on the island is ca. 8 m above sea level (asl) and 40% of the island lies below 5 m asl (D'Arcy, 1971).

The last census of plants in Puerto Rico undertaken by the U.S. Fish and Wildlife Service (2010) reported 226 individuals, including seedlings, saplings and mature plants with 68% of the reproductive individuals found within protected areas. The remaining reproductive individuals were on private lands within an area subject to development for residential housing. No signs of natural dispersal were found for plants surveyed in Puerto Rico (U.S. Fish and Wildlife Service, 2010).

The population size on Anegada is estimated to be between 500 and 1000 mature individuals (Hamilton *et al.*, 2015b), and the species was located in 69% of 104 sampling points along 27 random transects situated in and around the Western Salt Ponds of Anegada RAMSAR site (Clubbe *et al.*, 2004). All individuals outside the RAMSAR site face development pressures, particularly road and housing construction, and invasive species are spreading into native habitats on the island (Pollard and Clubbe, 2003; Clubbe *et al.*, 2004; McGowan *et al.*, 2006a).

Insect pests are causing extensive declines in populations of dry forest species in the Caribbean (Serra *et al.*, 2003; Malumphy *et al.*, 2012). The potential introduction of insect pests that might attack *V. rupicola* is a particular concern due to the limited AOO of the species as they could infest all existing plants on any of the islands very quickly. During the course of this

research (Figure 11), two new pest insects were found attacking *V. rupicola* across Anegada (Malumphy *et al.*, 2015). Increased biosecurity is urgently needed to reduce the potential spread of pests in the region.

Management practices (i.e. road, trail and powerline maintenance) within Puerto Rico's protected areas have also negetively impacted the species in the wild (U.S. Fish and Wildlife Service, 2010). This is often compounded by the morphological similarities, to the untrained eye, between *V. rupicola* and several other widespread dry forest species (e.g. Rubiaceae *Guettarda scabra* (L.) Vent.). Close collaboration between conservationists, land managers and maintenance practitioners is challenging but fundamentally necessary for the species long-term survival.

Figure 11: Dr Michele Sanchez examining a drought stressed and feral livestock damaged *Varronia rupicola* for pests on Middle Cay, Anegada. ©M.A. Hamilton.

1.5. Thesis outline

In order to effectively conserve *Varronia rupicola* and enable informed management decisions, investigations into the species distribution and its relationship with other taxa in the region as well as the size and genetic variability of the extant populations are required. These topics are the focus of the research presented in this thesis which is the synthesis of field activities undertaken and collections made between 2012 and 2015 in the Puerto Rican Bank.

Following the recommendation of Assis (2014) for researchers to clearly define methods employed, this research has used reciprocal illumination (Hennig, 1966), specifically anatomical, molecular, biogeographical and ecological evidence, as well as taxa sampling (Heath *et al.*, 2008) through additional species and sampling locations to test hypotheses. These are being employed as the most appropriate methods given the previous systematic work undertaken for the study group (Miller, 1985; Miller and Nowicke, 1989; Gottschling *et al.*, 2001; Gottschling, 2003; Gottschling *et al.*, 2005; Miller and Gottschling, 2007; de Stapf, 2010; Weeks *et al.*, 2010; Stutzman *et al.*, 2012), the species recorded distribution in the insular Caribbean and its ecological niche.

The specific aims for each chapter are followed by an introduction to the topics discussed before detailing the materials and methods employed by this research. Subsequently, the results are presented before these are discussed. Finally the main conclusions for the chapter are provided.

In Chapter Two, the biogeography of *V. rupicola* is explored to determine the species native range and ecological preferences as well as the impacts of on-going habitat modification and future sea level rise. The phylogenetic placement of *V. rupicola* and several other species of *Varronia* and *Cordia* native to the Caribbean are the focus of Chapter Three using ITS and *trnL-trnF* regions. Chapter Four explores the genetic variability and structure of *V. rupicola* populations. This is undertaken using nuclear microsatellites to determine if there are differences between and within the islands where the species occurs as well as determining if genetic diversity has been lost in the wild and captured in *ex-situ* collections. A general discussion of the findings presented across chapters two to four along with the conservation implications of the results obtained and further research opportunities follow in Chapter Five.

Chapter 2: Biogeography of *Varronia rupicola*

This chapter explores the biogeography of *Varronia rupicola* with the aim of answering several specific questions about the native range and ecological preferences of the species and the impacts of on-going habitat modification and future sea level rise.

- First, where is the species currently extant and does this differ from its historic native range?
- Second, do *V. rupicola* plants occur on any specific substrates across the native range of the species? If so, where are they located and how much area do they cover?
- Third, are *V. rupicola* plants associated with any specific land cover types overlying the substrates supporting the species across its native range? If so, where are they located and how much area do they cover?
- Fourth, are *V. rupicola* plants found within protected areas (proposed or established)? If so, how much area of the land cover types known to support *V. rupicola* plants do these protected areas contain?
- Fifth, what are the implications of past sea level rise on the native range of *V. rupicola*?
- Sixth, will proposed scenarios for future sea level rise have an impact on the native range of *V. rupicola*?

2.1. Introduction

The science of biogeography has advanced significantly in recent years through the development of Geographical Information System (GIS) software, remote sensing and free access to global as well as more refined regional datasets. GIS in particular has enabled large and varied datasets to be compiled, manipulated and queried to produce a wide range of outputs including land cover and geology maps as well as digital elevation models (DEMs). These outputs offer the opportunity to simulate and explore change, spatially and temporally, across a wide range of parameters (Lomolino *et al.*, 2006).

A species range and the size of its populations are seldom static. The expansion and contraction of a population are driven by abiotic and biotic factors. Among these are human induced and natural pressures, mating systems, gene pools, changing climate and available habitat. Studying biogeography of a species requires knowledge of the species interaction with its environment coupled with human and natural disturbances that effect the organism to be studied and comparison of historic and modern distribution data (Lomolino *et al.*, 2006; Lomolino, 2010).

Varronia rupicola grows in dry forest habitat with underlying limestone geology (Pollard and Clubbe, 2003; U.S. Fish and Wildlife Service, 2010; Wenger *et al.*, 2010; BCPeabody Construction Services Inc. and Coll Rivera Environmental, 2011) in the subtropical dry forest life zone (Ewel and Whitmore, 1973; Lugo *et al.*, 1999; Miller and Lugo, 2009). The recorded habitat ranges from open scrubland at the edge of the dune systems to hillside thickets and closed canopy forest, all of which occur at lower elevations (Pollard and Clubbe, 2003; U.S. Fish and Wildlife Service, 2010). In south-western Puerto Rico and Vieques where *V. rupicola* occurs, the soil is mostly found in pockets between exposed limestone and is high in organic matter with an alkaline pH (Murphy and Lugo, 1990; Lugo *et al.*, 1996; Monsegur, 2009). On Anegada's eastern side and the limestone areas within the western Ramsar site (Figure 12), the species grows in similar soils to those described for Puerto Rico. *Varronia rupicola* plants growing on the western side of Anegada, outside of the limestone cays, are found in sandy soils with organic matter (Clubbe *et al.*, 2004; Hamilton *et al.*, 2015b).

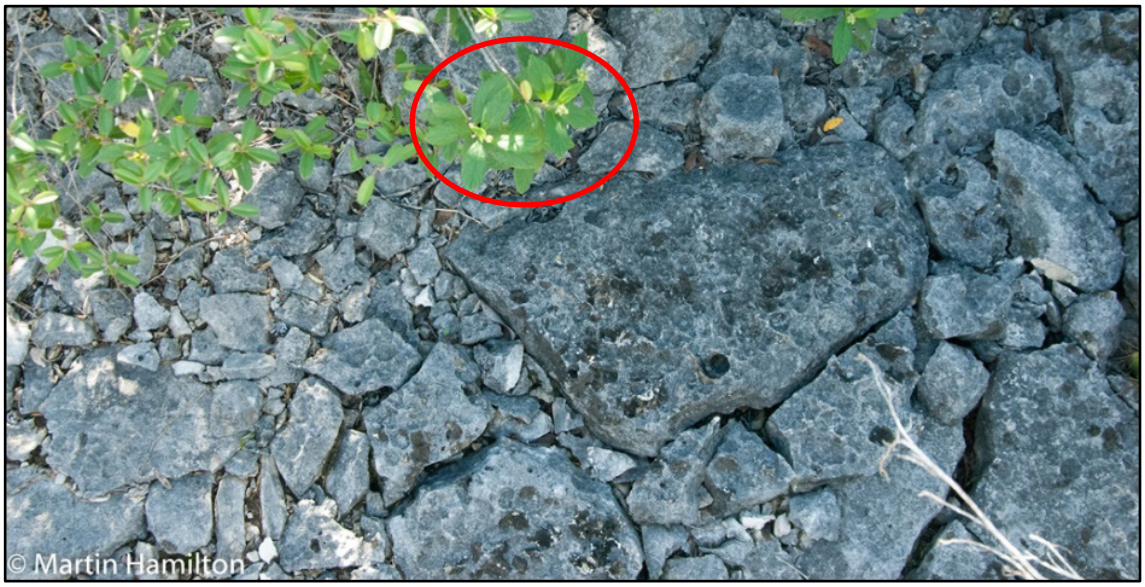

Figure 12: *Varronia rupicola* (highlighted with red oval) growing on exposed limestone within the Western Salt Ponds Ramsar site, Low Cay, Anegada. ©M.A. Hamilton.

The plant species associated with *V. rupicola* are mostly deciduous, at least during the driest months, less than 5m in height and generally form a single layer of vegetation (Ewel and Whitmore, 1973; U.S. Fish and Wildlife Service, 2010) as seen in Figure 13. *Varronia rupicola* is often associated with other rare, e.g. *Nashia inaguensis* (Verbenaceae) Millsp., and threatened, e.g. *Acacia anegadensis* Britton (Leguminosae), species across its range (Clubbe *et al.*, 2004; U.S. Fish and Wildlife Service, 2010).

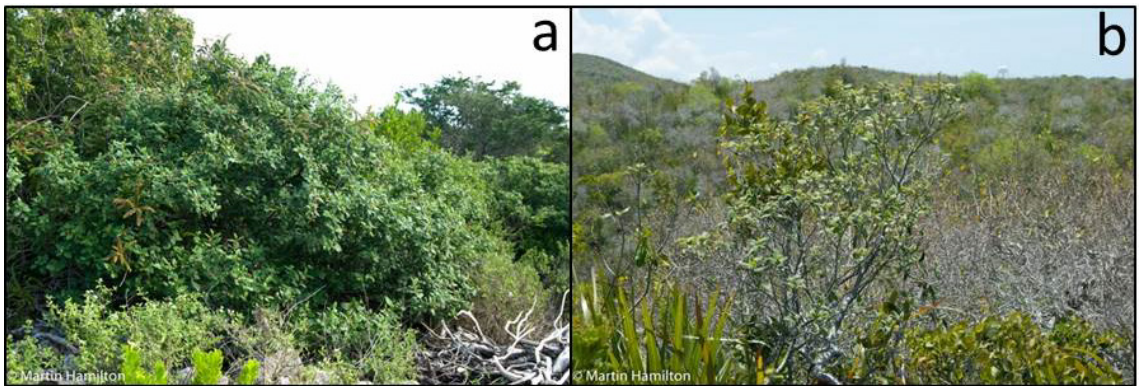

Figure 13: *Varronia rupicola* growing in dry forest habitat on a) Anegada as a large ca. 3 m rounded shrub and b) Puerto Rico as a small, emergent tree. ©M.A. Hamilton.

On the island of Puerto Rico the species is only known to occur in the south-west on coastal limestone (Axelrod, 2011) from the municipalities of Ponce, in the east, to Guánica, in the west. *Varronia rupicola* has been recorded on Vieques at two locations, Punta Jalova and Puerto Ferro, along the southern coast within the eastern tract of the Vieques NWR (Proctor, 1991; Breckon, 2007). The species has been recorded across most of the island of Anegada (Britton, 1916; D'Arcy, 1975; Pollard and Clubbe, 2003; Hamilton, 2005). The locations highlighted here are shown in Figure 14.

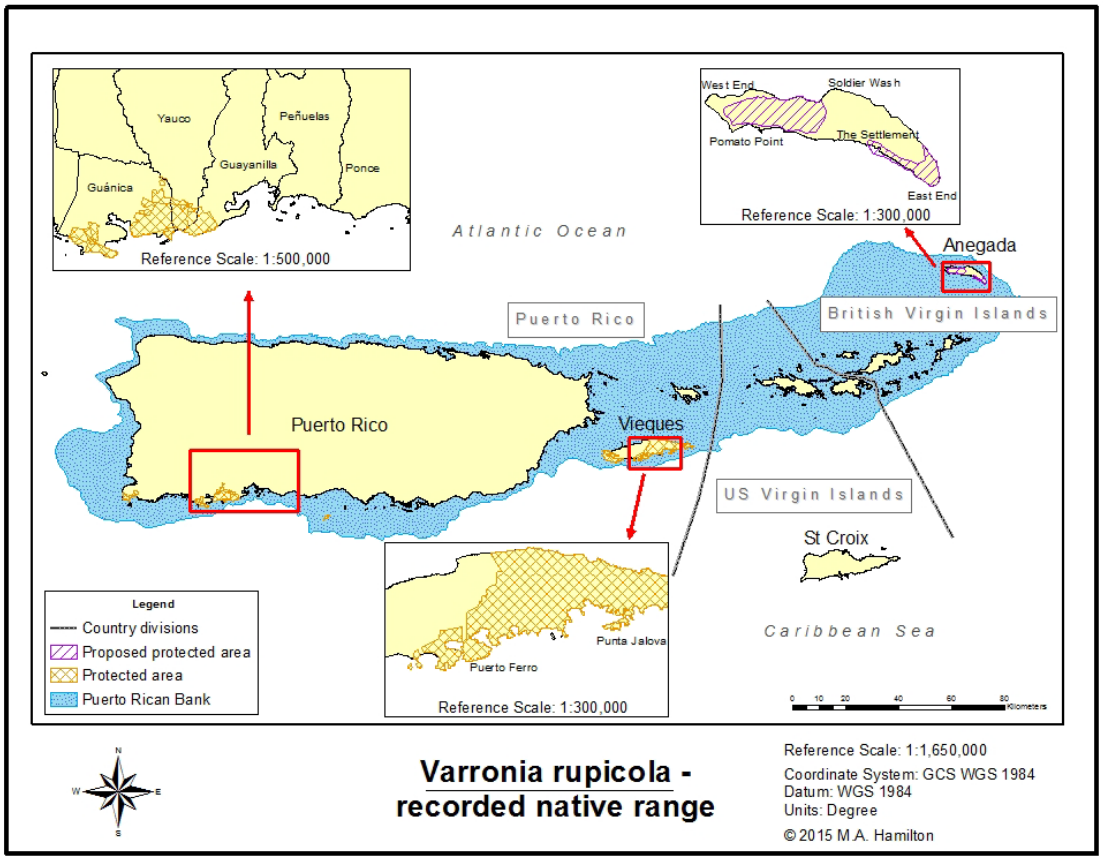

Figure 14: Map of *Varronia rupicola* collection localtities derived from all available species records prior to 2012 across the Puerto Rican Bank showing borders of proposed and existing protected areas in the vicinity of recorded localities. The species has been recorded across Anegada (inset top right), two locations on Vieques (bottom inset) and along the south-western coast of Puerto Rico (inset top left).

Little is known about the specific habitat requirements and ecological niche of *V. rupicola*. There are no known studies comparing the native range of the species to published land cover or geology maps and there are no studies exploring the possible implications of past sea level rise or impact of scenarios for future sea level rise. As such, the following sections provide an overview of the available information about the geology, land cover and sea level rise (past and future) of the species native range.

2.1.1. Geology

The geology of the Puerto Rican Bank (PRB) and specifically south-western Puerto Rico is complex due to tectonic and climatic events that led to subsidence, faulting and submersion/emersion (Masson and Scanlon, 1991; Graham, 2003; Mann *et al.*, 2005; Joyce, 2009). Except for Anegada which is composed exclusively of limestone, the PRB islands are, like their closest neighbours in the Greater Antilles, composed of ca. 115-60 million year old Lower Cretaceous to Palaeocene deposits (Graham, 2003; Acevedo-Rodríguez and Strong, 2008) overlaid on ca. 200 million year old Jurassic oceanic basement deposits (Acevedo-Rodríguez and Strong, 2008) that formed as an oceanic piece of the Pacific crust pushed between the North and South American plates (Stanek *et al.*, 2009). Many of the volcanic islands also have limestone deposits, especially along their coasts. These deposits formed during periods of submersion mostly caused by the glacial cycles, particularly from the Oligocene to the Pliocene (Graham, 2003). According to Lugo *et al.* (2001), 7.5% of the surface of Puerto Rico is covered by deposits of limestone. Unlike the predominantly volcanic islands of the rest of the PRB, Anegada formed during the last interglacial period of the Pleistocene as part of a coral reef system (Meyerhoff, 1926; Gore, 2013). Piecing together the evidence of the complex climatic and physical history of the region is an on-going area of research that, according to Graham (2003), may never provide a complete reconstruction that enables migration and speciation of individual plant taxa to be fully described.

Prior to the Eocene, the PRB islands were probably continuously submerged and of little relevance for the study of terrestrial biota (Santiago-Valentín *et al.*, 2004). During the Oligocene, Puerto Rico and Hispaniola separated (Graham, 2003) which undoubtedly played a role in speciation events through isolation and loss of genetic exchange (Santiago-Valentín *et al.*, 2004). This epoch was the beginning of a global period of reef building that lasted through the Miocene (Frost *et al.*, 1983). These reef systems became deposits of limestone as they were exposed through eustatic changes. Limestone deposits in the native range of *V. rupicola* formed during different epochs across the PRB. The oldest of these relevant deposits are on the island of Puerto Rico where the Juana Diaz limestone formed in the early Miocene prior to the formation of the Ponce limestone later in the same epoch (Krushensky and Monroe, 1975).

Limestone along the south and east of Vieques formed during the Pliocene (Renken *et al.*, 2002) whereas exposed limestone on Anegada formed the most recently during the Pleistocene (Howard, 1970). Following the deposition of the Anegada limestone, further eustatic changes driven by glacial cycles (see 2.1.3. Sea level rise) led to the exposure of the entire PRB as a single land mass during the latter part of the Pleistocene. The separation of the current islands was due to sea level rise during the Holocene (Santiago-Valentín *et al.*, 2004). Significant events relating to the formation of the PRB and deposits of limestone across geological time are shown in Table 1.

Table 1: Significant events in the formation of the Puerto Rican Bank and deposits of limestone across geological time based on the International Commission on Stratigraphy international chronostratigraphic chart (Cohen *et al.*, 2013)**; *Start date in millions of years ago.**

Era	Period	Epoch	Start date*	Event	Reference
Cenozoic	Quaternary	Holocene	0.012	Separation of the current Puerto Rican Bank islands	Santiago-Valentin and Olmstead, 2004
		Pleistocene	2.588	Formation of Anegada limestone	Howard, 1970
	Neogene	Pliocene	5.332	Formation of Vieques limestone	Renken *et al.* 2002
		Miocene	23.030	Formation of Juana Diaz and Ponce limestones	Krushensky and Monroe, 1975
	Palaeogene	Oligocene	33.900	Separation of Puerto Rico and Hispaniola	Graham, 2003
		Eocene	55.800	Emergence of modern Puerto Rican Bank islands	Santiago-Valentin and Olmstead, 2004
		Palaeocene	66.000	Formation of Puerto Rican Bank islands volcanic base	Acevedo-Rodríguez and Strong, 2008
Mesozoic	Cretaceous	Upper	100.500		
		Lower	~145.000		
	Jurassic	Upper	~163.500	~	~
		Middle	~174.100	~	~
		Lower	~201.300	Formation of Puerto Rican Bank oceanic basement deposits	Acevedo-Rodríguez and Strong, 2008

The surface geology of the island of Puerto Rico is well known due to the mapping work of the United States Geological Survey (USGS) during the 20th century (Renken *et al.*, 2002). Detailed quadrangle geological maps (1:20,000 map scale) have yet to be completed for a few areas, including the Guánica quadrangle (Addarich-Martínez, 2009); however, island wide geological maps were produced for the USGS by Briggs and Akers (1965) and updated by Bawiec (1999).

Overlain volcanic materials in southern Puerto Rico are covered mainly by Quaternary and Miocene deposits, with the latter dominated by the Juana Diaz formation and Ponce

limestone. The Juana Diaz formation is divided into an upper calcareous layer of Miocene origin and basal Oligocene beds of cobbles, pebbles and sand. Ponce limestone rests atop the Juana Diaz formation or Cretaceous rock along the southern coast of modern Puerto Rico (Monroe, 1980) and atop Eocene sandstone on the small off-shore island of Isla Caja de Muertos (Kaye, 1957; Monroe, 1980). Limestones of southern and northern Puerto Rico of similar age are very different, mainly due to faulting and climatic conditions (Monroe, 1980). Due to the lower rainfall levels in southern Puerto Rico, a layer of Quaternary derived caliche created by the evaporation of water from the slight solution of the surface limestone has developed into a thick cap that makes inspection of the bedrock difficult outside of road cuts and quarries (Monroe, 1980).

The geology of Vieques is less well understood due a lack of detailed mapping; however, the USGS produced a simplified 1:30,000 surface geology map for the island as part of a geochemical study (Learned *et al.*, 1973). Renken *et al.* (2002) produced the most recent geological map of the island based on the works of Briggs and Akers (1965) and Learned *et al.* (1973) which depict Pliocene limestone deposits. Meyerhoff (1927) stated that these deposits rest directly on the Cretaceous origin volcanic base of Vieques.

The limestone geology of Anegada is the least studied in the PRB with the most notable studies on the subject being those of Howard (1970), Atwater *et al*. (2012, 2014) and Spiske and Halley (2014). As sea levels lowered following the last interglacial period, the exposed coral reefs died forming a solid limestone slab that is today the island of Anegada (Howard, 1970). Gore (2013) used the geology and surface deposits of Anegada to divide it nearly in half calling the eastern portion the 'Anegada limestone formation' and the western portion the 'Anegada ridge plain formation'. Previous authors called the former the rocky or limestone plain and the latter the sandy plain (Britton, 1916; D'Arcy, 1975). The deposits of sand and alluvium (unconsolidated sediments) on Anegada that overlie the limestone bedrock are all thought to be of Quaternary origin (Howard, 1970; Spiske and Halley, 2014).

The available geological maps will be used to determine if *V. rupicola* is associated with any specific types of substrate across its native range.

2.1.2. Land cover

A series of studies led by the United States Forest Service (USFS) were undertaken to provide regional maps of the relationship of land cover to surface geology and topography across Puerto Rico and the Virgin Islands (PRVI). These studies used remote sensing-based maps derived from cloud-free optical satellite imagery (Landsat) processed into image mosaics first described by Helmer and Ruefenacht (2005). Mapping land cover and forest types was

undertaken with supervised classification of Landsat TM and/or ETM+ imagery using decision tree analysis software (Kennaway and Helmer, 2007; Kennaway *et al.*, 2008; Gould *et al.*, 2008). The classifications developed for PRVI are based on Helmer *et al.*'s (2002) hierarchical system for Landsat image classification that was adapted from Areces-Mallea *et al.* (1999) for the Caribbean based on the National Vegetation Classification Standard (FGDC, 1997). Predictor variable datasets were produced to assist classification of image pixels that included topography (derived from digital elevation models or digitised topographic maps), geology and precipitation where available. Training data, either from field survey or existing classification maps, were used to develop classified land cover maps. Image classification was checked for accuracy using expert review and reference IKONOS panchromatic sharpened imagery (Kennaway and Helmer, 2007; Kennaway *et al.*, 2008; Gould *et al.*, 2008).

Cloud-free optical satellite imagery mosaics were produced for Puerto Rico and Vieques using images captured in 1991 and 2000 to study land cover change, especially due to anthropogenic factors. Kennaway & Helmer (2007) produced land cover maps for Puerto Rico using a regional habitat classification with 29 classes (Figure 15).

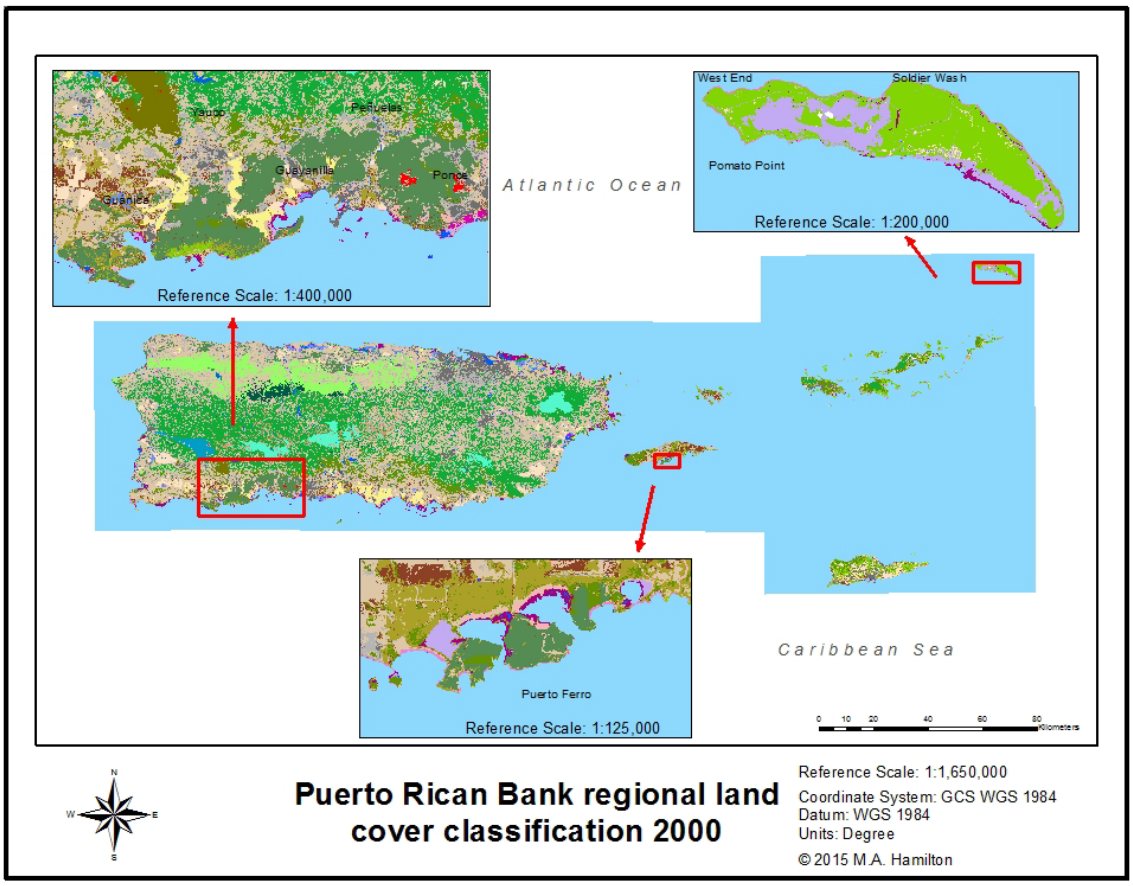

Figure 15: Regional habitat classifications for Puerto Rico and the Virgin Islands based on Kennaway & Helmer (2007) **for the country of Puerto Rico and Kennaway *et al.*** (2008) **for the U.S. and British Virgin Islands.**

Gould *et al.* (2008) produced detailed habitat types for the islands of Puerto Rico and Vieques using a refined habitat classification with 70 classes. Mapping across the Virgin Islands used cloud-free optical satellite imagery mosaics from 2000 to produce land cover maps showing refined habitat and regional habitat (see Figure 15) classifications for U.S. (USVI) and British Virgin Islands (BVI) that included 29 and 20 classes, respectively (Kennaway *et al.*, 2008).

Helmer and Ruefenacht (2005) found that between 1991 and 2000 built-up/urban areas on Puerto Rico increased by 7.2%. The proximity to urban areas and a lack of protection for older, species-rich forests on karst increased their likelihood of development (Kennaway and Helmer, 2007). Along the southern coast of Puerto Rico, Helmer *et al.* (2008) found that around the city of Ponce many older forests on unprotected areas of limestone geology were converted to built-up/urban areas or quarries. This is of particular concern for *V. rupicola* as it is known to occur in this area (U.S. Fish and Wildlife Service, 2014b). Between 1991 and 2000, Vieques saw a 49% increase in built-up/urban areas (Helmer and Ruefenacht, 2005). No published data are available for the same period on Anegada.

The aforementioned land cover maps will be used in this research to explore habitat preference for *V. rupicola* in relation to geology and protected areas across the PRB.

2.1.3. Sea level rise

The USGS produced bathymetric and topographic data for PRVI to assist in disaster management planning in the past decade. These data available varies from a strictly topographic 1/12 arc-second (2.5 m) digital elevation model (DEM) for the island of Anegada (DDM, 2014) to bathymetric and topographic 1/3 arc-second (10 m) DEMs for the USVI (Love *et al.*, 2015) and Puerto Rican municipalities of Arecibo, Fajardo, Guayama, Mayaguez, Ponce and San Juan (Taylor *et al.*, 2008) as well as bathymetric and topographic 1 arc-second (30 m) DEMs (Figure 16) for the country of Puerto Rico (Taylor *et al.*, 2008) and all of the Virgin Islands (Love *et al.*, 2015). The Anegada 1/12 arc-second DEM is derived from a Light Detection and Ranging (Lidar) survey via small aircraft undertaken in January 2014 (DDM, 2014). The 1/3 arc-second DEMs derived for specific islands and municipalities as well as the 1 arc-second DEMs for PRVI were derived from a range of sources described in detail by Taylor *et al.* (2008) for Puerto Rico and Love *et al.* (2015) for the Virgin Islands. Access to these high quality data afford the opportunity to explore past sea level rise and the implications for *V. rupicola* as well as the possible impact of future sea level rise scenarios on the long-term survival of the species across the PRB.

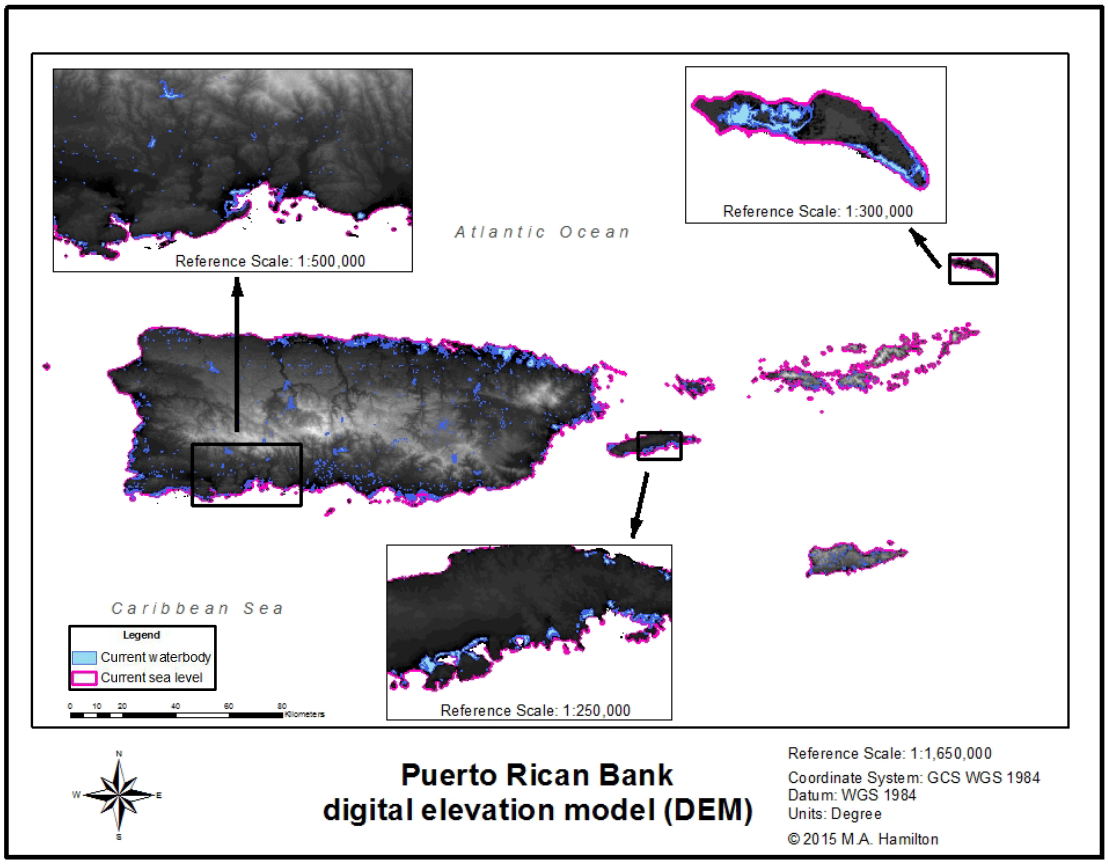

Figure 16: 1 arc-second (30 m) digital elevation model (DEM) data for Puerto Rico and the Virgin Islands available for the country of Puerto Rico (Taylor *et al.*, 2008) and the Virgin Islands (Love *et al.*, 2015) as well as 1/12 arc-second (2.5 m) DEM for the island of Anegada (inset top right) classified to exclude all data below 0 m and showing 32 natural breaks using 1 m, 2 m, 3 m, 6 m, 25 m and then 50 m increments up to the maximum 1330 m asl. The native range of *Varronia rupicola* is highlighted on south-western Puerto Rico (inset top left), Vieques (inset bottom middle) and Anegada (inset top right).

Sea level changes at the local level can vary from that of the global mean due to background geological processes, gravitational distribution of ice and water as well as dynamics of the atmosphere and ocean (Kopp *et al.*, 2015). Hansen *et al.* (2007) suggested that based on palaeoclimatic data, the Earth's climate can be quickly flipped due to global sensitivity to forcing. Since integrating such variable processes across temporal and spatial scales to reconstruct past sea level and project future changes is an active area of research, many gaps still exist and debate is active (Gregory *et al.*, 2014; Horton *et al.*, 2014).

Past sea level rise

Sea level has fluctuated significantly in the past; however, precise information about the extent varies widely, especially for more distant periods. This is mainly due to the complicated nature of determining sea levels above and below current sea level on constantly moving and shifting land masses (Martinson *et al.*, 1987; Bard *et al.*, 2002). Glacial and interglacial climate oscillations cause the transfer of water between the oceans and ice sheets which can result in rapid changes in sea level (Lambeck *et al.*, 2002a). The speed of these changes is controlled by

complex interactions between the land, oceans, atmosphere and ice sheets that are influenced by changes in insolation and internal feedback (Lambeck *et al.*, 2002a).

The PRB underwent a general period of uplift during the transition period from the Eocene, when it is thought that the modern islands first appeared, to the Oligocene. This was followed by higher sea levels during the latter parts of the Oligocene and during the Miocene (Santiago-Valentín *et al.*, 2004). During the middle Pliocene, sea level was ca. 25 (±10) m higher than today (Hansen *et al.*, 2007). Between 160,000 and 140,000 ybp, sea levels were around 120 m lower than current levels during the penultimate glacial maximum (Bard *et al.*, 2002). The Last Interglacial Period (LIG) lasted from 130,000 to 115,000 ybp (Dahl-Jensen *et al.*, 2013) and sea levels reached no more than 10 m higher than present levels (Church *et al.*, 2013) with strong evidence from corals on tectonically stable coasts that sea level was 4-6 m above current levels (Overpeck *et al.*, 2006).

The last glacial period, 114,000 to 19,000 ybp, saw sea levels drop overall with five periods of higher sea levels occurring from 60,000 to 32,000 ybp (Lambeck *et al.*, 2002a). In the lead up to the Last Glacial Maximum (LGM), a rapid fall in sea level of around 50 m in less than 1000 years has been suggested by Lambeck *et al.* (2002b) that implies rapid growth and decay of ice sheets is possible. During the LGM that lasted from 30,000 to 19,000 ybp (Lambeck *et al.*, 2002a) sea levels reached at least 120 m below those of today (Fairbanks, 1989; Siddall *et al.*, 2003). These eustatic changes driven by glacial cycles led to the exposure of the Puerto Rican Bank as a single land mass during the latter part of the Pleistocene.

Following the LGM and the onset of the melting of the ice sheets, sea level began to rise globally for around 13,000 years (Masson-Delmotte *et al.*, 2013). During the latter part of the Pleistocene, sea level rose around 20 m by ca. 17,000 ybp (Fleming *et al.*, 1998) and around 14,000 ybp had risen another ca. 25 m due to the deglaciation of the West Antarctic Ice Sheet before the start of the Holocene (Clark *et al.*, 2009). Fairbanks (1989) stated that sea level had risen a further ca. 25 m by around 10,000 ybp; therefore, the PRB would have been a single land mass for ca. 20,000 years. Over the next ca. 1,500 years (8,500 ybp), sea level rose ca. 25 m (Fairbanks, 1989). Fleming *et al.* (1998) stated that another ca. 22 m sea level rise was experienced over the following 1,500 years (ca. 7,000 ybp). Relative sea level stability has been experienced over the past ca. 3,000 years up to the middle of the 19th century (Williams, 2013). Since the turn of the 20th century, annual mean sea level rise rates increased from a background of 1.7 mm starting in 1900 to 3.2 mm between 1993 and 2010 (Rhein *et al.*, 2013).

Available sea level rise data and DEMs will be used to explore the potential impacts of past sea level rise on *V. rupicola*.

Future sea level rise

The greenhouse gas (GHG) emissions produced since the Industrial Revolution have increased global mean temperature which is responsible for current sea level rise through melting of the ice sheets and thermal expansion (Williams, 2013). Hansen *et al.* (2007) listed the three most important of these GHG emissions, in order of impact, as carbon dioxide (CO_2), methane (CH_4) and tropospheric ozone (0_3). To enable long-term modelling, four Representative Concentration Pathways (RCPs) were developed by a wide range of emission and modelling experts to produce a comprehensive dataset using radiative forcing values in Watts per square metre (W/m^2) through 2100 (van Vuuren *et al.*, 2011). The RCPs selected from forcing values in the literature ranged from 2.6 to 8.5 W/m^2 (Table 2) and resulted in one mitigation scenario (RCP2.6) with a very low level of forcing, two scenarios (RCP 4.5 and RCP 6) with medium stabilisation and one scenario (RCP8.5) with a very high level of forcing (van Vuuren *et al.*, 2011). The RCPs are supplemented by Extended Concentration Pathways (ECPs), or RCP extensions (Meinshausen *et al.*, 2011), that allow modelling through 2300 to be undertaken using the same radiative forcing values of 2.6, 4.5, 6.0 and 8.5 W/m^2 (van Vuuren *et al.*, 2011).

Table 2: Global mean sea level rise in metres (m) for the period 2020-2100 per Representative Concentration Pathway (RCP) with median values followed by likely range in brackets. Table modified from IPCC (2013a).

Year	RCP2.6	RCP4.5	RCP6.0	RCP8.5
2020	0.08 [0.06 to 0.10]	0.08 [0.06 to 0.10]	0.08 [0.06 to 0.10]	0.08 [0.06 to 0.11]
2030	0.13 [0.09 to 0.16]	0.13 [0.09 to 0.16]	0.12 [0.09 to 0.16]	0.13 [0.10 to 0.17]
2040	0.17 [0.13 to 0.22]	0.17 [0.13 to 0.22]	0.17 [0.12 to 0.21]	0.19 [0.14 to 0.24]
2050	0.22 [0.16 to 0.28]	0.23 [0.17 to 0.29]	0.22 [0.16 to 0.28]	0.25 [0.19 to 0.32]
2060	0.26 [0.18 to 0.35]	0.28 [0.21 to 0.37]	0.27 [0.19 to 0.35]	0.33 [0.24 to 0.42]
2070	0.31 [0.21 to 0.41]	0.35 [0.25 to 0.45]	0.33 [0.24 to 0.43]	0.42 [0.31 to 0.54]
2080	0.35 [0.24 to 0.48]	0.41 [0.28 to 0.54]	0.40 [0.28 to 0.53]	0.51 [0.37 to 0.67]
2090	0.40 [0.26 to 0.54]	0.47 [0.32 to 0.62]	0.47 [0.33 to 0.63]	0.62 [0.45 to 0.81]
2100	0.44 [0.28 to 0.61]	0.53 [0.36 to 0.71]	0.55 [0.38 to 0.73]	0.74 [0.53 to 0.98]

The fifth and most recent Intergovernmental Panel on Climate Change (IPCC) assessment report (AR5) of Working Group I by Church *et al.* (2013) provided RCP scenarios (see Table 2) that suggest that the 21st century rate of mean sea level rise globally will exceed the observed 1971-2010 rate. The increases in sea level rise projections of the AR5 compared to AR4 are primarily due to model improvements (Church *et al.*, 2013), but these are still below the projections of many other studies (Overpeck *et al.*, 2006; Hansen *et al.*, 2007; Rahmstorf, 2007; Pfeffer *et al.*, 2008; Grinsted *et al.*, 2010; Nicholls and Cazenave, 2010; Wetzel *et al.*, 2012) and have been criticised as too conservative (Horton *et al.*, 2014). However, Church *et al.* (2013) stated that there is a lack of sufficient evidence to support projections of higher sea level rise than those presented in the AR5 for the 21st century. Pfeffer *et al.* (2008) concluded that it is physically untenable for sea level rise in excess of 2 m by 2100 and that climate

variables would need to climb to extremely high levels at an accelerated speed in order to achieve a 2 m sea level rise by 2100 under physically possible conditions.

Beyond the 21st century, sea level rise of meters higher than present levels is possible if GHG emissions are not lowered (Pfeffer *et al.*, 2008; Church *et al.*, 2013). The 2300 sea level rise projections of AR5 range from 0.41-0.85 m for ECP2.6 to the ECP8.5 levels of 0.92-3.59 m (Church *et al.*, 2013; Horton *et al.*, 2014). Therefore, without mitigation to reduce GHG emission levels in the atmosphere, sea level rise is expected to continue and accelerate for centuries (Williams, 2013) with regional, possibly extreme, variations (Church *et al.*, 2013) and surpass the 1 m 'guardrail' (Horton *et al.*, 2014) that has been suggested to minimise impact on human society and global biodiversity.

Higher sea levels will contribute to the destruction of terrestrial habitats that are not adapted to saline conditions through seawater inundation associated with periodic high tides (e.g. centennial tides) or caused by cyclonic activity (Courchamp et al., 2014) that is expected to increase in severity (IPCC, 2012). Williams (2013) advised governmental bodies to prepare adaptation plans for sea level rise between 0.5 and 2.0 m by 2100 and suggested that between 4 and 8 m is possible in the following centuries. Such rises in global sea level will have major impacts on the coastal zones and human infrastructure. Adaptation and forced movements will undoubtedly put pressure on inland and upland areas further exacerbating the biodiversity conservation issues of today. As such, Courchamp *et al.* (2014) urged the integration of climate change, especially future sea level rise scenarios, into management plans and research of island biodiversity across the 180,000 islands worldwide.

Bellard *et al.* (2014) undertook a study using 1, 2, 3 and 6 m sea level rise scenarios for 2100 to 2300 across 4447 islands in ten insular biodiversity hotspots, including the Caribbean. The study found that between 6% and 19% of the islands would be completely submerged with the Caribbean being one of the three most impacted hotspots (Bellard et al., 2014). These values along with the IPCC (2013a) RCP values for 2100 will be explored in this research to determine the potential impact of future sea level rise on the native range of *V. rupicola.*

2.2. Materials and Methods

The research undertaken into the biogeography of *V. rupicola* included field survey and sampling across the PRB as well as desk based studies as described in the following sections.

2.2.1. Survey and sampling of wild populations

To enable planning of field survey and baseline data acquisition, all available *V. rupicola* specimen and observation data were incorporated into a database using BRAHMS software

(Botanical Research and Herbarium Management System, version 7.6, Oxford University). Records lacking GPS coordinates were georeferenced using the gazetteer available in the UKOTs Species and Specimens Database (Hamilton *et al.*, 2015a) to facilitate spatial visualisation. Using these data, maps were produced to facilitate planning and discussions with regional colleagues ahead of surveys. A full list of observations and vouchers held globally for the species is provided in Appendix 1: *Varronia rupicola* records.

Data recorded during surveys comprised substrate type, phenology, flower morphology, recruitment and observed/implied threats to the species in the immediate area. Methods for collecting DNA samples from wild populations of *V. rupicola* for population studies (see Chapter 4: Conservation genetics of *Varronia rupicola*) varied between the countries of the British Virgin Islands and Puerto Rico due to the different numbers of extant individuals; however, every plant of the species encountered during surveys between 2012 and 2015 was recorded as an observation to provide the records necessary to address the biogeographic questions posed for the species.

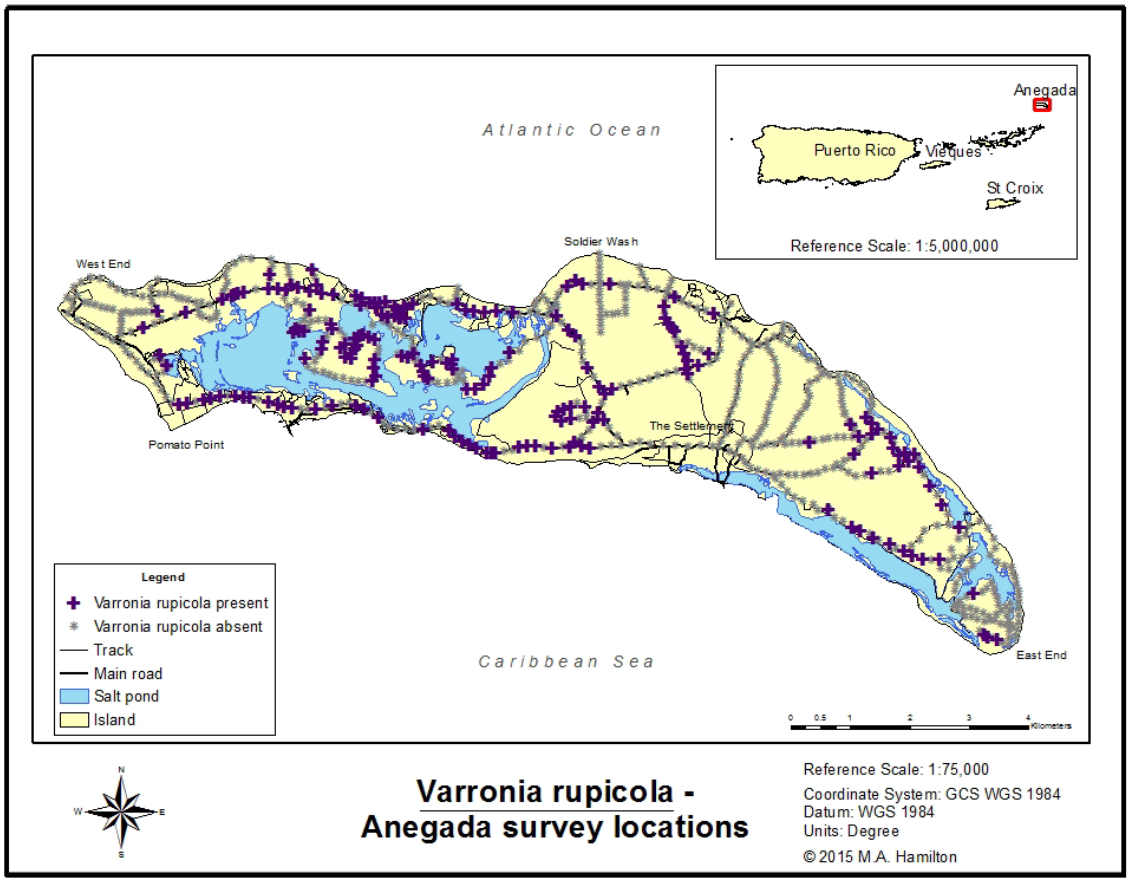

Figure 17: *Varronia rupicola* survey locations on Anegada showing species presence (purple plus) and absence (grey asterisk) recording locations.

On the island of Anegada (Figure 17), sampling was undertaken using transects (Bullock, 2006) with presence/absence recording of *V. rupicola* within a 10x10 m plot every 100 m and an

alternating left or right recording location across the island and its habitats. Recording was undertaken using the existing network of roads, trails and tracks as well as transects through intact vegetation to minimise sampling bias (Greenwood and Robinson, 2006). If *V. rupicola* was present at the 100 m recording location, a DNA sample was taken. Every 500 m, leaf morphology measurements (petiole length, blade length and blade width) were recorded from two randomly selected leaves on old growth (i.e. brown/black, not green, stem) and a DNA sample taken. Every kilometre a herbarium specimen was collected (if the removal of the specimen would not be detrimental to the survival of the individual) with a DNA sample and leaf morphology measurements. If a plant was not encountered within 500 m, the next individual found was sampled regardless of the distance from the nearest presence/absence sampling point.

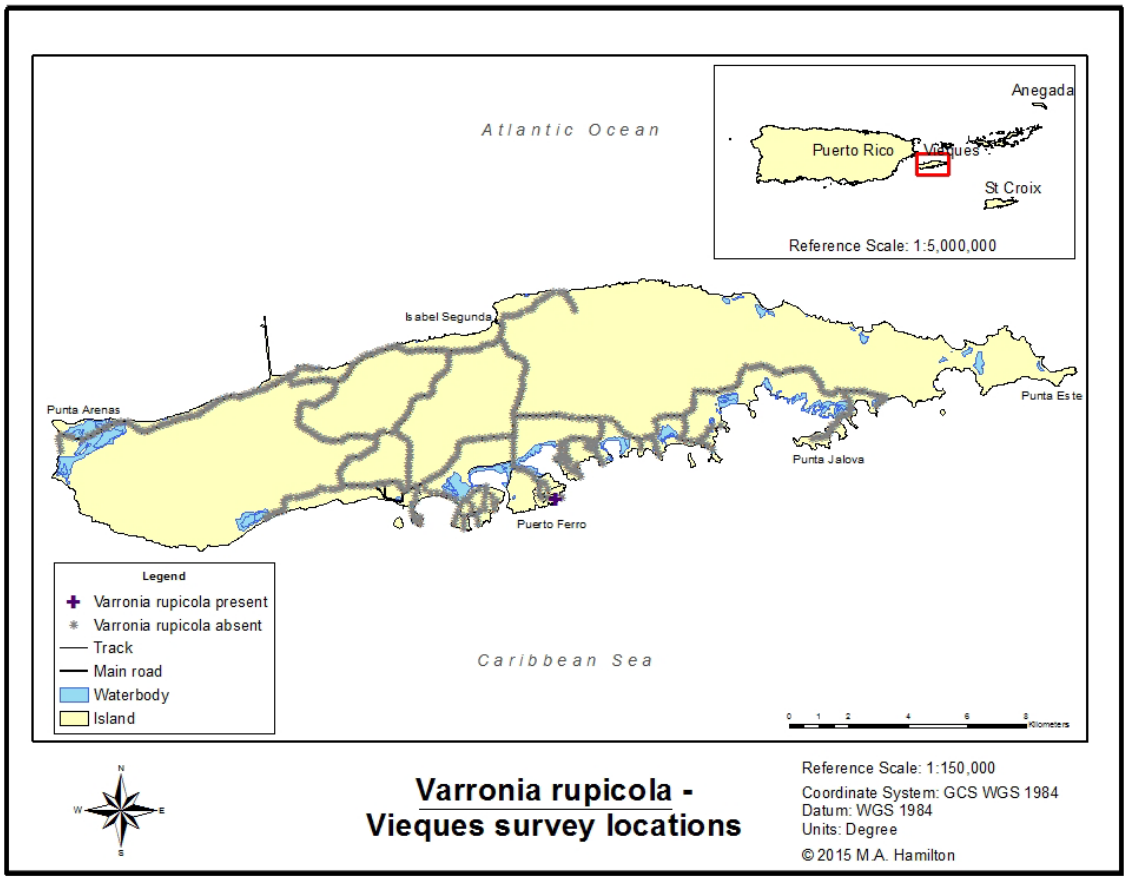

Figure 18: *Varronia rupicola* survey locations on Vieques showing species presence (purple plus) and absence (grey asterisk) recording locations.

On the islands of Vieques (Figure 18) and Puerto Rico (Figure 19), every known individual identified through recent survey work of Puerto Rican colleagues and historic records with precise locality information was sampled. To help ensure sampling consistency, presence/absence recording was undertaken along transects within a 10x10 m plot every 100 m; however, every new individual encountered was sampled due to the low number of extant individuals on Puerto Rico and Vieques. Sampling was undertaken in areas of suitable habitat

identified through discussions with Puerto Rican colleagues, interpretation of satellite imagery and ground-truthing of dry forest vegetation overlying limestone on the south-west coast of Puerto Rico and Vieques islands. Transects were also undertaken in areas and habitats not known to support the species to help reduce sampling bias. When an individual was encountered, adaptive sampling (Greenwood and Robinson, 2006) using 10x10 m plots was undertaken until no new individuals were encountered. All sampling on Puerto Rico and Vieques included collection of a DNA sample and recording leaf morphology measurements (petiole length, blade length and blade width) from two randomly selected leaves on old growth (i.e. brown/black, not green, stem). Herbarium voucher specimens were collected if the location was a new locality for the species and the removal of the specimen would not be detrimental to the survival of the individual.

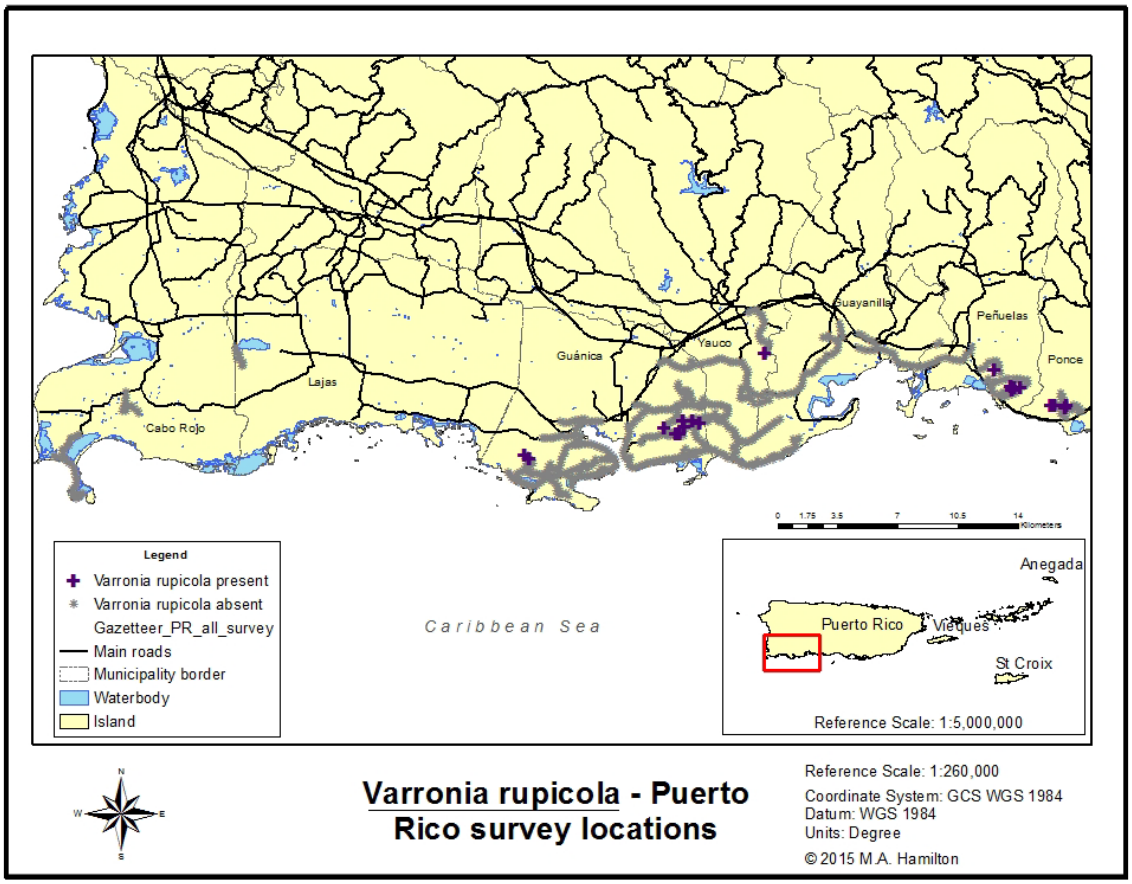

Figure 19: *Varronia rupicola* survey locations on Puerto Rico showing species presence (purple plus) and absence (grey asterisk) recording locations.

All survey data was captured using a handheld computer with built-in GPS (Trimble™ Juno® 3, Trimble Navigation Limited, Sunnyvale, CA, USA) running ArcPad® software (ESRI™, version 10.0, Redlands, CA, USA). Data collection and processing standards followed Hamilton (2008). Opportunistic species observations were recorded using a digital camera with built-in GPS (Sony™, model A77V, Tokyo, Japan). Images were processed using LightRoom® software (Adobe®, version 5.1, Seattle, Washington) and metadata was exported for use in BRAHMS

software using the plug-in LR/Transporter (developed by Timothy Armes). All field data were captured with GPS devices set to record the default geographic coordinate system (GCS) using World Geodetic System 1984 (WGS 1984) datum, jointly abbreviated as 'GCS WGS 1984'. Observation and sample data was incorporated into the BRAHMS software developed database for further processing and subsequent export for use in other software programs. All voucher specimens collected from wild plants are deposited in the herbarium (K) of the Royal Botanic Gardens, Kew with various duplicate vouchers lodged at other herbaria (see Appendix 1: *Varronia rupicola* records) and all DNA samples are deposited in the Kew DNA Bank (Royal Botanic Gardens, Kew, DNA Bank, http://www.kew.org/data/dnaBank/).

2.2.2. Mapping geology, land cover and sea level rise

Using ArcGIS® for Desktop software (ESRI™, version 10.1, Redlands, CA, USA), a GIS was developed with the GCS WGS 1984 map coordinate system using existing data sourced from the British Virgin Islands National GIS, USGS, USFS and the UKOTs Species and Specimens Database. Existing data available comprised layers for roads, boundaries (*i.e.* protected areas, municipalities and islands), waterbodies and geology as well as DEMs, processed land cover imagery and digital copies of printed geological and topographical maps. Field data generated by the author was added to the GIS following processing.

The existing available data varied considerably in scale, accuracy, resolution and content across the PRB; therefore, the author was required to undertake heads-up, or on-screen, digitising to create missing or uniform scale layers across the PRB. Much of the data variation was due to less accurate feature identification performed by recognition algorithms or on-screen digitising at low resolution that resulted in map scales greater than 1:50,000 which had limited use for the purposes of this research. Although heads-up digitising can be a very time consuming process, the results of visual interpretation by a user at high resolution is usually more accurate (Horning *et al.*, 2010).

Digitising was undertaken directly in the GIS using ArcGIS® software at 1:5000 or 1:100,000 map scale, as discussed in the following sections, or in Google Earth™ (Google Inc., Pro version 7.1, Mountain View, CA, United States). Google Earth™ digitising was undertaken using the Keyhole Markup Language (.kml file type) and imported into the GIS using ArcToolbox within ArcGIS® to convert .kml files into shapefiles (.shp), the native file type for vector data in ArcGIS®. Google Earth™ digitising was performed using a 500 m eye level to increase accuracy. Satellite imagery viewed in Google Earth™ for digitisation of features was provided by either Spot Image™ (Airbus Group NV, Leiden, Netherlands) or DigitalGlobe™ (DigitalGlobe Corporate, Westminster, CO, USA). Spot Image™ imagery provides a 2.5 m resolution and 10–

15 m positional accuracy for each pixel (Airbus Defence and Space, 2011) whereas DigitalGlobe™ imagery provides product resolution of 1m and positional accuracy of 5 m (DigitalGlobe, 2011, 2015). Recent studies by Mohammed *et al*. (2013) and Paredes-Hernández (2013) from India and Mexico, respectively, have shown that Google Earth™ imagery meets the American Society for Photogrammetry and Remote Sensing (ASPRS) positional accuracy standards for the production of ASPRS Class 1, 1:20,000 maps (ASPRS, 1990, 2015); however, archived imagery available from Google Earth™ was shown by Potere (2008) to have much less accurate positional values, especially for developing countries. Dates of images used in this research were recent, although they varied from island to island, as follows: Anegada (11/10/2011 to 26/03/2013); Vieques (25/10/2014 to 18/06/2015); and Puerto Rico (13/11/2014 to 10/02/2015).

Geology

Existing geological layers and digital copies of printed maps available in the GIS were compared for resolution and accuracy following any necessary georeferencing or re-projecting. Field survey data from this research was overlaid on the geological layers to determine which areas and types of geological deposits were of relevance to *V. rupicola*. Geological deposits that required digitisation in south-western Puerto Rico, Vieques and Anegada were all performed directly in the GIS at a 1:5,000 map scale using the maps listed in Table 3. *Varronia rupicola* records generated by this research were overlaid on the bespoke geological layers in the GIS to associate the underlying geology using the tool **'**Spatial join'. This enabled subsequent calculations for the number of records per geological type and map production showing the distribution of geological features. The results of the geological mapping are presented in 2.3.1. Geology.

Table 3: Geological maps arranged by date and title pertaining to the native range of *V. rupicola* and used in this research to digitise limestone and other geological deposits on the islands of Anegada, Puerto Rico and Vieques.

Author(s)	Date	Title	Scale	Area covered
Briggs and Akers	(1965)	USGS Map HA-197	1:240,000	Puerto Rico (country)
Howard, J.	(1970)	Reconnaissance geology of Anegada Island	1:75,000	Anegada Island
Glover and Mattson	(1973)	USGS Map I-735	1:20,000	Rio Descalabrado Quadrangle
Learned *et al.*	(1973)	USGS Map 73-155	1:30,000	Vieques Island
Krushensky and Monroe	(1975)	USGS Map I-863	1:20,000	Ponce Quadrangle
Monroe, W.	(1976)	USGS Map 899	1:140,000	Puerto Rico Island
Glover *et al.*	(1977)	USGS Map MF-886	1:20,000	Playa de Ponce and Santa Isabel Quadrangles
Krushensky and Monroe	(1978)	USGS Map I-1042	1:20,000	Peñuelas and Punta Cuchara Quadrangles

Author(s)	Date	Title	Scale	Area covered
Krushensky and Monroe	(1979)	USGS Map I-1147	1:20,000	Yauco and Punta Verraco Quadrangles
Monroe, W.	(1980)	USGS Map 953	1:160,000	Puerto Rico Island
Volckmann, R.P.	(1984a)	USGS Map I-1557	1:20,000	Cabo Rojo-Parguera Quadrangle
Volckmann, R.P.	(1984c)	USGS Map I-1558	1:20,000	San German Quadrangle
Volckmann, R.P.	(1984b)	USGS Map I-559	1:20,000	Puerto Real Quadrangle
Bawiec, W.J.	(1999)	USGS Map 98-038	1:100,000	Puerto Rico (country)
Renken *et al.*	(2002)	USGS Map 1419	1:100,000	Puerto Rico (country)
Addarich-Martínez, L.	(2009)	The geologic mapping and history of the Guánica Quadrangle	1:20,000	Guánica Quadrangle
Atwater *et al.*	(2012)	Geomorphic evidence for an unusual tsunami a few centuries ago at Anegada	1:75,000	Anegada Island
Spiske and Halley	(2014)	A coral-rubble ridge as evidence for hurricane overwash, Anegada	1:75,000	Anegada Island

Land cover

Regional habitat types raster data of Kennaway & Helmer (2007) for Puerto Rico and Vieques and Kennaway *et al.* (2008) for BVI (see Figure 15) were converted to polygons using the 'Raster to Polygon' tool in ArcToolbox and then merged to create a PRVI regional habitat vector layer. Habitats from the regional habitat layer for the islands of Puerto Rico (including southern offshore cays), Vieques and Anegada were exported to generate a *V. rupicola* habitats layer. All *V. rupicola* records generated by this research were overlaid on the habitats layer to assign the appropriate regional land cover classification to each species record using 'Extract values to points' in ArcToolbox. All records of *V. rupicola* generated by this research were also overlaid on the detailed habitat types raster data of Gould *et al.* (2008) for the islands of Puerto Rico and Vieques and Kennaway *et al.* (2008) for Anegada to assign the appropriate detailed land cover classification to each record using 'spatial join' in ArcToolbox.

For Puerto Rico, any habitat occurrences other than 'Mature secondary lowland dry limestone semi-deciduous forest' of Gould *et al.* (2008) were examined to determine if the occurrence was due to the positional accuracy error of the GPS unit (± 10 m) or the pixel size of the habitat map. If necessary, corrections were made to the habitat classification following interpretation of the data in ArcGIS™ and/or Google Earth™. This process was repeated for each island where incorrect habitat types (e.g. 'golf courses' on Anegada) were associated with any *V. rupicola* records. These steps enabled the calculation of number of records per habitat type on each island.

Historical agriculture features on Anegada were digitised at a 1:5,000 map scale directly in the GIS from a Directorate of Overseas Surveys (DOS) digitised map (DOS, 1966) following georeferencing. The Anegada 1/12 arc-second topographic DEM was used to digitise further historical agriculture features near East End not captured in the DOS map. All *V. rupicola* records generated by this research from Anegada were overlaid on the newly created historical agriculture features layer to explore the impact of historical anthropogenic disturbance on the species.

Modern anthropogenic disturbance across the native range of *V. rupicola* and waterbodies, permanent and seasonal, on Anegada were digitised in Google Earth™ and then imported into the GIS. The latter features were used strictly for mapping purposes, whereas the former were used to explore the impact of current anthropogenic disturbance on the species habitat. This was undertaken by first converting the detailed habitat types raster data of Gould *et al.* (2008) for the islands of Puerto Rico and Vieques and Kennaway *et al.* (2008) for Anegada to polygons using the 'Raster to Polygon' tool in ArcToolbox. The new detailed habitat layers were then clipped to the relevant geology layer(s) for each island. Finally, the anthropogenic feature layers were used to erase the overlaid areas between the 2000 detailed habitat types and the modern anthropogenic features. This resulted in a layer depicting the modern extent (*i.e*. 2014) of the detailed habitat types that support *V. rupicola* per geology type.

The global data set of forest change available from Hansen *et al.* (2013) was subsequently added to the GIS. These raster data were converted to polygons and then clipped to geological layers created during this research to enable calculations to be made for forest change across the native range of *V. rupicola* as a comparison to the values derived from heads-up digitisation undertaken in this research.

The modern extent of the detailed habitat types that support *V. rupicola* were finally clipped to the existing and proposed protected areas found across the PRB to enable calculations of remaining habitat within protected areas. The results of the methods above and maps produced will be presented in 2.3.2. Land cover.

Sea level rise

The Anegada Island coastline available in the GIS did not provide the required accuracy for this research and was, therefore, recreated using Google Earth™ before importing into the GIS. The Anegada 1/12 arc-second topographic DEM (Figure 20) was then used to make refinements to the coastline based on Lidar recorded elevation data. All other coastlines, supplied by USFS, were found to be of adequate accuracy for sea-level rise mapping. All *V. rupicola* records made during this research were overlaid on the available DEMs (raster data) for each island to record

the precise elevation using the tool 'Extract values to points'. This enabled elevational ranges to be established for the species on each island.

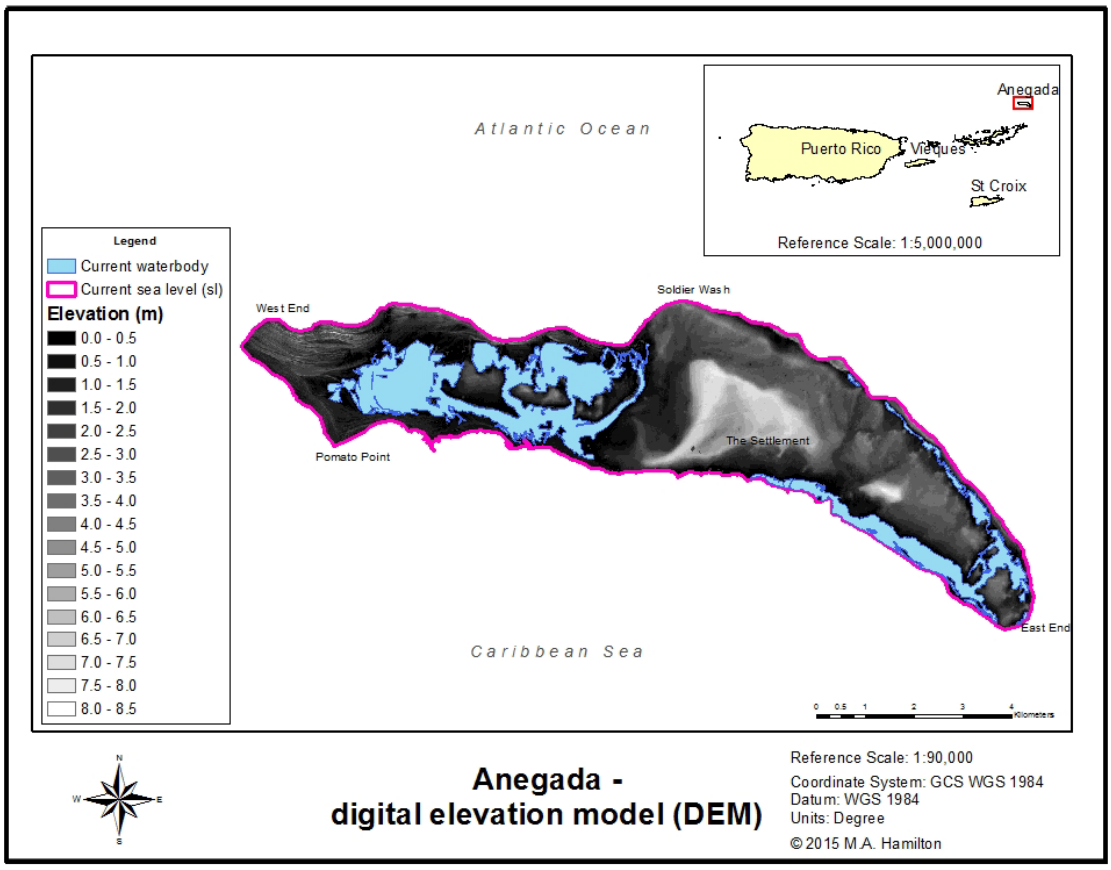

Figure 20: Anegada 1/12 arc-second (2.5 m) digital elevation model (DEM) showing 0.5 m classification as well as current sea level and waterbodies.

Past sea level rise

The USGS produced bathymetric and topographic 1 arc-second (30 m) DEMs (see Figure 16) for the country of Puerto Rico (Taylor *et al.*, 2008) and the Virgin Islands (Love *et al.*, 2015) were jointly classified in the GIS to exclude all data below -120m and above 0m of current sea level with four natural breaks using -50 m, -25 m, -3 m and 0 m to depict sea level rise across the PRB since the end of the Last Glacial Maximum (see Table 5). Using these jointly classified 1 arc-second DEMs, sea level boundaries at -120 m for the PRB and St Croix were digitised at 1:100,000 scale before a past sea level rise map was produced.

Future sea level rise

The four scenarios used by Bellard *et al.* (2014) and based on published sea level rise studies by Overpeck *et al.* (2006), Hansen *et al.* (2007), Rahmstorf (2007), Pfeffer *et al.* (2008), Grinsted *et al.* (2010), Nicholls and Cazenave (2010) and Wetzel *et al.* (2012) were used to explore the potential impacts of future sea level rise (2100 to 2300) on *V. rupicola* habitat in the PRB. This was undertaken using the USGS produced bathymetric and topographic 1 arc-second (30 m) DEMs (see Figure 16) for the country of Puerto Rico (Taylor *et al.*, 2008) and the

Virgin Islands (Love *et al.*, 2015) as well as the 1/12 arc-second (2.5 m) DEM for the island of Anegada (DDM, 2014). The three DEMs were jointly classified in the GIS to exclude all data below 0 m of current sea level with five natural breaks using 1 m, 2 m, 3 m, 6 m and 1330 m asl (the maximum height in the DEMs) to depict the future sea level rise scenarios across the PRB between 2100 and 2300.

The Anegada 1/12 arc-second topographic DEM was also used to produce a future sea level rise map using the four IPCC RCP scenarios for 2100 (IPCC, 2013a). The DEM was classified in the GIS to exclude all data below 0 m of current sea level with five natural breaks using 0.44 m (RCP2.6), 0.53 m (RCP4.5), 0.55 m (RCP6.0), 0.74 m (RCP8.5) and 8.6 m (highest point in DEM). The IPCC scenarios were not explored for the rest of the PRB due to the resolution of the DEMs available being too course for the RCP sea level rise values.

2.3. Results

To determine the species current extant range and how this differs from its historical native range, all available historical records and the survey data gathered by this research were used. Based on examination of 41 historical vouchers, *V. rupicola* has been recorded along the south-west coast of the island of Puerto Rico in the municipalities of Guánica, Guayanilla, Peñuelas and Ponce; however, the localities of many of these records are poorly defined. The species has also been recorded on Vieques Island at two locations, Punta Jalova and Puerto Ferro, and is only known from the latter location in recent records. On Anegada, the species is found across the island and predominantly recorded on the western side.

Survey at 1940 points was undertaken between 2012 and 2015 with *V. rupicola* present at 179 (9%) points. On the island of Vieques, the species was only found present at one recording point, whereas the islands of Puerto Rico and Anegada had considerably more presence records with 26 and 152, respectively. In total, 822 observations (presence and opportunistic records) were made across the native range of the species between 2012 and 2015 with observations on Vieques (n = 6), the island of Puerto Rico (n = 165) and Anegada (n = 651) varying considerably (see Table 4 and Appendix 2: Survey points by substrate and regional habitat class).

Table 4: The total number of survey points, absence records and presence records as well as all observations (presence and opportunistic records) recorded per island for *Varronia rupicola* between 2012 and 2015.

Island	Survey points	Absence records	Presence records	All observations
Anegada	666	514	152	651
Vieques	514	513	1	6
Puerto Rico	760	734	26	165
Total	**1940**	**1761**	**179**	**822**

Review of the locality information for all observations recorded during this research found that the species is extant across the PRB where it grows on the islands of Puerto Rico (south-western coastal municipalities of Guánica, Yauco, Peñuelas and Ponce) and Vieques (Puerto Ferro) in the country of Puerto Rico and across the island of Anegada in the British Virgin Islands (Figure 21).

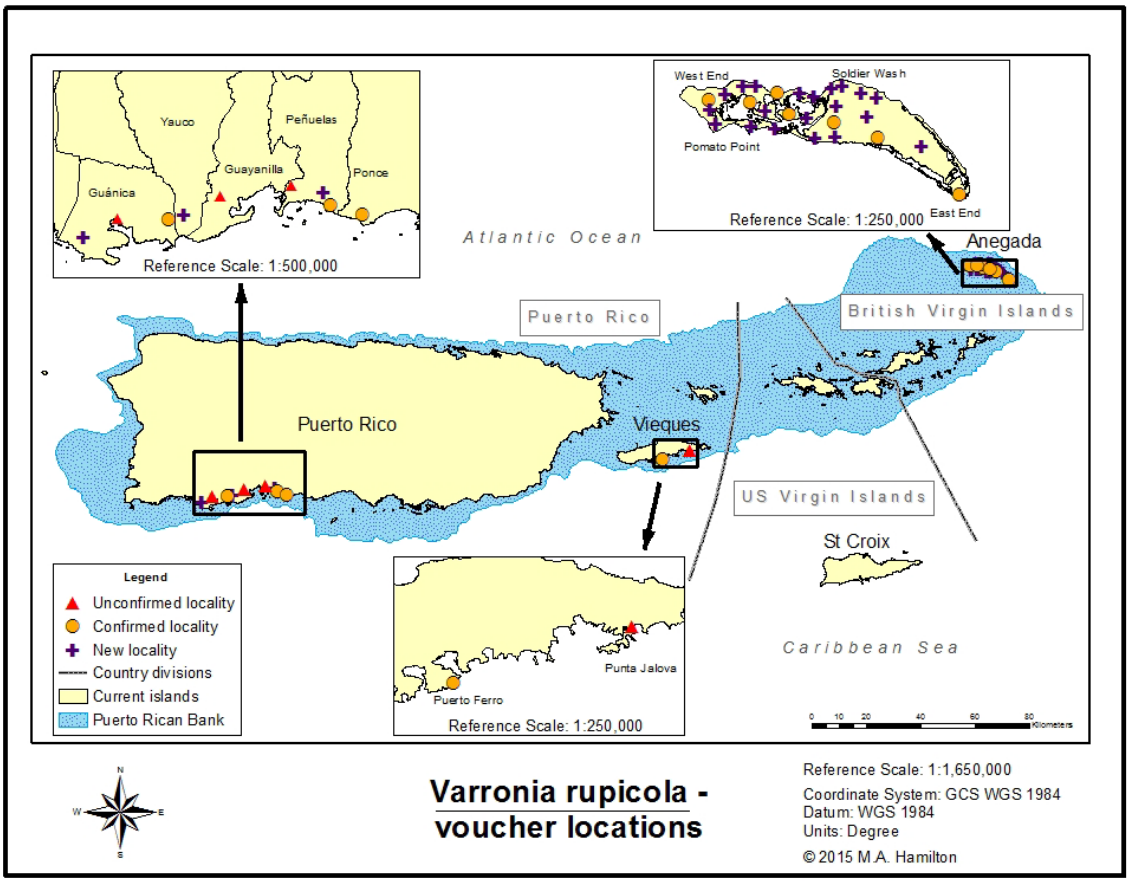

Figure 21: Map showing unconfirmed localities (red triangle) from records made prior to 2012 as well as confirmed localities (orange circle) where historical (pre-2012) and recent (2012-2015) records have been made and new localities (purple plus) where records were made between 2012 and 2015.

Within the Puerto Rican municipalities, the species is currently known to occur in specific wards as follows: Guánica municipality in Carenero and Montalva wards; Yauco municipality in Barina ward; Peñuelas municipality in Tallaboa and Encarnación wards; and Ponce municipality in Canas ward. No records were made during the course of this research in the Guayanilla municipality where significant developments (*i.e.* highway and oil refinery) have occurred in the past half-century. The species was only found at a single location on the limestone peninsula of Puerto Ferro on Vieques Island. Across the island of Anegada, the species has been recorded at 27 localities: Bones Bight, Bones Low Point, Bumber Well Cay, Capt. Auguste George Airport, Citron Bush, Cow Wreck Bay, East End, Flamingo Pond, Jack Bay, Keel Point, Low Cay, Middle Cay, North Raibin Slob, Nutmeg Point, Pearl Point, Pomato Point, Sambeal

Slob, Setting Point, Soldier East Point, Soldier Point, Saltheap Point, The Settlement, Vagabond Pond, Warner, West End, Windlass Bight and Windlass Low Point. Of particular note is a >1.75 km gap between observations in the centre of the island. The native range will be explored further in 2.4. Discussion and conclusions.

2.3.1. Geology

Review of available literature and subsequent digitisation of published maps were used to identify any specific substrates on which *V. rupicola* plants occur, where these substrates are located and how much area the substrates cover across the species native range. The production of a dated list of significant events relating to the formation of the Puerto Rican Bank and deposits of limestone across geological time (see Table 1) was followed by identification of relevant geological deposits. Considerable variation exists in the quality and quantity of information available for the relevant geological deposits across the native range of the species. Discrepancies between the USGS 1:20,000 quadrangle maps for the island of Puerto Rico were observed at several map intersections and required courser scale maps to be used in order to determine appropriate adjustments for the intersecting areas. A detailed USGS map for the Guánica Quadrangle is lacking which required the use of Addarich-Martínez (2009) that has not been adopted by the USGS. Considerable difference was observed between Addarich-Martínez (2009) and courser scale USGS maps (Briggs and Akers, 1965; Monroe, 1980; Bawiec, 1999), particularly in the Montalva ward. The limestone deposition of Monroe (1980) was followed for the Guánica Quadrangle given the focus of that author in the area over a long period of time.

On Anegada, *V. rupicola* plants were found to be growing on Pleistocene limestone and Quaternary deposits of sand. A total of 157 records were made on the later and 494 on the former. No *V. rupicola* plants were found on Quaternary deposits outside Anegada. Pleistocene limestone deposits above current sea level on Anegada total 19.80 km^2 (Figure 22). The remaining land area is covered by 7.43 km^2 of Quaternary sand deposits where *V. rupicola* plants have been recorded and 4.38 km^2 of Quaternary alluvium deposits that are not known to support the species.

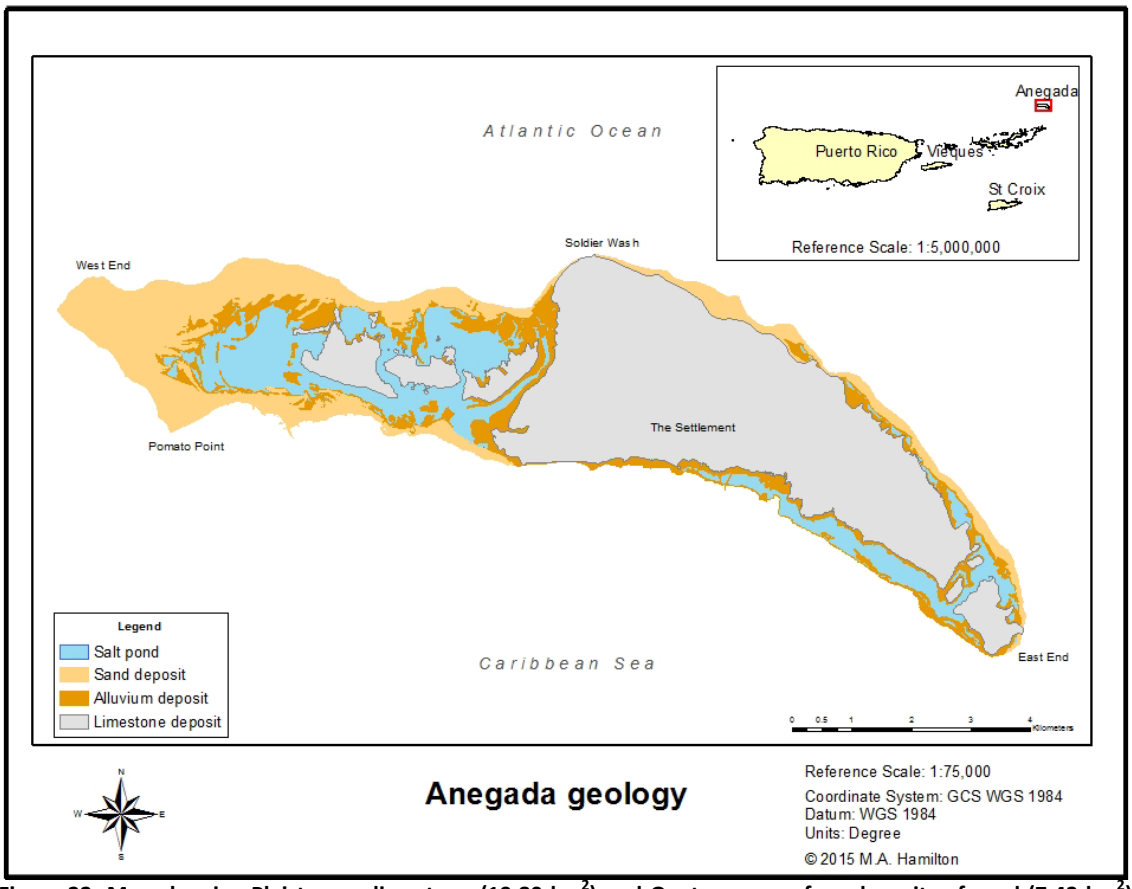

Figure 22: Map showing Pleistocene limestone (19.80 km^2) and Quaternary surface deposits of sand (7.43 km^2) and alluvium (4.38 km^2) on the island of Anegada.

On the island of Vieques, the limestone formation that supports *V. rupicola* was deposited during the Pliocene and is limited to the central part of the southern coast and the eastern tip of the island (Figure 23). The Pliocene limestone deposits on Vieques total 6.28 km^2. Various other deposits, not known to support the species, cover the remainder of the island. Pliocene limestone is not found in the Punta Jalova area; however, there are Quaternary surface deposits of sand along the coast in adjacent bays.

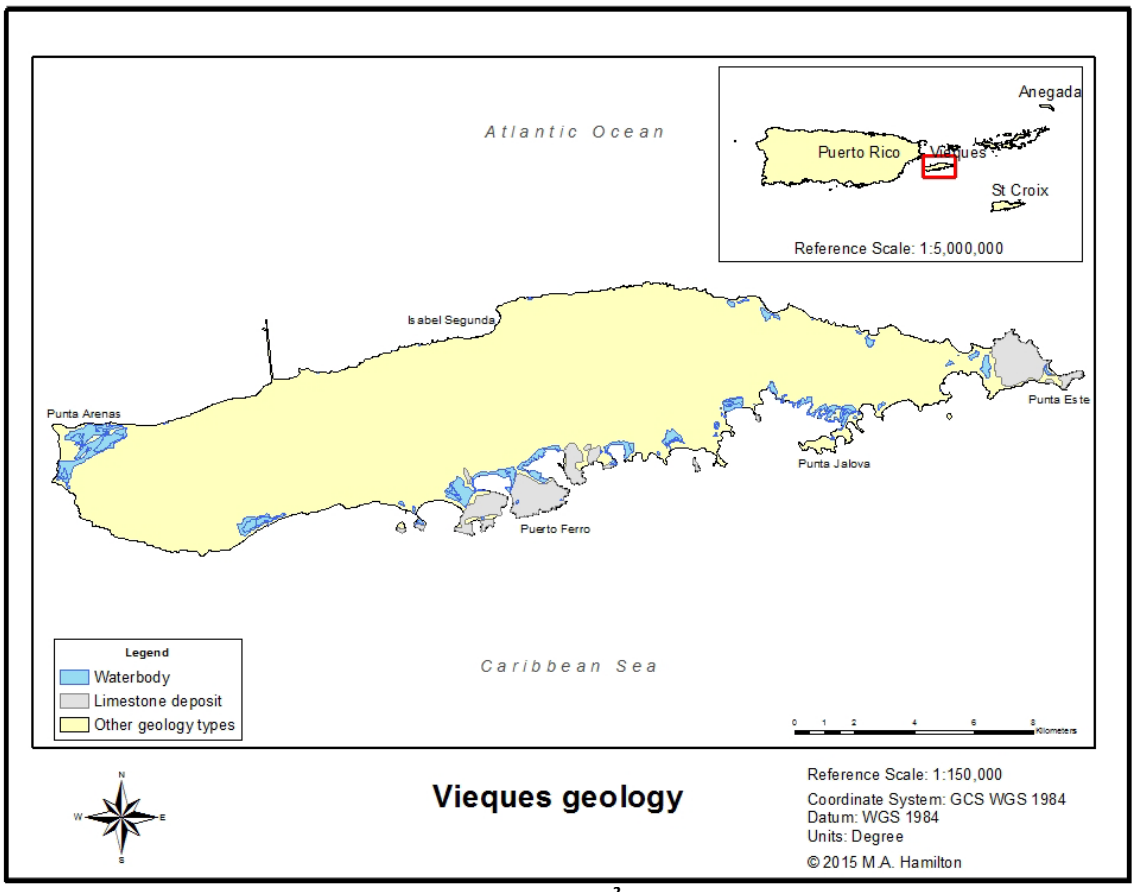

Figure 23: Map showing Pliocene limestone deposits (6.28 km^2) on the island of Vieques.

The southern limestone formations on the island of Puerto Rico (Figure 24) that support *V. rupicola* were deposited during the Neogene period with Juana Diaz limestone forming early in the Miocene followed by the formation of Ponce limestone later in the same epoch (Krushensky and Monroe, 1975; Addarich-Martínez, 2009). The total area of Miocene limestone formations that are known to support *V. rupicola* plants is only 165.82 km^2 with 67.83 km^2 of Juana Diaz limestone and 97.99 km^2 of Ponce limestone. The latter occurs in nine municipalities along the south-western coast of Puerto Rico from Santa Isabel in the east to Cabo Rojo in the west. Juana Diaz limestone is restricted to only six of these municipalities, Juana Diaz to Guánica, from east to west. Neither of these limestone formations nor Quaternary surface sand deposits is known to occur in the locality described on the Woodbury (1959) collection from Ensenada ward of Guánica municipality.

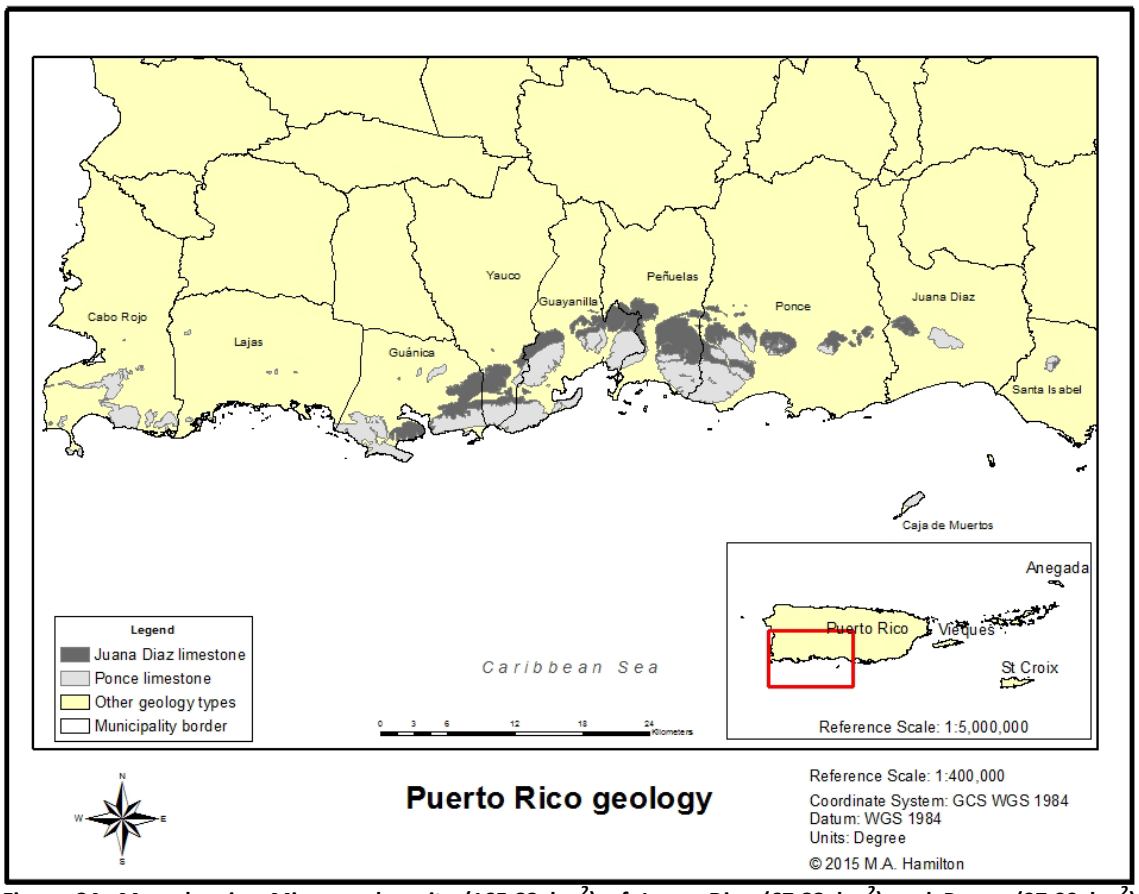

Figure 24: Map showing Miocene deposits (165.82 km^2) of Juana Diaz (67.83 km^2) and Ponce (97.99 km^2) limestone on the island of Puerto Rico.

2.3.2. Land cover

To determine if *V. rupicola* plants are associated with any specific land cover types overlying the substrates supporting the species across its native range and if the species is found within protected areas (proposed or established), compilation of existing data, digitisation of new features and incorporation of field survey data was undertaken. This was followed by data manipulation and extraction using the GIS developed for this research. These steps enabled the identification of specific land cover types, where these are located and how much area they cover as well as how much area of the land cover types known to support *V. rupicola* plants occur within protected areas.

The examination of historical records suggests that the species typically occurs in areas of dry forest that are intact or with only moderate disturbance. Using survey and observational data derived from this research and the detailed habitats of Kennaway *et al.* (2008) identified for Anegada in 2000, the habitat 'Deciduous, Evergreen and Mixed Forest and Shrubland with or without Succulents' totalled 18.34 km^2 in 2000, primarily on limestone (16.32 km^2), in which 479 *V. rupicola* observations were made. By 2014, this habitat had decreased to 17.09 km^2 with only 3.09 km^2 falling within the boundaries of the proposed protected areas on the island. The second highest number of observations (n = 100) were made in 'Evergreen Coastal

Shrubland' that totalled 3.60 km^2 in 2000 that mostly exists on sand (2.73 km^2). This habitat decreased to 2.96 km^2 by 2014 due mainly to a reduction in the area on sand to 2.12 km^2. Only 0.83 km^2 of this habitat falls within proposed protected areas. The remaining species records were from 'Low Density Urban', 'Pasture, Hay, Abandoned Agriculture or Other Grassy Areas' and 'High-Medium Density Urban' with 59, nine and four records, respectively. The intact, and obviously preferred, habitats of the species are 'Deciduous, Evergreen and Mixed Forest and Shrubland with or without Succulents' and 'Evergreen Coastal Shrubland' on the island of Anegada. These habitats classified in 2000, clipped to the underlying substrates of Pleistocene limestone and Quaternary sand deposits and overlaid with modern anthropogenic disturbances, are presented in Figure 25.

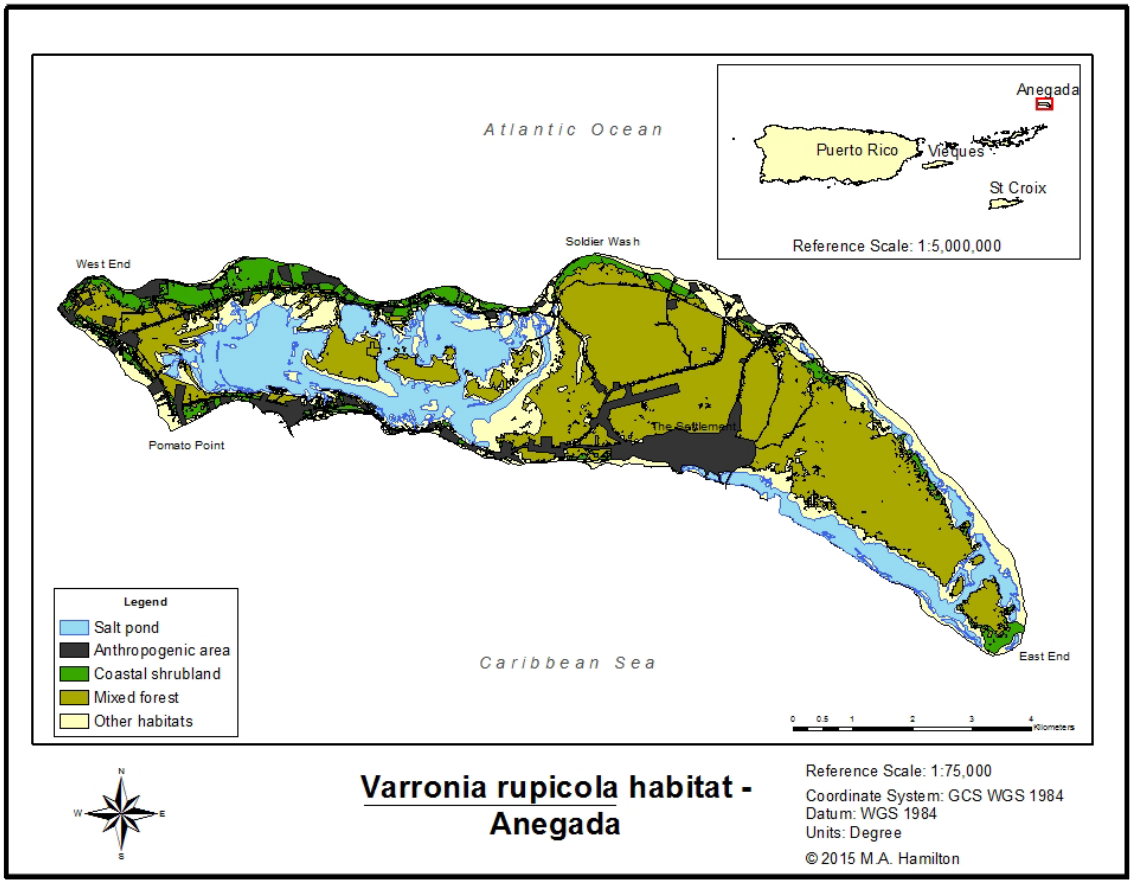

Figure 25: Map showing the two intact, refined habitat types of Kennaway *et al.* (2008), 'Coastal shrubland' (2.96 km^2) and 'Mixed forest' (17.09 km^2), in 2014 that support extant *Varronia rupicola* overlying Pleistocene limestone and Quaternary sand deposits on the island of Anegada along with anthropogenic disturbance digitised from 2011-2013 imagery. Note: 'Evergreen Coastal Shrubland' = 'Coastal shrubland'; 'Deciduous, Evergreen and Mixed Forest and Shrubland with or without Succulents' = 'Mixed forest'.

Extant plants (n = 6) on Vieques are only found within the Vieques NWR. All plants were recorded in the refined 'Mature secondary lowland dry limestone semi-deciduous forest' habitat type of Gould *et al.* (2008) at a single location**.** In 2000, the area of this habitat was a mere 3.55 km^2 and only 3.04 km^2 was found to overlie Pliocene limestone deposits. Between

2000 and 2014, the forest area decreased on Vieques to only 2.86 km^2. The 'Mature secondary lowland dry limestone semi-deciduous forest' habitat on the island of Vieques remaining in 2000, clipped to the underlying Pliocene limestone substrate and overlaid with modern anthropogenic disturbances, is presented in Figure 26.

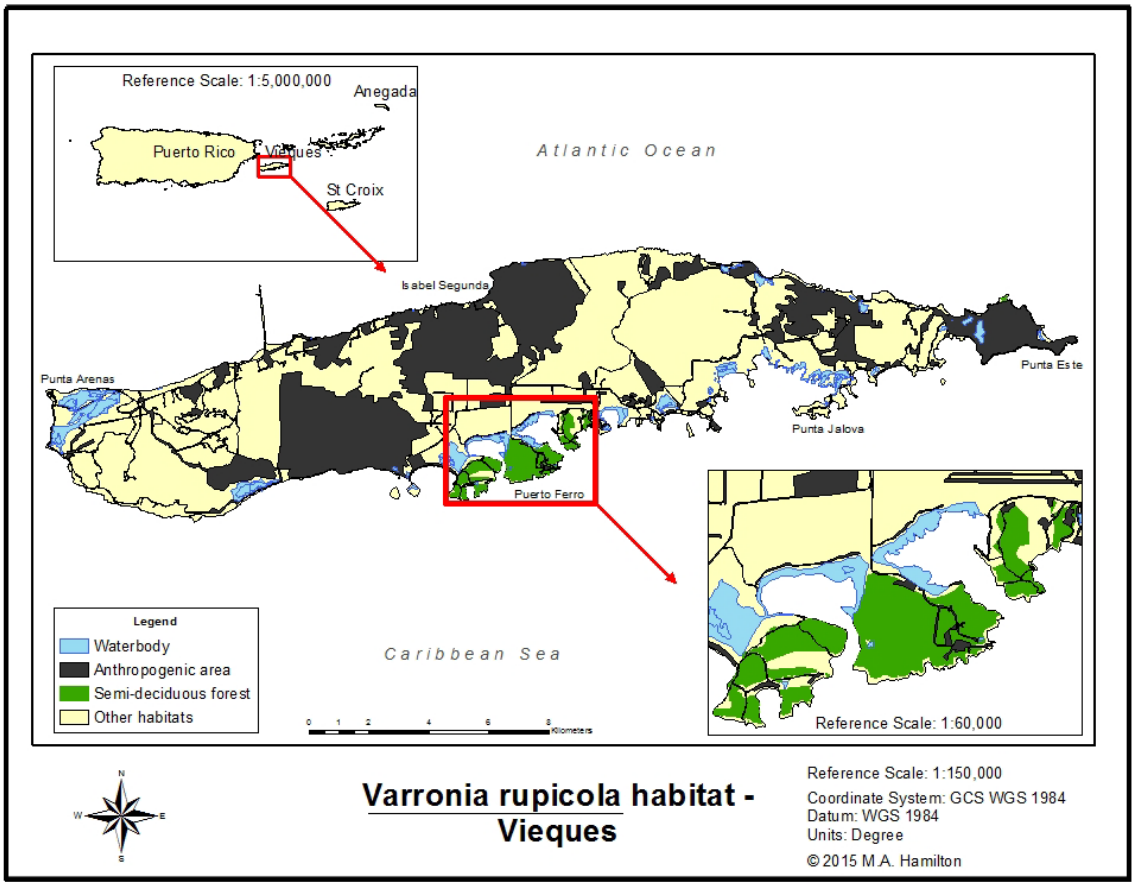

Figure 26: Map showing the only habitat type of Gould *et al.* (2008), 'Semi-deciduous forest' (2.86 km^2), in 2014 that supports extant *Varronia rupicola* overlying Pliocene limestone deposits on the island of Vieques with anthropogenic disturbance digitised from 2014-2015 imagery. Note: 'Mature secondary lowland dry limestone semi-deciduous forest' = 'Semi-deciduous forest'.

On the island of Puerto Rico, five sterile individuals, in the same locality, were found within 'Young secondary lowland dry limestone semi-deciduous forest' habitat type of Gould *et al.* (2008) that totalled 13.66 km^2 in 2000 on either Juana Diaz or Ponce limestone formations. All other *V. rupicola* plants on Puerto Rico Island, as on Vieques, were found in 'Mature secondary lowland dry limestone semi-deciduous forest' habitat type of Gould *et al.* (2008). On mainland Puerto Rico, 95.34 km^2 of the latter habitat was remaining in 2000; however, only 72.66 km^2 is found on either Juana Diaz or Ponce limestone formations with 33.64 km^2 and 39.02 km^2, respectively.

By 2014, the preferred habitat on either Juana Diaz or Ponce limestone formations had been further reduced on mainland Puerto Rico to 65.06 km^2. Both Juana Diaz and Ponce limestone

formations lost habitat during the period such that the remaining forest on each was 31.06 km^2 and 34.00 km^2, respectively. The 'Young secondary lowland dry limestone semi-deciduous forest' habitat type of Gould *et al.* (2008) had also decreased between 2000 and 2014 to 8.16 km^2 with 5.67 km^2 and 2.49 km^2 on Juana Diaz and Ponce limestone formations, respectively. The remaining area of the preferred habitat, 'Mature secondary lowland dry limestone semi-deciduous forest', on the island of Puerto Rico classified in 2000, clipped to the underlying Miocene limestone substrates and overlaid with modern anthropogenic disturbances, is presented in Figure 27.

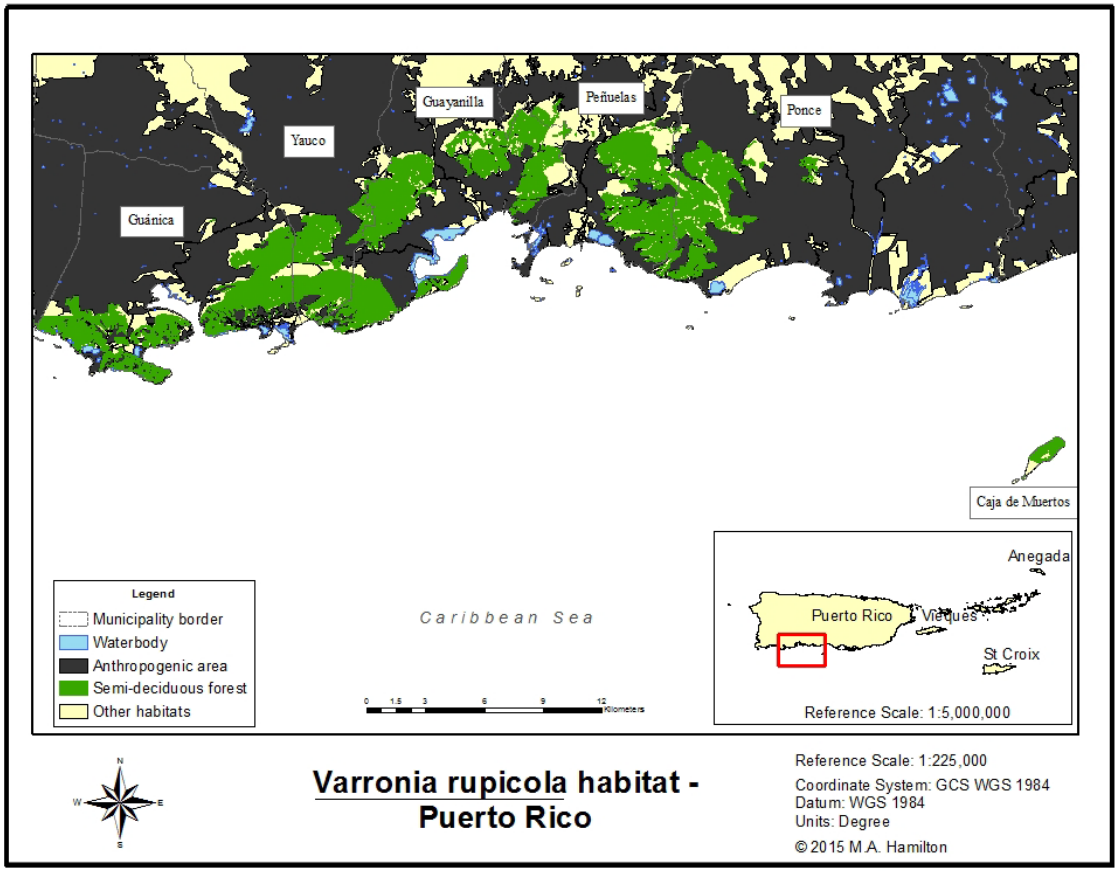

Figure 27: Map showing the intact habitat type of Gould *et al.* (2008), 'Semi-deciduous forest' (65.06 km^2), in 2014 that supports extant *Varronia rupicola* overlying Miocene deposits of Juana Diaz (forest area = 31.06 km^2) and Ponce (forest area = 34.00 km^2) limestone on the island of Puerto Rico with anthropogenic disturbance digitised from 2014-2015 imagery. Note: 'Mature secondary lowland dry limestone semi-deciduous forest' = 'Semi-deciduous forest'.

The largest amount of observed land development in Puerto Rico occurred in the limestone areas of the main island to the west of Ponce where 1.71 km^2 was developed between 2000 and 2014 across the Juana Diaz (0.69 km^2) and Ponce (1.02 km^2) limestone formations. This is 3% of the Juana Diaz and 4% of Ponce limestone formations in this area. Based on the area digitised by the author, 46.19 km^2 (47%) of the vegetation covering the Ponce limestone formation has been impacted by anthropogenic disturbance.

The area of preferred habitat found within protected areas across the PRB varies considerably between the islands. Only 3.09 km^2 of the total area (20.04 km^2) of the intact, preferred habitat on Anegada is proposed for protection (Figure 28). Conversely, the entire habitat on Vieques is within established protected areas; however, the Vieques NWR is undergoing extensive clearance and modification due to the remedial works to remove unexploded ordinance (UXO) within the reserve and anthropogenic impacts caused by recreational use in the reserve and the nearby DRNA protected area. Of the remaining forest overlying Juana Diaz or Ponce limestone formations on the island of Puerto Rico, 22.08 km^2 is found within existing protected areas. Of this, 8.42 km^2 is forest on Juana Diaz limestone and a further 13.66 km^2 is forest on Ponce limestone. The total area under protection includes the small (1.54 km^2), offshore cay of Caja de Muertos where *V. rupicola* has not been previously recorded. The cay includes 1.24 km^2 of Ponce limestone and 0.91 km^2 of 'Mature secondary lowland dry limestone semi-deciduous forest' (Gould *et al.*, 2008) under protection (see Figure 27).

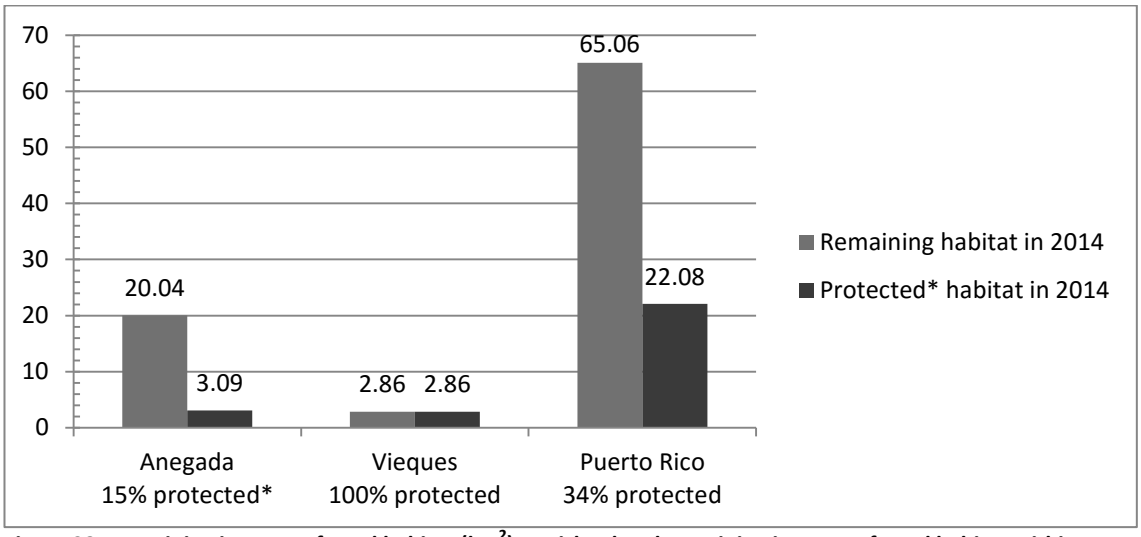

Figure 28: Remaining intact preferred habitat (km^2) per island and remaining intact preferred habitat within protected areas* (km^2) for *Varronia rupicola* per island across the PRB; Note: (*) denotes that the areas on Anegada are proposed for protection.

The regional habitat classifications of Kennaway & Helmer (2007) for Puerto Rico and Vieques and Kennaway *et al.* (2008) for BVI (see Figure 15) were used to undertake comparative analyses of the species habitat occurrence and preference across the PRB. 'Mature secondary lowland dry limestone semi-deciduous forest' of Gould *et.al.* (2008) is included in the broader 'Semi-Deciduous and Drought Deciduous Forest on Karst/limestone (includes semi-evergreen forest)' of Kennaway & Helmer (2007). All *V. rupicola* plants recorded on Puerto Rico (n = 165) and Vieques (n = 6) occur in the latter broad habitat type and only on limestone. Plants on Anegada fall into one of four regional habitat types of Kennaway *et al.* (2008): 'Deciduous, Evergreen Coastal and Mixed Forest or Shrubland with Succulents' (n = 579) across limestone (82%) and sand (18%); 'Low-Medium Density Urban' (n = 59) across limestone (20%) and sand

(80%); Pasture, Hay or Inactive Agriculture (n = 9) across limestone (78%) and sand (22%); and 'High-Medium Density Urban' (n = 4) only on sand.

The 'Deciduous, Evergreen Coastal and Mixed Forest or Shrubland with Succulents' habitat that the species occurs in on the island of Anegada was present on Puerto Rico (3.81 km^2) and Vieques (0.42 km^2) on deposits of limestone in 2000; however, the species has not been recorded in that habitat on the last two islands during this research. The 'Semi-Deciduous and Drought Deciduous Forest on Karst/limestone (includes semi-evergreen forest)' that the species occurs in on Vieques (2.79 km^2 in 2000) and Puerto Rico (105.48 km^2 in 2000) is not found on Anegada. The results of calculations for the number of *V. rupicola* observations per habitat and substrate using both the refined and regional classifications are provided in Appendix 3: *Varronia rupicola* observations made between 2012 and 2015.

The area (km^2) of intact forest habitats across the native range of *V. rupicola* was calculated for each island in 2000 and 2014 to determine the loss of habitat per island by substrate (Figure 29). Between 2000 and 2014, the largest percentage loss (12%) was experienced for intact forest on Anegada's Quaternary sand deposits while the largest area of loss was experienced on Puerto Rico's Ponce limestone where 5.02 km^2 (5% loss) was converted to other land cover types. These findings were subsequently compared to the global data set of forest change generated by Hansen *et al.* (2013).

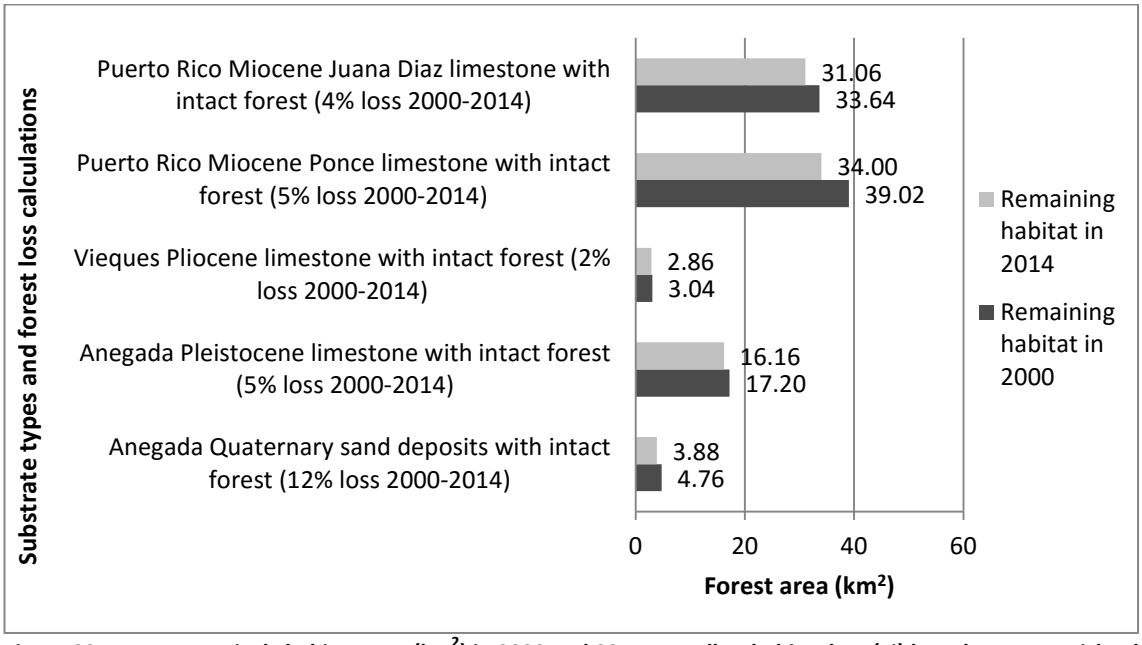

Figure 29: Intact *V. rupicola* habitat area (km^2) in 2000 and 2014 as well as habitat loss (%) by substrate per island between 2000 and 2014 using the regional classifications 'Semi-Deciduous and Drought Deciduous Forest on Karst/limestone (includes semi-evergreen forest)' of Kennaway & Helmer (2007) **for Puerto Rico and Vieques and 'Deciduous, Evergreen Coastal and Mixed Forest or Shrubland with Succulents' of Kennaway *et al.* (2008) for BVI.**

The Hansen *et al.* (2013) global data set of forest change provided a much less accurate measure of forest change across the native range of *V. rupicola* when compared to the values derived from heads-up digitisation of this research (Figure 30). The differences observed are easily explained by the course resolution (30 m) of the data used by Hansen *et al.* (2013).

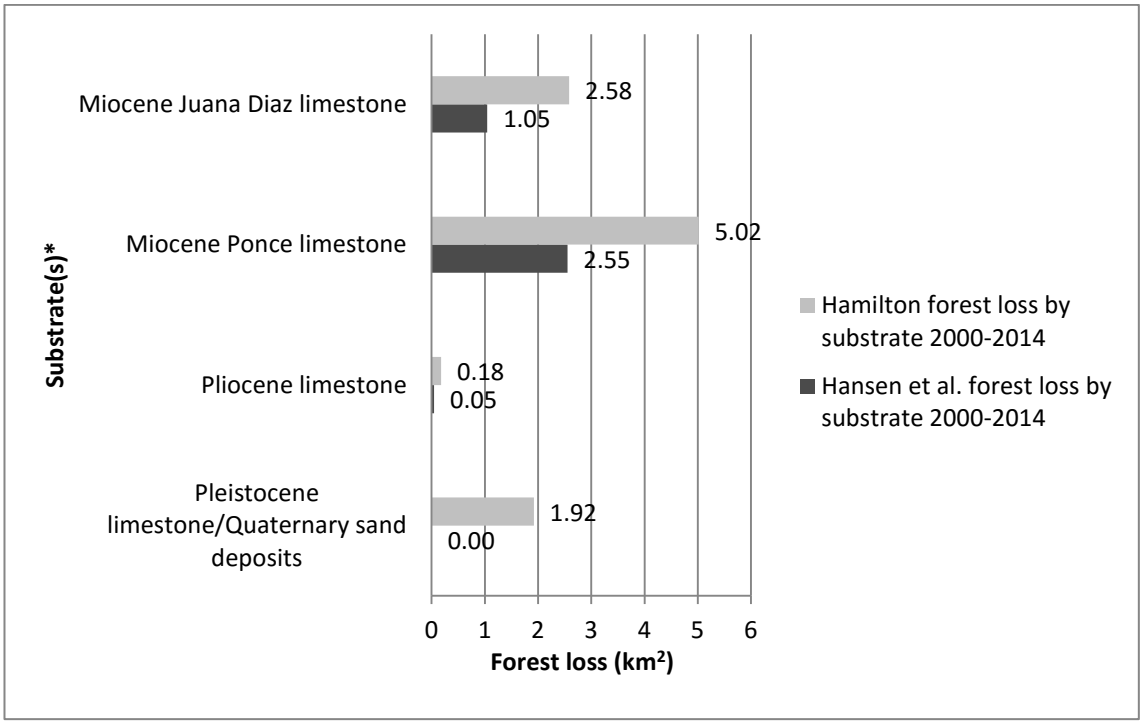

Figure 30: Comparison of the findings of this research compared to the global data set of Hansen *et al.* (2013) for forest loss between 2000 and 2014. Note: (*) denotes that substrates are classed as 1) Anegada Pleistocene limestone/Quaternary sand deposits, 2) Vieques Pliocene limestone, 3) Puerto Rico Miocene Ponce limestone and 4) Puerto Rico Miocene Juana Diaz limestone.

Historical agriculture features on the island of Anegada were digitised using a Directorate of Overseas Surveys (DOS) map (DOS, 1966) and the 1/12 arc-second topographic DEM for Anegada (DDM, 2014) to explore the impact of historical anthropogenic disturbance on the vegetation and specifically explore how this may have impacted *V. rupicola*. The majority of the agriculture features were in close proximity to The Settlement where the human population was traditionally centred. The digitised historical agriculture features on Anegada are shown in Figure 31 and further discussed in 2.4.2. Land cover.

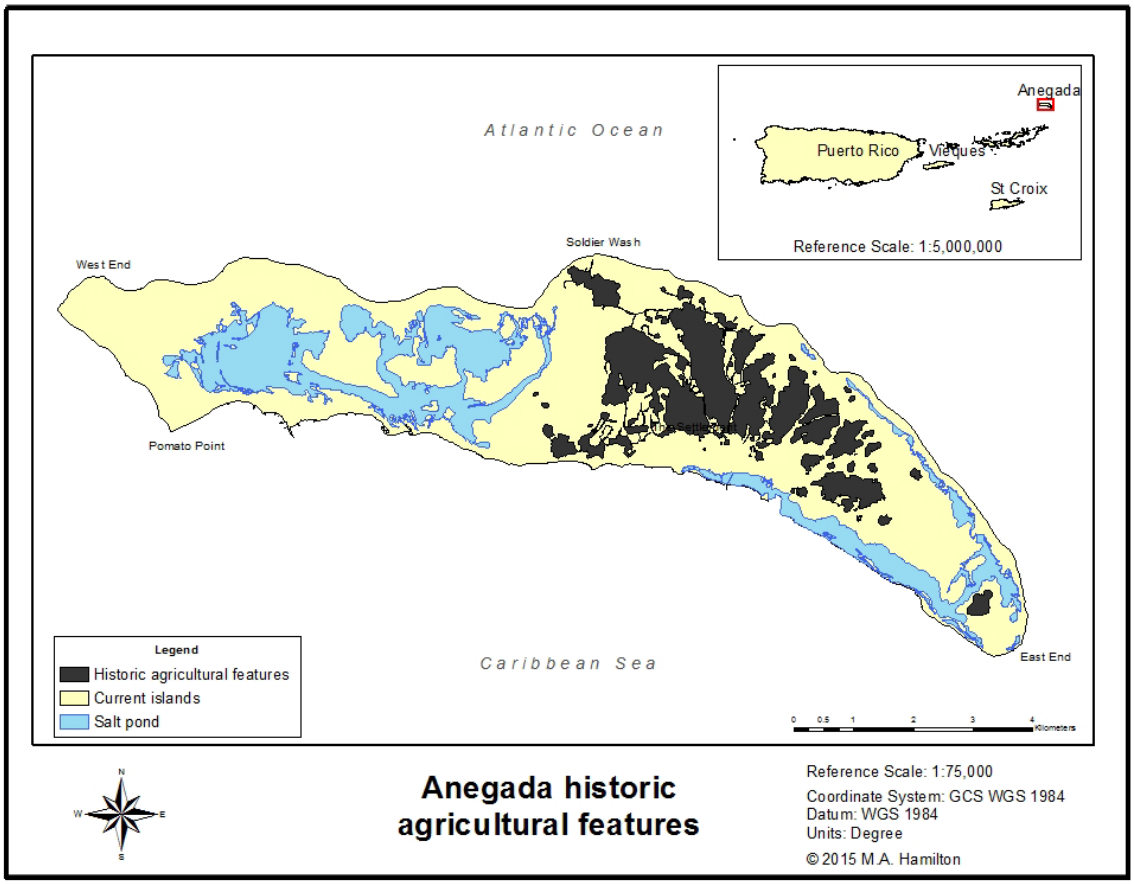

Figure 31: Map showing historical agriculture features on Anegada digitised from a Directorate of Overseas Surveys (DOS) printed map (DOS, 1966) and the Anegada 1/12 arc-second topographic DEM.

2.3.3. Sea level rise

The current total area of land across the three islands with *V. rupicola* populations is 8902.63 km^2. Puerto Rico is by far the largest island in the PRB with 8737.97 km^2. The other two islands, Vieques and Anegada, are considerably smaller with only 133.06 km^2 and 31.60 km^2, respectively. Based on examination of historical records, the species has been recorded below 200 m asl. This research found extant *V. rupicola* plants between 0.90 m and 7.45 m asl on Anegada, at a single location 12.00 m asl on the island of Vieques and at much higher elevations on the island of Puerto Rico where records ranged from 60.00 to 214.00 m asl (Figure 32). These results have potentially interesting links to the underlying geology as well as *V. rupicola* populations across the PRB and are discussed further in 4.4.4. Main conclusions.

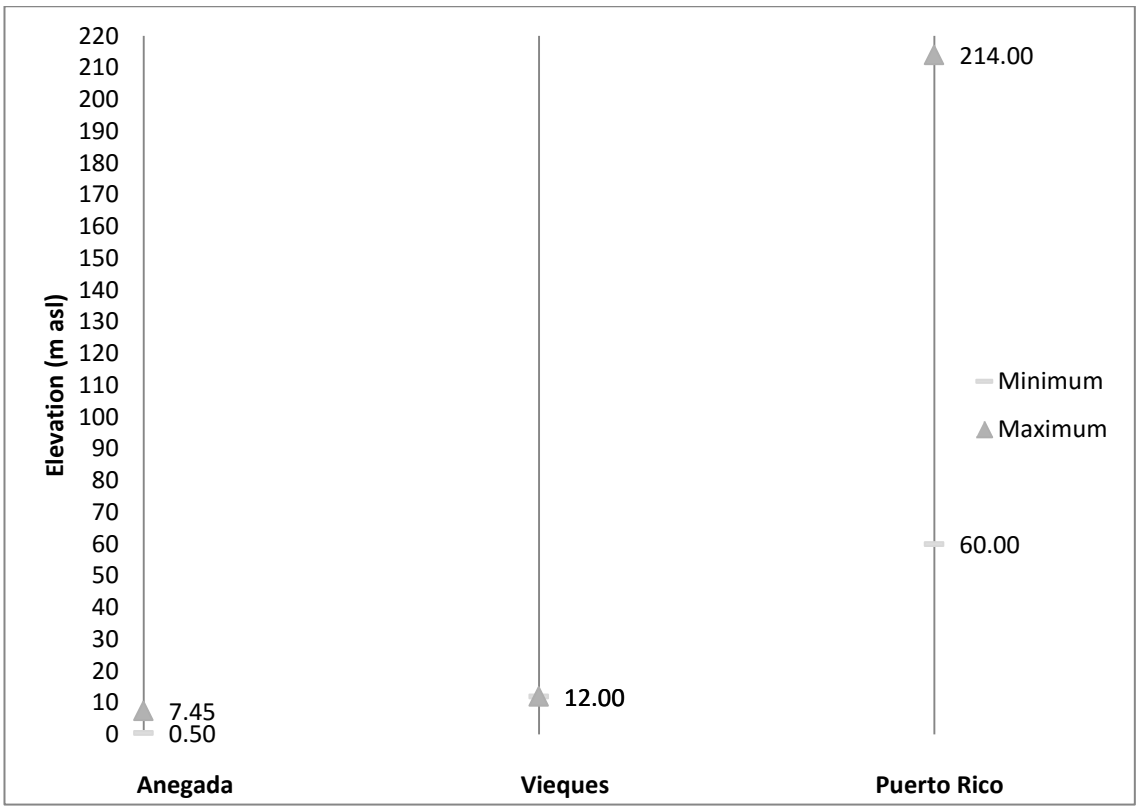

Figure 32: Altitudinal range of extant *Varronia rupicola* plants across the species native range on the islands of Anegada, Vieques and Puerto Rico with elevation shown in meters above sea level (m asl).

Past sea level rise

The fifth question posed for this biogeographic research, "What are the implications of past sea level rise on the native range of *V. rupicola*?", was addressed following extensive literature review, production of a dated sea level rise chronology of the PRB for the past 20,000 years (Table 5) and subsequent map production.

Table 5: Significant events related to sea level rise across the Puerto Rican Bank since the Last Glacial Maximum (LGM), ~20,000 years before present (k ybp) to the present. Note: the Pleistocene lasted from 2.588 mya to 11,700 years ago when the Holocene started.

Epoch	Start date	Sea level	Event	Reference
Holocene	~3k ybp	0 m	Relative sea level stability	Rhein *et al.*, 2013
	~7k ybp	-3 m	Islands isolated across PRB	Fleming *et al*., 1998
	~8.5k ybp	-25 m	Virgin Islands separated from Puerto Rico, Vieques and Culebra	Fairbanks, 1989
	~10k ybp	-50 m	Sea level had risen ~25m in 4k years; PRB single land mass for ~20k years	Fairbanks, 1989
Pleistocene	~14k ybp	-75 m	Sea level had risen ~25m in 3k years	Clark *et al.*, 2009;
	~17k ybp	-100 m	Sea level had risen ~20m in 3k years	Fleming *et al*., 1998
	~20k ybp	-120 m	Onset of ice sheets melting following LGM; PRB single land mass for ~11k years	Siddall *et al*., 2003

During the latter part of the Pleistocene, eustatic changes driven by glacial cycles led to the exposure of the entire Puerto Rican Bank as a single land mass (Figure 33). Sea level began to rise following the LGM due to the onset of the melting of the ice sheets. The PRB had been a single land mass for ca. 11,000 years at this point in time. By around 10,000 ybp, sea level had risen a further ca. 25 m (Fairbanks, 1989); therefore, the PRB would have been a single land mass for at least 20,000 years. Sea level rose a further ca. 25 m by ca. 8,000 ybp (Fairbanks, 1989) and separated the Virgin Islands from Puerto Rico, Vieques and Culebra. Over the next 1,500 years (ca. 7,000 ybp), another ca. 22 m sea level rise was experienced (Fleming *et al.*, 1998) that isolated the individual islands across the PRB with slightly more land area than today. The current islands of the PRB have had nearly the same land area for ca. 3,000 years. This is due to relative sea level stability with annual mean sea level rise rates increasing from a background of 1.7 mm from 1900 to 3.2 mm between 1993 and 2010 (Rhein *et al.*, 2013). The implications of these findings are discussed in 2.4.3 Sea level rise.

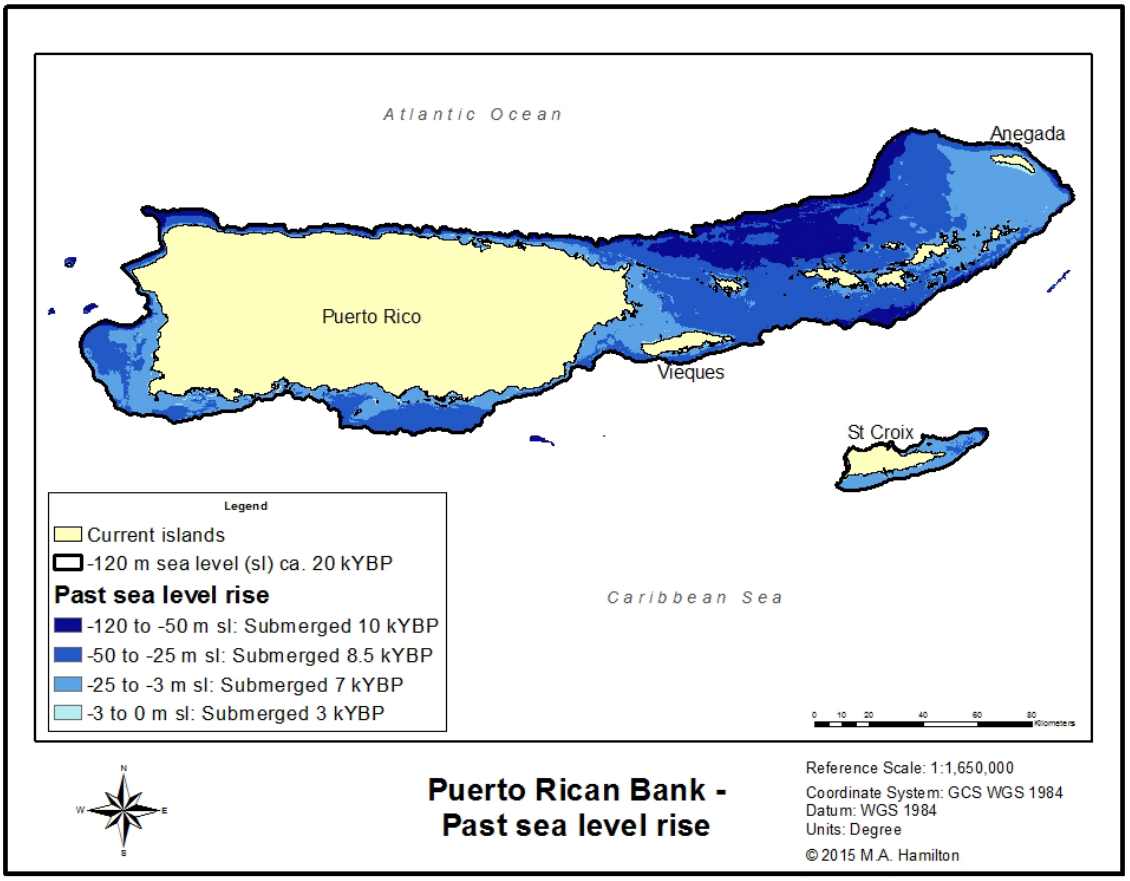

Figure 33: Sea level rise across the Puerto Rican Bank since the end of the Last Glacial Maximum (LGM) showing significant and dated sea levels: -120 m ca. 20,000 years before present (20 kYBP); -50 m ca. 10,000 years before present (10 kYBP); -25 m ca. 8,500 years before present (8.5 kYBP); -3 m ca. 7,000 years before present (7 kYBP); and 0 m ca. 3,000 years before present (3 kYBP).

Future sea level rise

Exploring the impact of proposed future sea level rise scenarios on the native range of *V. rupicola* required extensive desk study of this controversial and highly debated topic in order to develop appropriate sea level rise scenarios for 2100 and beyond to 2300 using available DEMs. Map production was undertaken to depict future sea level rise between 2100 and 2300 for the entire PRB (Figure 34) using the 1 m, 2 m, 3 m and 6 m scenarios of Bellard *et al.* (2014). Fortunately, the 1 m, 2 m, 3 m and 6 m scenarios for 2100 to 2300 of Bellard *et al.* (2014) will have no direct impact on extant *V. rupicola* plant locations on Puerto Rico or Vieques; however, these scenarios will have significant impact on the current locations of the species across Anegada. Individual maps for Anegada, Vieques and Puerto Rico are provided in 2.4.3 Sea level rise to illustrate the discussion.

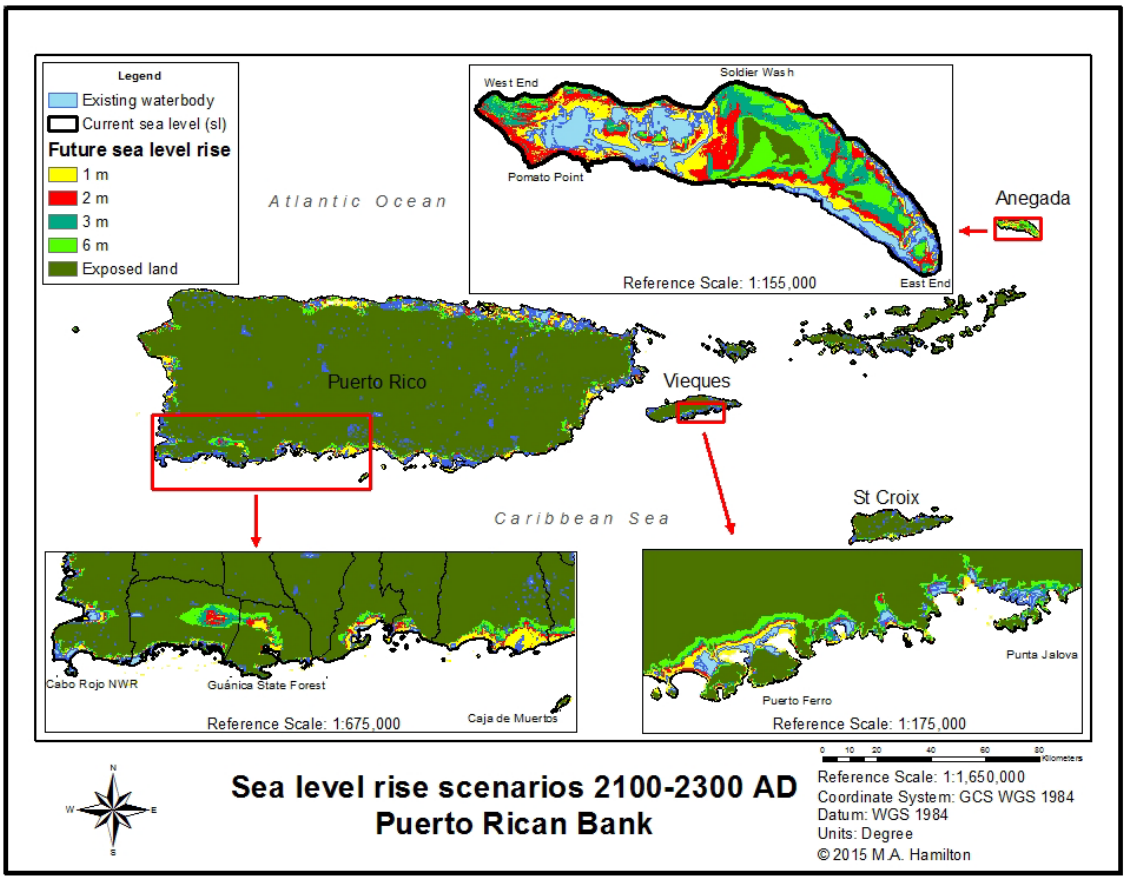

Figure 34: Map showing future sea level rise scenarios of 1 m, 2 m, 3 m, and 6 m for 2100 to 2300 AD (Bellard *et al.*, 2014) **across the Puerto Rican Bank with map insets showing impact to the native range of *V. rupicola*.**

The four IPCC RCP scenarios for 2100 (IPCC, 2013a) were only mapped for Anegada (Figure 35) as the resolution of the DEMs available for the rest of the PRB was too course for the RCP sea level rise values: 0.44 m (RCP2.6), 0.53 m (RCP4.5), 0.55 m (RCP6.0) and 0.74 m (RCP8.5).

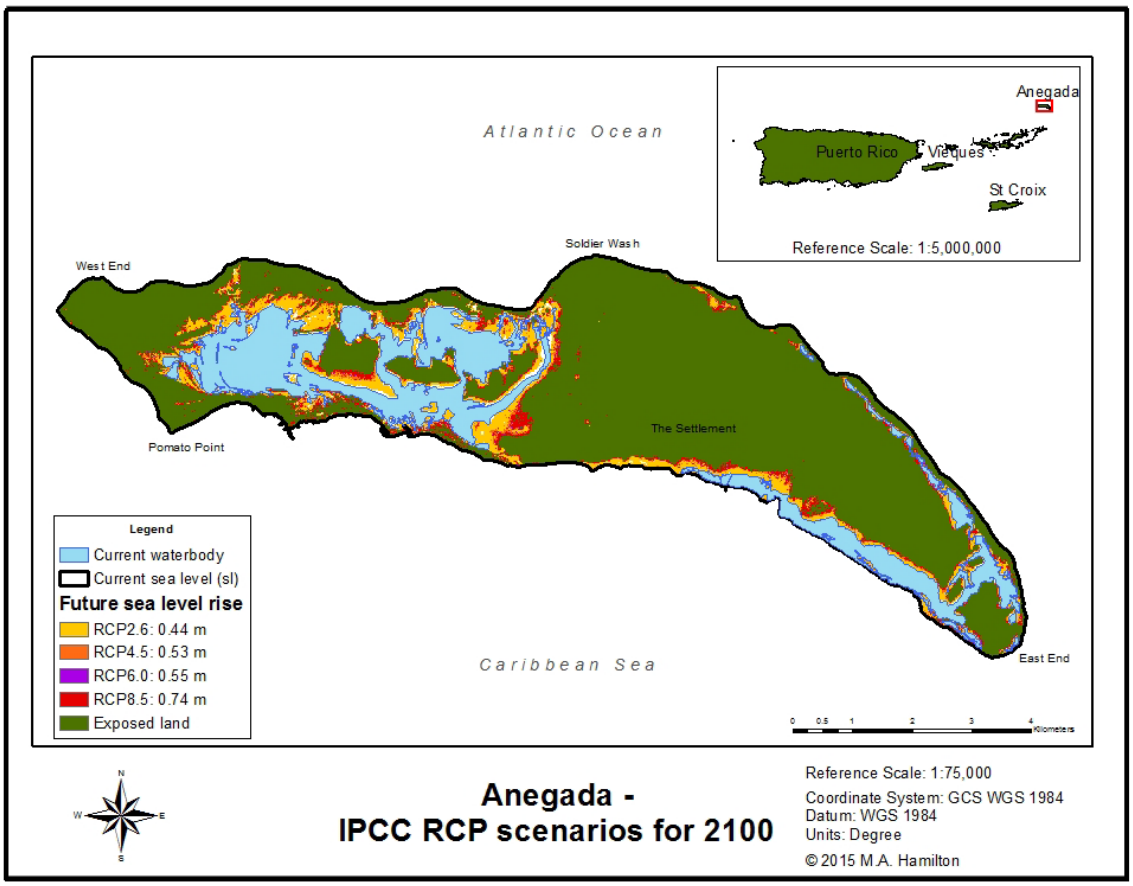

Figure 35: Map showing future sea level rise scenarios for 2100 AD on the island of Anegada using median values of the IPCC (2013a) **Representative Concentration Pathways (RCP): 0.44 m (RCP2.6), 0.53 m (RCP4.5), 0.55 m (RCP6.0) and 0.74 m (RCP8.5).**

The highest point on Anegada is a mere 8.56 m asl with 94% of the island (29.62 km^2) lying below 6 m asl and 75% of the island (23.69 km^2) lying below 3 m asl. Using the median values of the four IPCC RCPs, sea level rise by 2100 will have a minimal direct impact on *V. rupicola* that ranges from a 4% (29 individuals) to 12% (75 individuals) loss using locations of extant plants to evaluate the impact. The post-2100 scenarios will have significant impact on the species current locations across Anegada with losses of extant individual locations varying from 20% to 99% between the 1 m and 6 m scenarios (Figure 36). The direct impact on Anegada and the indirect impacts of future sea level rise scenarios across the PRB will be discussed in 2.4.3 Sea level rise.

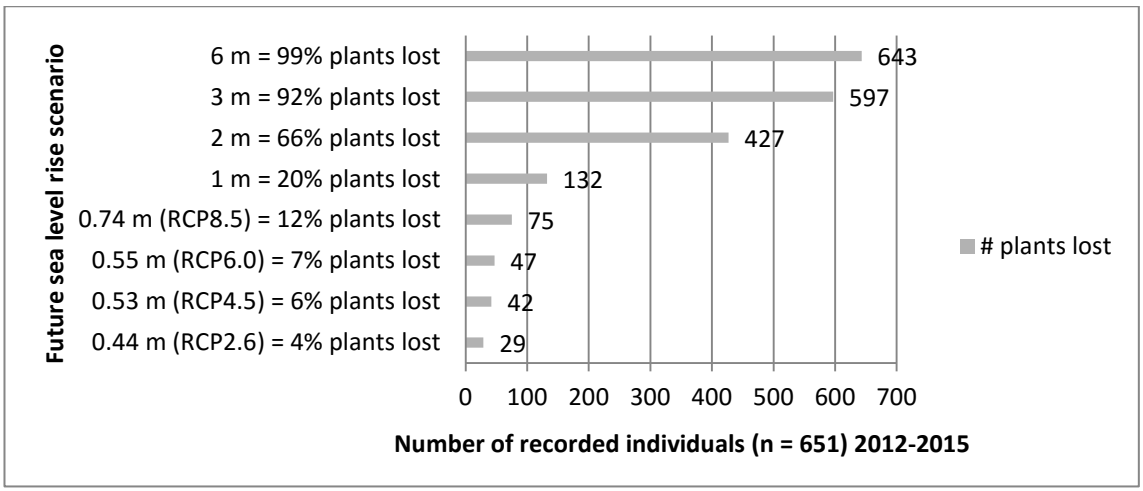

Figure 36: Impacts of future sea level rise on extant *Varronia rupicola* plants growing on the island of Anegada by scenario, up to 2300 AD. Scenarios based on IPCC (2013a) **Representative Concentration Pathways (RCP) for 2100, 0.44 m (RCP2.6), 0.53 m (RCP4.5), 0.55 m (RCP6.0) and 0.74 m (RCP8.5), as well as 1 m, 2 m, 3 m and 6 m scenarios of Bellard *et al.* (2014) for 2100 to 2300 AD.**

2.4. Discussion and conclusions

To address the first biogeographic question about the species current range, survey data gathered across the native range of the species between 2012 and 2015 in this research was processed and spatially viewed alongside all available historical records. Surveys for this research (Table 4) showed the species to be extant on each of the islands in the PRB that are part of its historical range with considerable variation in the area occupied between the islands. The single location on Vieques is in stark contrast to several south-western coastal municipalities (Guánica, Yauco, Peñuelas and Ponce) on Puerto Rico and across almost the entire extent of Anegada with 27 specific localities discernible. The historical recorded range of the species includes localities where no modern records exist including the Ensenada ward of Guánica municipality, the Indios ward in the Guayanilla municipality (both on Puerto Rico Island) and Punta Jalova on Vieques (see Figure 21). All records from the Guayanilla municipality are poorly defined and at least one collected in 1913, Shafer #1989, could represent another lost locality. The notes suggest the specimen was collected between Guayanilla and Tallaboa on a limestone outcrop. The road that once traversed these two areas crossed through Ponce limestone where an oil refinery was constructed leaving little habitat in the area for the species. Although the species has not been recorded in these locations in several decades, its existence cannot be ruled out due to survey limitations in these areas. Much of the remaining dry forest habitat on limestone in Guayanilla municipality is found on privately owned land that is difficult, or even impossible, to access. The Ensenada ward locality is problematic due to a lack of underlying Miocene limestone in the area; however, this is within the Guánica Quadrangle which lacks a USGS produced/adopted geology map. The Punta Jalova area on Vieques is part of the former Eastern Maneuver Area of the U.S. Navy that is

undergoing extensive clearance and modification due to unexploded ordinance (UXO) clearance resulting in very limited access and the inability to undertake surveys of many areas. This area lacks the Pliocene limestone that is known to support the species; however, Quaternary deposits of sand are found in the area. The lack of detailed information for the two Woodbury vouchers from Ensenada (1959) and Punta Jalova (1978) make confirmation of these locations very challenging. The military use of the areas in the species native range on Vieques could potentially be the cause of the limited numbers (six individuals at a single location) observed through direct (e.g. habitat fragmentation and degradation) or indirect impacts (e.g. deterrence of dispersers). Further survey and collaboration with ornithologists and herpetologists are warranted and will be discussed further in 5.3.5. Reproduction biology and dispersal.

This research made new formal voucher records in three Puerto Rican municipalities (see Figure 21). The first was collected in the Montalva ward of the Guánica municipality within the western tract of the Guánica State Forest. In the Barina ward of Yauco, vouchers were collected in the eastern tract of the Guánica State Forest and the Catala Farm, a privately owned area that was accessed with permission of the owner. The final new formal voucher was collected in Tallaboa ward of the Peñuelas municipality. These locations were surveyed following discussions with Puerto Rican colleagues that had either seen the species, but not formally vouchered the location, or agreed survey was warranted when reviewing maps due to the presence of suitable habitat. The positive result of surveys in areas of suitable habitat where no historical records existed support the methodology used and highlight the importance of local, expert input. Further survey based on the detailed geology and habitat maps presented in this research is warranted as previously unknown plants/populations may exist, especially on Puerto Rico.

Relatively few historical vouchers (n = 14) from Anegada exist (Appendix 1: *Varronia rupicola* records) and most do not provide detailed locality information; therefore, the majority of vouchers made from that island represent new localities for the species. Seven specific localities recorded in previous vouchers were confirmed and a further 20 localities were defined by this research across the island (see Figure 21).

Overall, the native range and detailed localities of the species have been better defined by this research. There are 506 vouchered records collected between 1886 and 2015 known to exist for *Varronia rupicola* that include 177 herbarium sheets, 459 DNA samples, two spirit collections, and one wood sample. Prior to the start of this research, only 41 vouchers existed; therefore, this research has generated 424 new vouchers for the species from wild and

cultivated material. There are a further 784 georeferenced observation records for the species collected between 2004 and 2015. Prior to the start of this research, 223 georeferenced observations existed with all but one, recorded in Puerto Rico, originating from Anegada. This research has generated 561 new observations for the species from across its native range that will be used in conjunction with population genetics findings (see 4.4.4. Main conclusions) to undertake a re-assessment of the species conservation status based on these new data.

2.4.1. Geology

In addressing the second biogeography question about substrates that support *V. rupicola*, it was necessary to interpret existing data and digitise printed maps to produce geological features across the PRB. Discrepancies were found to exist between available data and maps. This was true for several intersections of the USGS 1:20,000 quadrangle maps for the island of Puerto Rico. According to Albrecht (2007), these discrepancies are commonly encountered in GIS and require the user to resolve these using data that provides an appropriate scale for the product required; therefore, courser scale (*i.e.* <1:240,000) maps were interrogated to determine adjustments where discrepancies were found. A major issue encountered was a lacking USGS map for the Guánica Quadrangle. There are only a few map quadrangles not completed for Puerto Rico and the Guánica Quadrangle is of particular significance given the presence of the Guánica State Forest. A map produced as part of an MSc thesis by Addarich-Martínez (2009) does exist for the quadrangle; however, considerable difference is observed when compared to earlier, courser scale USGS maps. For this reason, Monroe (1980) was followed for the quadrangle as he focused on the limestone deposits of the area over a long period of time and is usually referenced by subsequent USGS workers in their maps of the southern coast. There are several consequences to this decision and require some caveats to be attached to the results presented here, beyond the normal issues encountered when digitising due to error associated with projections and resolution of source data. First, *V. rupicola* observations are shown to occur on Ponce limestone in the Montalva ward when following Monroe (1980); however, the map of Briggs and Akers (1965) suggests that these plants may be growing on Juana Diaz limestone similar to all other plants found within the Guánica forest, but elevation data do not support this as all other plants at a similar altitude are recorded on Ponce limestone. Second, following Monroe (1980) results in a large area of Juana Diaz limestone in the Monte Las Pardas area between the western and eastern tracts of the Guánica forest, whereas the map of Addarich-Martínez (2009) shows this area as a large Ponce limestone deposit. The species has not been found in this area through recent surveys; however, the area contains a significant area of intact habitat without protection. Determining the correct substrate classification is necessary to assist further survey and research into *V.*

rupicola. For this reason, an officially adopted/produced USGS map for the Guánica Quadrangle is desperately needed.

Assessing the geological mapping results of this study in relation to species observations and underlying substrate has shown the species is limited to deposits of specific substrates that vary considerably in size and distribution across the PRB. Anegada was found to have two substrates, Pleistocene limestone and Quaternary sand deposits (overlying Pleistocene limestone), supporting the species with 494 and 157 observations of the species, respectively (Figure 37). These findings demonstrate that the species is able to grow on recently deposited sand even though it has not been formally recorded growing on the substrate elsewhere across its native range. This provides an opportunity to explore population reinforcement or assisted colonisation (IUCN/SSC, 2013) of the species into protected areas with suitable substrates across the species range (see 5.2. Conservation strategies).

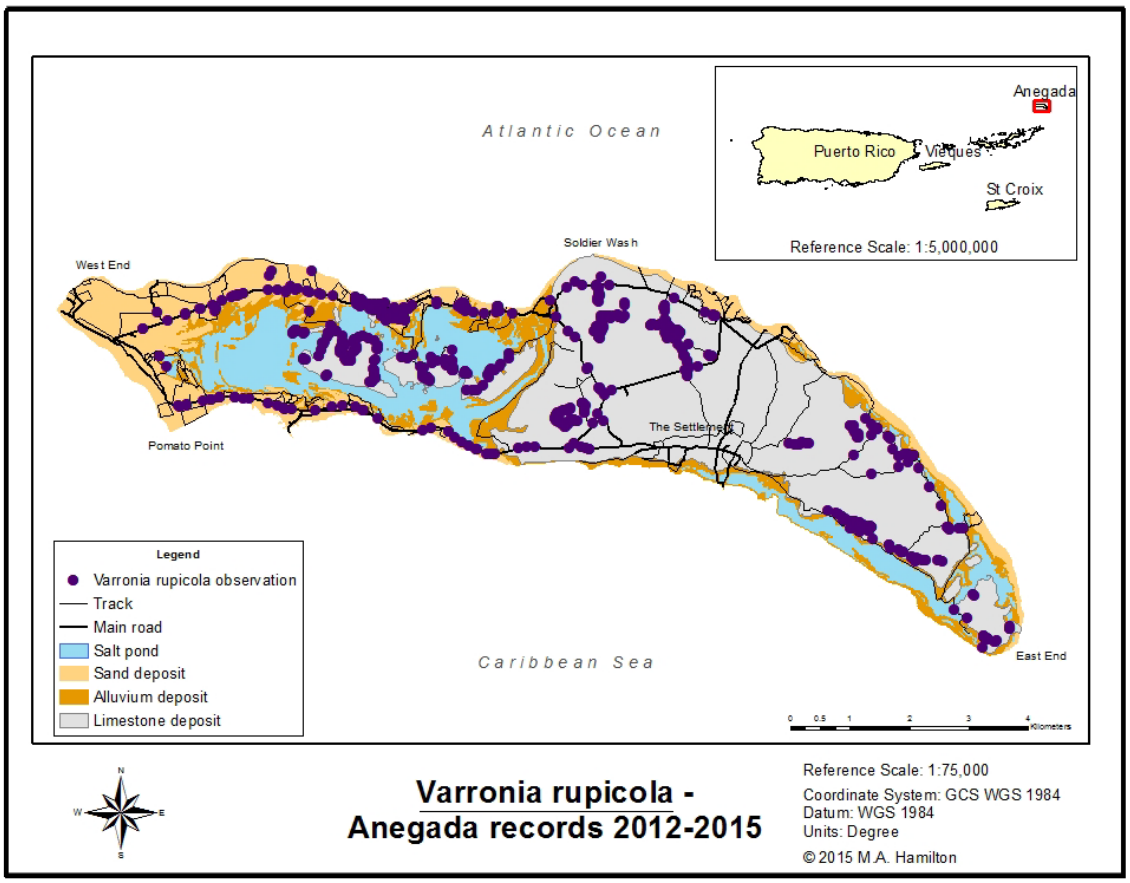

Figure 37: Map showing all recorded observations (n = 651) of *Varronia rupicola* between 2012 and 2015 growing on Quaternary sand deposits (157 observations) and Pleistocene limestone deposits (494 observations) across the island of Anegada.

Vieques, with the smallest area of substrate supporting the species, was also found to be supporting the least number (n = 6) of extant *V. rupicola* plants (Figure 38). The Puerto Ferro

limestone peninsula was the only locality with extant plants even though Pliocene limestone deposits occur in several areas.

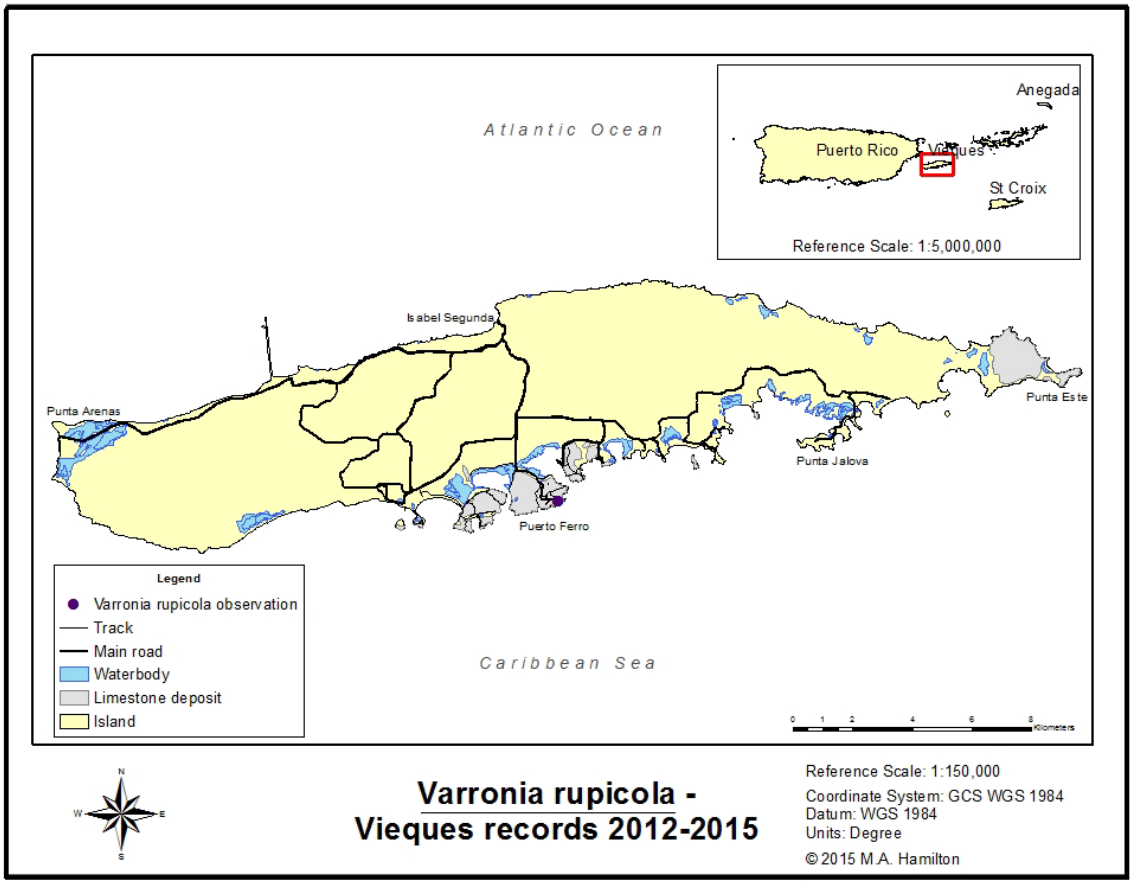

Figure 38: Map showing all recorded observations (n = 6) of *Varronia rupicola* between 2012 and 2015 growing on Pliocene limestone at a single locality, Puerto Ferro, on the island of Vieques.

The Miocene limestone formations on Puerto Rico were the largest area of substrate supporting *V. rupicola* across its native range but with only 165 observations (Figure 39) split between Juana Diaz and Ponce limestone, where 110 and 55 observations were made, respectively. The age of these deposits and the considerable elevation difference observed between deposits on Puerto Rico compared to Vieques and Anegada suggest that the species may have originated on Puerto Rico and subsequently colonised progressively younger limestone formations from west to east. This will be discussed further in relation to the findings of the phylogenetic placement and population genetics of the species in Chapter 5: Discussion, conservation implications and research opportunities.

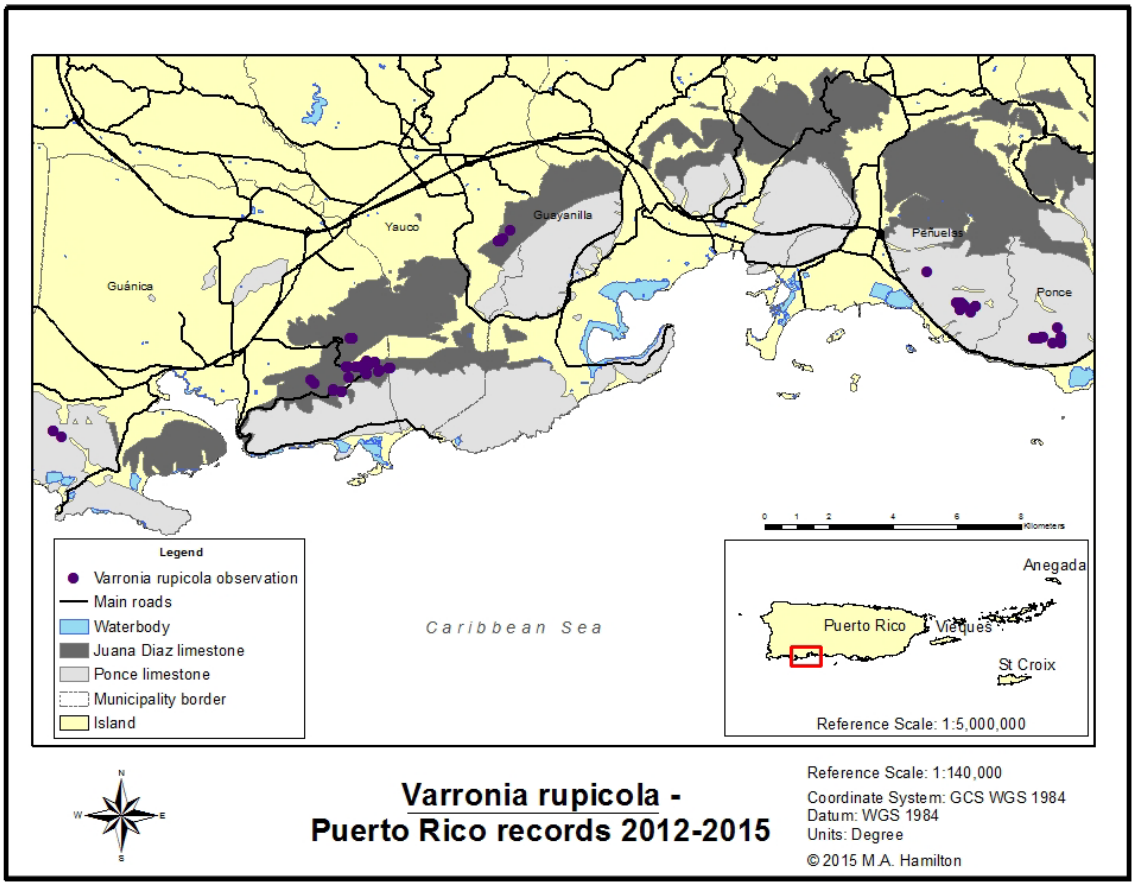

Figure 39: Map showing all recorded observations (n = 165) of *Varronia rupicola* between 2012 and 2015 growing on Miocene deposits of Juana Diaz (110 observations) and Ponce (55 observations) limestone along the south-western coast of the island of Puerto Rico.

2.4.2. Land cover

In determining that *V. rupicola* plants are associated with a relatively few land cover types that overlie the specific substrates that were found to support the species, a much clearer picture of the reduction and fragmentation of the species remaining habitat has been drawn. The variation in habitats between the two countries and across the substrates highlights the species ability to adapt to its environment; however, the limited number and area of habitats involved and the complete absence of the species in heavily disturbed habitats other than on Quaternary sand substrate on Anegada suggests that the species struggles to adapt to anthropogenic disturbance, particularly on limestone substrates where moisture retention is a major impedance to establishment (McLaren and McDonald, 2003). Following identification of habitats and substrates supporting the species, the assessment of protected area coverage shed light onto the current *in-situ* conservation measures and the gaps that exist (see 5.2. Conservation strategies). Maps below include no new data and are provided to assist visualisation of the discussion.

Two refined habitat types of Kennaway *et al.* (2008), overlying specific substrates that support the species were found to be the preferred habitats for *V. rupicola* on Anegada. Anegada

currently lacks designated protected areas, but two new protected areas are proposed for the island. These are the Eastern Ponds which is to be designated as a National Park and the Western Ponds that includes the existing Ramsar site which is to be designated as a Protected Landscape. Once established, these protected areas would only include 15% of the intact, preferred habitat for the species (Figure 40). On-going anthropogenic disturbance, even within the boundary of the existing Ramsar site, is of major concern and protected area designation and regulation is urgently needed. The proposed protected area coverage will be discussed further in 5.1. Conservation implications following the presentation of the population analyses results in Chapter 4: Conservation genetics of *Varronia rupicola*.

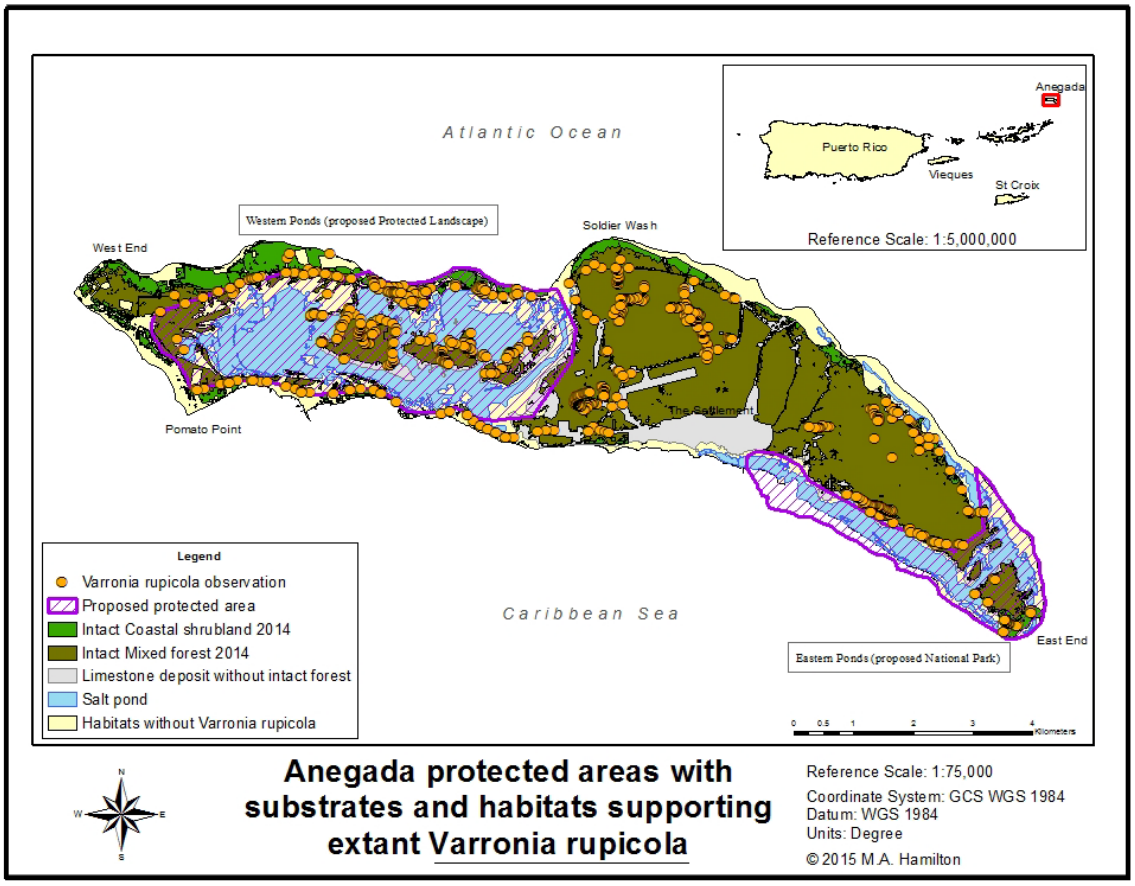

Figure 40: Map showing proposed protected areas with Quaternary deposits of sand and Pleistocene limestone covered by intact 'Deciduous, Evergreen and Mixed Forest and Shrubland with or without Succulents' (aka 'Mixed forest') and 'Evergreen Coastal Shrubland' (aka 'Coastal shrubland') of Kennaway *et al.* (2008) supporting extant *Varronia rupicola* on Anegada.

Although all of the preferred habitat of the species classified by Gould *et al.* (2008) and substrates known to support it is found within the DRNA reserve or the Vieques NWR (Figure 41), both of these areas experience on-going disturbance and degradation across much of their area. The eastern tract of the Vieques NWR that is home to the six extant *V. rupicola* includes the former Eastern Maneuver Area of the U.S. Navy that underwent extensive modification and was used for military exercises that left UXO in the landscape, particularly at Punta Este

(CH2M HILL, 2012a) on the eastern tip of the island. The UXO is now being identified and removed which often requires clear-cutting of the vegetation with some subsequent restoration planned (CH2M HILL, 2012b). Many areas not subjected to UXO remediation work within the refuge are open to the public and suffer from disturbance and degradation due to recreational use and arson as does the entire DRNA reserve. Road improvement works on the Puerto Ferro peninsula of Vieques were thought to have extirpated *V. rupicola* from the island until it was rediscovered during this research. These factors coupled with the species limited distribution put it in a very precarious situation and suggest urgent and sustained management is required to bolster wild populations and develop *ex-situ* collections (see 5.2. Conservation strategies).

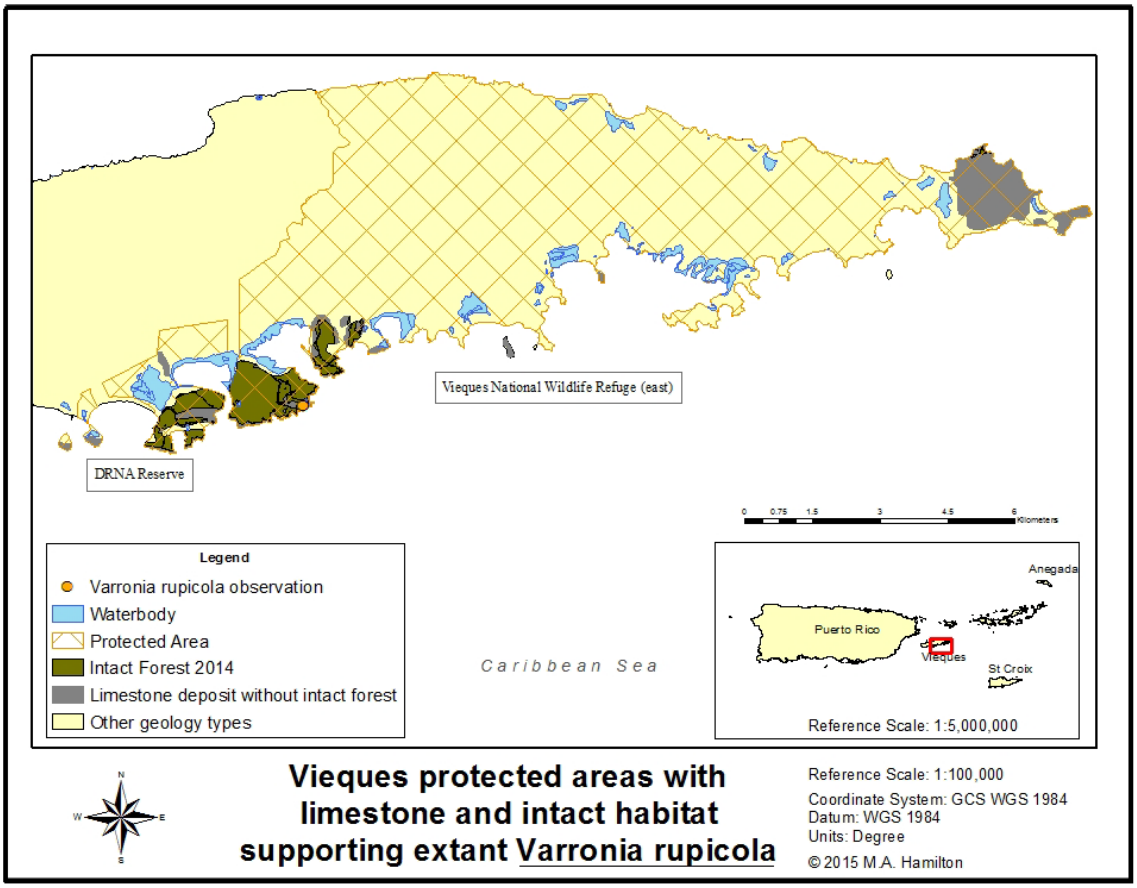

Figure 41: Map showing protected areas with Pliocene limestone deposits and intact 'Mature secondary lowland dry limestone semi-deciduous forest' (aka 'Semi-deciduous forest') of Gould *et al.* (2008) supporting extant *Varronia rupicola* on Vieques.

Protection for the preferred habitat of the species classified by Gould *et al.* (2008) and substrates known to support it on Puerto Rico (Figure 42) is limited to the Guánica State Forest. This situation leaves a large proportion of the extant individuals, especially those on Ponce limestone, unprotected and subject to anthropogenic disturbance. This is of particular concern near Ponce as this research found 3% of the Juana Diaz and 4% of Ponce limestone formations in this area were developed between 2000 and 2014. More generally, this research

found that 47% of the vegetation covering the Ponce limestone formation in south-western Puerto Rico has been impacted by anthropogenic disturbance. Research by Martinuzzi *et al.* (2007) found urban expansion in Puerto Rico between 1977 and 1994 to be associated with proximity to existing urbanisation and lower elevations, whereas Kennaway and Helmer (2007) found that between 1991 and 2000 limestone deposits underwent >20% of the anthropogenic disturbance on the island with the forests impacted being of an older age. The 'Mature secondary lowland dry limestone semi-deciduous forest' habitat type of Gould *et al.* (2008) corresponds to the dry forest age class 4 of Helmer *et al.* (2008) that is 50-64+ years old; therefore, *V. rupicola* is found in some of the oldest and most threatened forest in Puerto Rico.

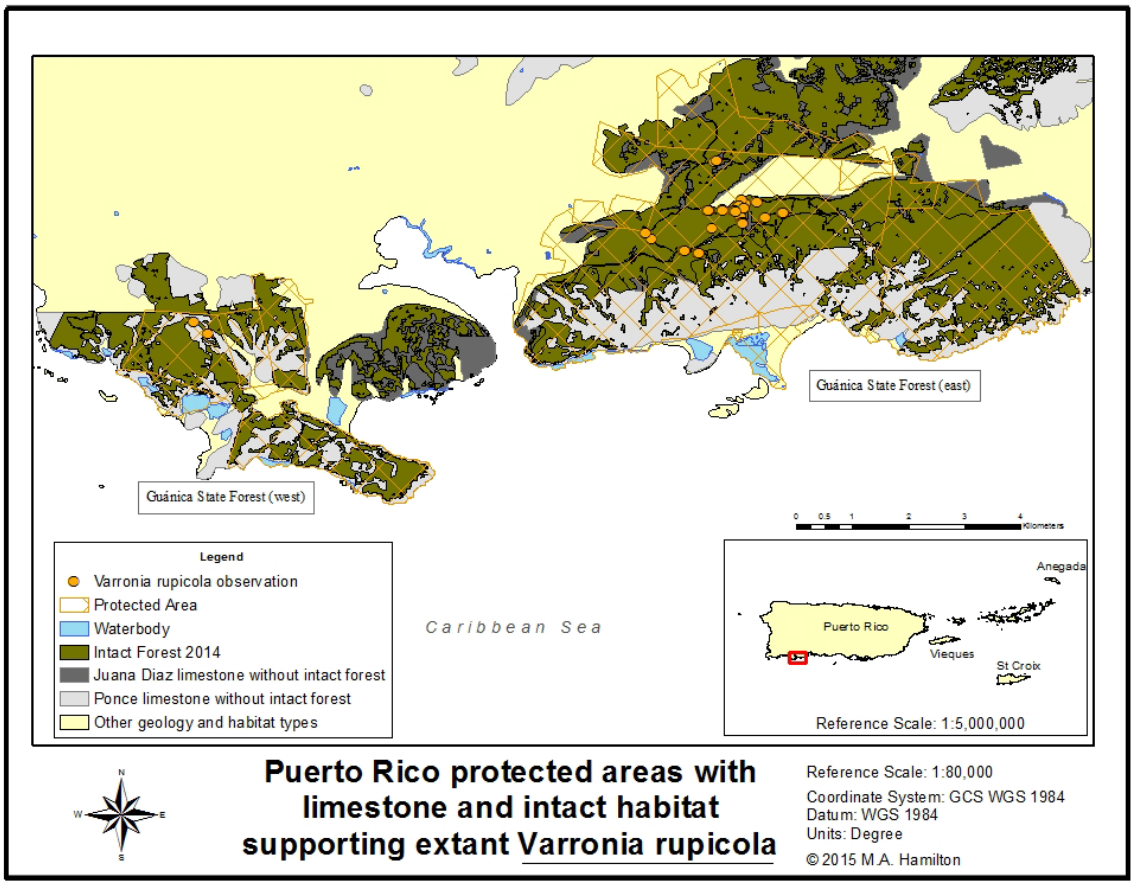

Figure 42: Map showing protected areas with Miocene limestone deposits and intact 'Mature secondary lowland dry limestone semi-deciduous forest' (aka 'Semi-deciduous forest') of Gould *et al.* (2008) supporting extant *Varronia rupicola* on Puerto Rico.

Outside the species native range, opportunities exist for the assisted colonisation of the species to establish populations that include currently unprotected genetic diversity. One such example is on the small, offshore cay of Caja de Muertos where both Ponce limestone and 'Mature secondary lowland dry limestone semi-deciduous forest' (Gould *et al.*, 2008) are under protection. Other examples are along the southern coast of Puerto Rico where Ponce limestone rests atop Cretaceous rock at the protected area Los Morrillos de Cabo Rojo

(Monroe, 1980; Weaver and Schwagerl, 2009) and Cabo Rojo NWR (Monroe, 1980; U.S. Fish and Wildlife Service, 2013a). These opportunities with reference to population genetics will be further explored in 5.2.3. Conservation introductions.

There are several localities in south-western Puerto Rico with Miocene limestone geology and 'Mature secondary lowland dry limestone semi-deciduous forest' habitat type of Gould *et al.* (2008) that have not had intensive survey and appear to have suitable habitat for *V. rupicola*. Among these areas are: Cabo Rojo municipality – Boquerón; Guánica municipality – Caño, Susúa Baja; Yauco municipality - Jácana; Guayanilla municipality – Boca, Cedro, Jaguas, Macaná, Magas, Playa, Quebradas; Peñuelas municipality – Coto, Cuebas, Santo Domingo, Tallaboa Alta, Tallaboa Poniente, Tallaboa Saliente; Ponce municipality – Ponce, Magueyes, Magueyes Urbano, Marueño, Portugués Urbano, Segundo, Sabana Llana; and Caja de Muertos. These areas represent a relatively small proportion of the 78 municipalities and 875 wards found on the island of Puerto Rico; however, traversing the often steep limestone hills and dense vegetation can be an arduous task with access extremely difficult for the predominantly private land. The issue of privately owned land is the most challenging and often results in the greatest impact to species and their habitats through modification of the landscape with little or no regulation (Yackulic *et al.*, 2011).

Within the Guánica forest boundary, Kennaway and Helmer (2007) observed no land development between 1991 and 2000. During the same period on Vieques within the NWR, the only change observed occurred on the eastern end of the island (Kennaway and Helmer, 2007) on Pliocene limestone (Learned *et al.*, 1973; CDC, 2007) which was inside the live impact area of the Atlantic Fleet Weapons Training Facility (CDC, 2007) that had already undergone significant levels of disturbance. Once opened for access, there are several areas on Vieques that require thorough survey. Among these are Punta Jalova and Yellow Beach conservation area as well as the eastern tip of the island, Punta Este.

Historical agriculture features on Anegada (see Figure 31) suggest historical anthropogenic disturbance has had a significant impact on the species habitat and the localities of extant individuals. A large area in the centre of the island was under active management for many decades or centuries (Schomburgk, 1832), creating a nearly complete transect of disturbance through the middle of the island. The consequences of this historical disturbance in relation to population genetics are discussed further in Chapter 5: Discussion, conservation implications and research opportunities.

2.4.3 Sea level rise

Eustatic changes have caused significant variations in the amount of submerged and exposed land across the PRB in the past (see Table 1) and are projected to continue to do so into the future. How these changes have impacted individual species is poorly known and has never been assessed for *V. rupicola*. The following sections summarise the findings of research into past and future scenarios of sea level rise on the PRB and the potential implications to, and impacts on, *V. rupicola*. Maps below include no new data and are provided to assist visualisation of the discussion.

Past sea level rise

Eustatic changes driven by glacial cycles led to the exposure of the entire Puerto Rican Bank as a single land mass more than once in the past and submersion of larger parts of the islands that led to the formation of limestone deposits. Sea levels following the deposition of Miocene limestone formations on Puerto Rico appear to have been lower, suggesting that at least parts of the Juana Diaz and possibly Ponce limestones were never submerged again and would have been exposed substrates for colonisation. Following the deposition of the Pliocene limestone formation on Vieques, sea levels do not appear to have exceeded the levels experienced during the formation of that deposit. Thus the limestone subsequently exposed by lower sea levels would have been available for colonisation from Puerto Rican propagules that might have been avian dispersed during island isolation (high sea levels) or dispersed by a mixture of avian/non-avian organisms during PRB exposure (low sea levels). During the Pleistocene, limestone on Anegada was formed and subsequently exposed. Since its exposure, Anegada limestone has never been submerged again and was, therefore, available for colonisation from propagules originating further west in the PRB by methods previously described.

Barker *et al.* (2012) found that data from amphibians supported an 'Eastern Dispersal Hypothesis' in which the eastern islands of the PRB were colonised from mainland Puerto Rico subsequent to the penultimate interglacial period. Although the study species of Barker *et al.* (2012) is only thought to be able to disperse during periods of low sea level due to salt intolerance, the findings are nonetheless useful here due to the premise used in the study that suggests higher extinction rates in small, low lying islands require colonisers from larger, higher elevation islands. The results from other studies vary widely from low levels of endemism and high gene flow (Heatwole and MacKenzie, 1967) to low gene flow and high endemism (Malone *et al.*, 2003), suggesting that sea level fluctuations play a diverse role in insular, tropical island biogeography. Further discussion in relation to *V. rupicola* populations will be provided in 4.4.4. Main conclusions.

Future sea level rise

Hurricanes and tsunamis have had major impacts in the PRB (Committee on Natural Disasters, 1994), especially on Anegada as documented by several authors (Atwater *et al.*, 2012, 2014; Spiske and Halley, 2014). Higher sea levels that are thought to be a certain result of climate change will exacerbate future events (Nicholls and Cazenave, 2010). To compound the issue, Parsons and Geist (2009) found a higher hazard for a >0.5 m tsunami run-up for Puerto Rico and the Virgin Islands due to the proximity of the islands to the subduction zone between the North American and Caribbean plates. Due to the low-lying nature of Anegada, the island will probably be the most impacted in the native range of *V. rupicola* by future events.

The median values of the four IPCC RCP scenarios for 2100 (IPCC, 2013a) showed that *V. rupicola* will experience minimal direct sea level rise impacts on Anegada (see Figure 35) with losses between 4% and 12% of the locations of the current extant individuals (see Figure 36). However, the indirect impacts on the species may be significantly higher due to anthropogenic change (e.g. development further inland and up slope) and the effects of natural disasters previously discussed.

Unfortunately, the species current locations across Anegada, where the largest numbers of observations were made, will experience significant impact with >1 m sea level rise (see Figure 36). Only 7.91 km^2 of Anegada's limestone lies above 3 m asl and a mere 1.98 km^2 are above 6 m asl. A portion of the current dune system along the north and west coasts has peaks above 3 m asl; however, rising seas will undoubtedly wash away these Quaternary deposits overlying limestone that is below 3 m asl through tidal and storm related events. With a nearly complete loss of extant localities (99% loss) using the 6 m sea level rise scenario, the majority of extant localities of the species will surely be lost (Figure 43). Even the 20% loss suggested by the 1 m scenario will have a significant impact on the species globally calling into question the current conservation measures for the species and the need for assisted migration and/or other conservation measures to be put into place.

The indirect impacts of future sea level rise scenarios across the PRB and the direct impact on Anegada will be discussed further in relation to population genetics (5.1.2. Sea level rise) and to mitigation options (5.2. Conservation strategies).

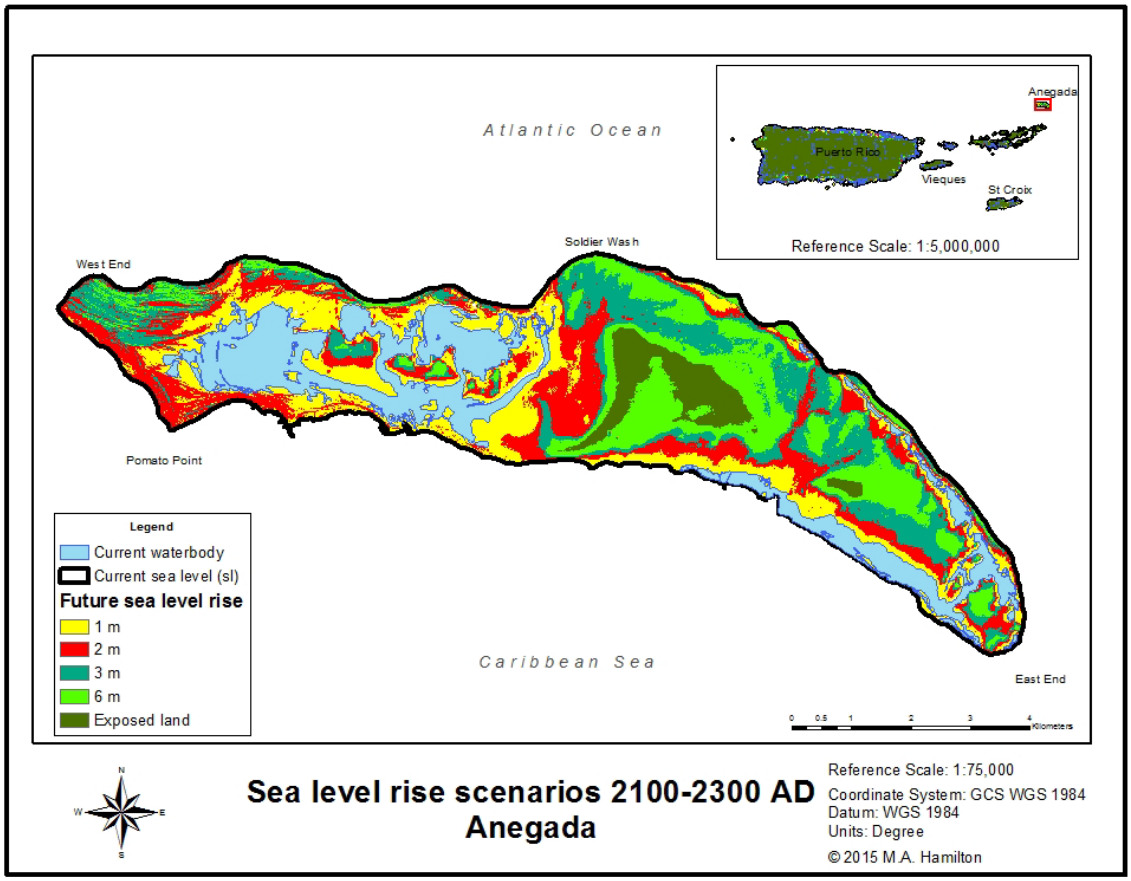

Figure 43: Map showing future sea level rise scenarios of 1 m, 2 m, 3 m, and 6 m for 2100 to 2300 AD (Bellard *et al.*, 2014) **on Anegada.**

The future sea level rise scenarios for 2100 to 2300 of 1 m, 2 m, 3 m and 6 m (Bellard *et al.*, 2014) will, fortunately, have no direct impact on extant *V. rupicola* plant locations on Vieques (Figure 44) or Puerto Rico (Figure 45); however, the indirect impacts could be significant, especially on Puerto Rico outside of the areas of protected habitat due to anthropogenic disturbance. By 2100, the Caribbean could see massive population displacement due to the prohibitive costs associated with coastal mitigation projects (Nicholls *et al.*, 2011). If low-lying, coastal areas are abandoned, available land (unprotected) at higher elevation but adjacent to existing infrastructure will be the obvious choice for anthropogenic disturbance. The preferred habitat of *V. rupicola* that is outside of existing protected areas meets these criteria and is, therefore, under severe threat in the face of sea level rise.

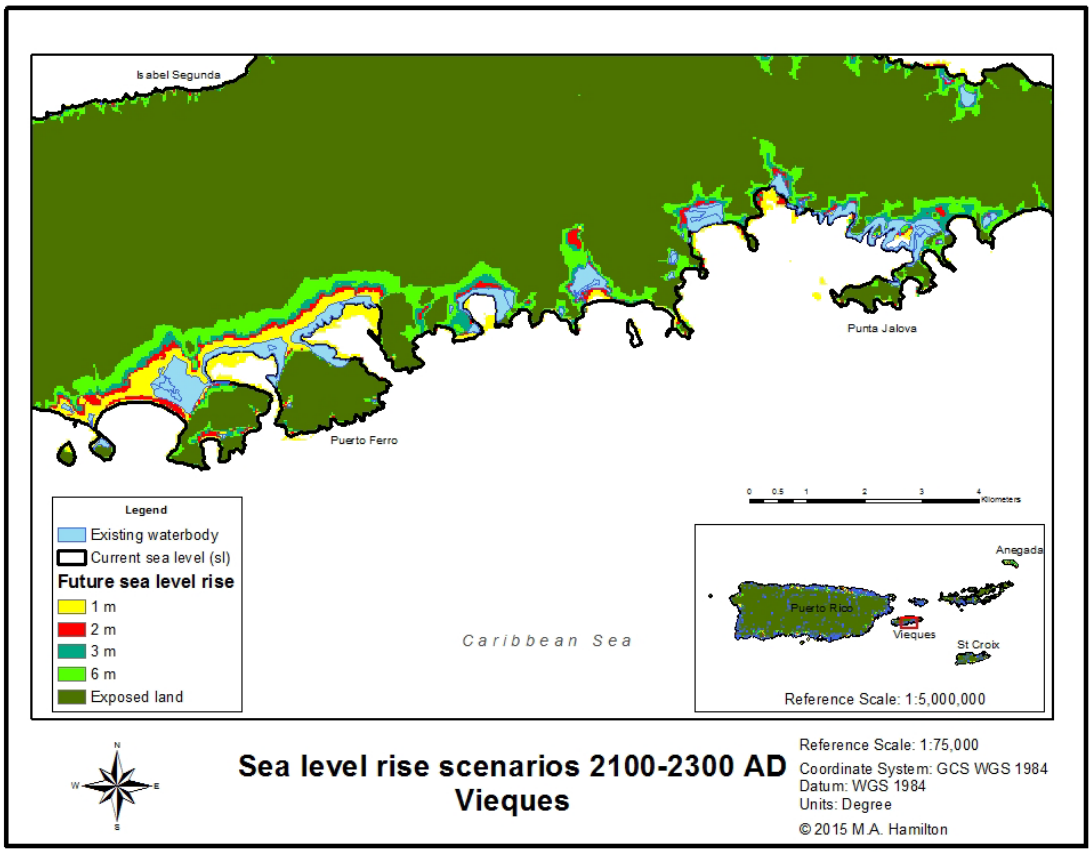

Figure 44: Map showing future sea level rise scenarios of 1 m, 2 m, 3 m, and 6 m for 2100 to 2300 AD (Bellard *et al.*, 2014) **on southern Vieques.**

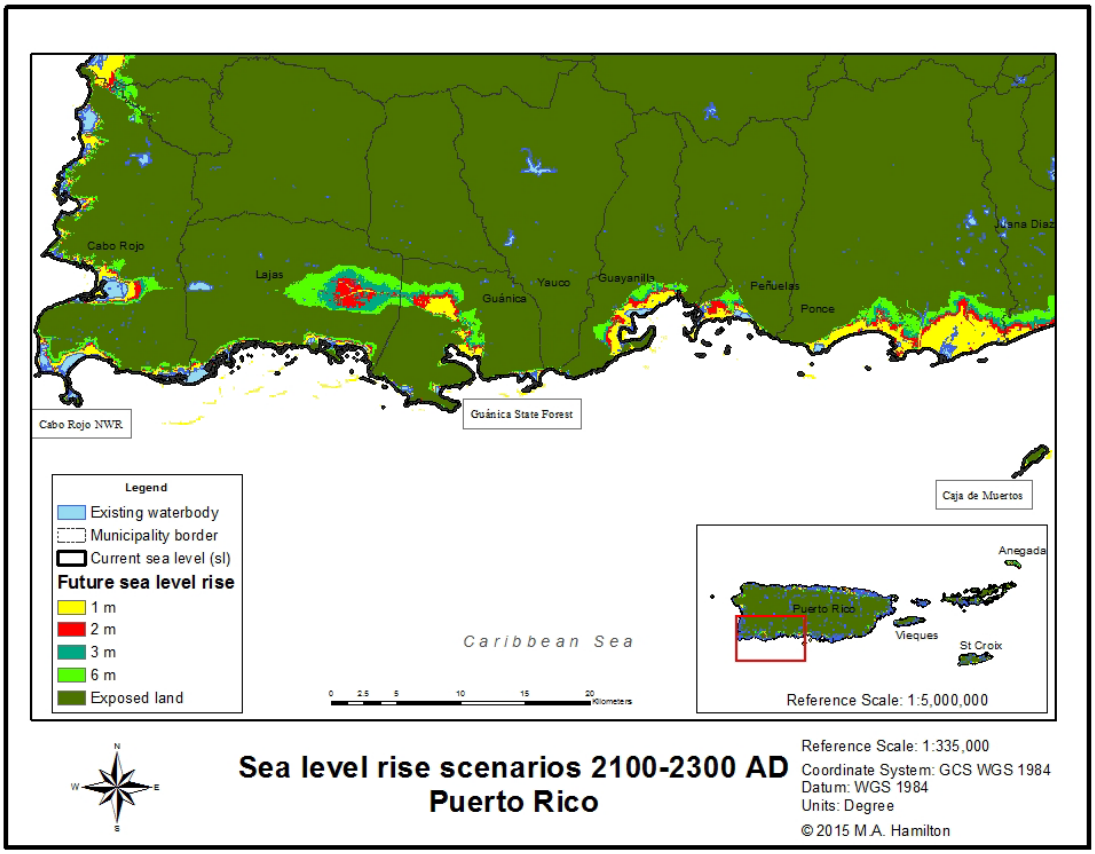

Figure 45: Map showing future sea level rise scenarios of 1 m, 2 m, 3 m, and 6 m for 2100 to 2300 AD (Bellard *et al.*, 2014) **on south-western Puerto Rico.**

2.4.4. Main conclusions

This research was undertaken to explore the biogeography of *V. rupicola* with the aim of answering several specific questions. These queries involved definition of the native range and ecological preferences of the species as well as the impacts of on-going habitat modification and future sea level rise. The original questions and answers provided by this research are as follows:

- **First, where is the species currently extant and does this differ from its historical native range?** The species is extant on the islands of Puerto Rico (south-western coastal municipalities of Guánica, Yauco, Peñuelas and Ponce); Vieques (Puerto Ferro); and Anegada (across the island in 27 localities). Within the Puerto Rican municipalities the species is currently known to occur in specific wards, as follows: Guánica municipality (Carenero, Montalva); Yauco municipality (Barina); Peñuelas municipality (Tallaboa, Encarnación); and Ponce municipality (Canas). The historical range of the species includes the Ensenada ward in Guánica municipality and Guayanilla municipality (Indios ward and undefined localities) on Puerto Rico and Punta Jalova on Vieques. The species has not been recorded in these locations in several decades; however, its existence cannot be ruled out due to survey limitations. The species was not previously recorded by formal voucher in Puerto Rico at Montalva ward (Guánica municipality), Barina ward (Yauco municipality) or Tallaboa ward (Peñuelas municipality); or in Anegada at twenty new localities.
- **Second, do *V. rupicola* plants occur on any specific substrates across the native range of the species? If so, where are they located and how much area do they cover?** Yes. The species has only been found on specific substrates during this research. Across the PRB, these substrates are located:
    - On Anegada (see Figure 22) - Pleistocene limestone (total area of 19.80 km^2) mainly on the eastern half of the island and Quaternary deposits of sand (total area of 7.43 km^2) only on the western half of the island;
    - On Vieques (see Figure 23) - Pliocene limestone (total area of 6.28 km^2) only along the central part of the southern coast and the eastern tip;
    - On Puerto Rico (see Figure 24) – Miocene limestone formations (total area of 165.82 km^2) of Juana Diaz limestone (67.83 km^2) and Ponce limestone (97.99 km^2) only along the south-western coast with Juana Diaz limestone restricted to six municipalities from Juana Diaz in the east to Guánica in the west and Ponce limestone in nine municipalities from Santa Isabel in the east to Cabo Rojo in the west.

- **Third, are *V. rupicola* plants associated with any specific land cover types overlying the substrates supporting the species across its native range? If so, where are they located and how much area do they cover?** Yes. The species has only been found within a limited number of habitats that vary based on the use of regional or refined classifications. Across the PRB, these habitats and their areas are:
    - On Anegada, two refined habitat types of Kennaway *et al.* (2008), 'Evergreen Coastal Shrubland' and 'Deciduous, Evergreen and Mixed Forest and Shrubland with or without Succulents' were found to be overlying specific substrates that support the species with 2.96 km^2 and 17.09 km^2 remaining in 2014, respectively (see Figure 25). These two habitats are combined in the regional habitat classification of Kennaway *et al.* (2008), 'Deciduous, Evergreen Coastal and Mixed Forest or Shrubland with Succulents', resulting in 20.04 km^2 remaining in 2014. The species was also recorded in 'Low Density Urban', 'Pasture, Hay, Abandoned Agriculture or Other Grassy Areas' and 'High-Medium Density Urban' overlying specific substrates that support the species with 59, nine and four records, respectively. The species was not found in these habitats although they do occur on the other islands. The refined/regional habitats the species was found in on other islands are not found on Anegada.
    - On Vieques, only 2.86 km^2 (see Figure 26) of the refined 'Mature secondary lowland dry limestone semi-deciduous forest' habitat type of Gould *et al.* (2008) which is included in the regional 'Semi-Deciduous and Drought Deciduous Forest on Karst/limestone (includes semi-evergreen forest)' habitat of Kennaway & Helmer (2007) was found to be overlying specific substrates that support the species and remained in 2014.
    - On Puerto Rico, 65.06 km^2 (see Figure 27) of the refined 'Mature secondary lowland dry limestone semi-deciduous forest' habitat type of Gould *et al.* (2008) was found to be overlying specific substrates that support the species remained in 2014. Five sterile individuals were also recorded in the refined 'Young secondary lowland dry limestone semi-deciduous forest' habitat type of Gould *et al.* (2008) with 8.16 km^2 overlying specific substrates that support the species remaining in 2014. These habitats are combined in the regional 'Semi-Deciduous and Drought Deciduous Forest on Karst/limestone (includes semi-evergreen forest)' habitat of Kennaway & Helmer (2007) meaning that 73.22 km^2 of this broader habitat overlying specific substrates that support the species remained in 2014.

- **Fourth, are *V. rupicola* plants found within protected areas (proposed or existing)? If so, how much area of the land cover types known to support *V. rupicola* plants do these protected areas contain?** Yes. The species has been found to occur within protected areas, both existing and proposed, in both countries and all three islands (see Figure 28). Across the PRB, these sites and the area of land cover types known to support *V. rupicola* plants are:
    - On Anegada, 'Deciduous, Evergreen and Mixed Forest and Shrubland with or without Succulents' and 'Evergreen Coastal Shrubland' only have 3.09 km^2 and 0.83 km^2, respectively, falling within the boundaries of the two proposed protected areas on the island (see Figure 40);
    - On Vieques, all 2.86 km^2 of the 'Mature secondary lowland dry limestone semi-deciduous forest' refined habitat type of Gould *et al.* (2008) is protected in the Vieques NWR (see Figure 41);
    - On Puerto Rico, 22.08 km^2 of the 'Mature secondary lowland dry limestone semi-deciduous forest' habitat type of Gould *et al.* (2008) is found within existing protected areas (see Figure 42). The area under protection is mainly within the Guánica State Forest; however, 0.91 km^2 of the total is included in the offshore cay of Caja de Muertos where *V. rupicola* has not been previously recorded.
- **Fifth, what are the implications of past sea level rise on the native range of *V. rupicola*?** Past sea level rise has caused both isolation of and connectivity between the current areas where *V. rupicola* plants are extant. This has potentially allowed colonisation of habitat on islands where the species had not previously existed, probably from west to east, through dispersal by the fauna. Periods of lower sea levels potentially led to connection between previously isolated populations and opportunities to colonise new areas of habitat. Subsequent periods of higher sea levels fragmented populations limiting dispersal by non-avian organisms and submerged suitable habitat in areas below 0 m asl.
- **Sixth, will proposed scenarios of future sea level rise impact the native range of *V. rupicola*?** The median values of the four IPCC RCP scenarios for 2100 (IPCC, 2013a) could only be mapped for Anegada (see Figure 35) due to the DEMs available having resolution that was too course for the rest of the PRB. On Anegada, IPCC RCP sea level rise scenarios by 2100 (see Figure 36) will have a minimal direct impact on *V. rupicola* ranging from a loss of 29 (4%) to 75 (12%) individual localities using locations of extant plants to evaluate the impact. The 1 m, 2 m, 3 m and 6 m scenarios for 2100 to 2300 (see Figure 34) will have no direct impact on extant *V. rupicola* plant locations on

Vieques (see Figure 44) or Puerto Rico (see Figure 45); however, these scenarios will have significant impact on the species current locations across Anegada (see Figure 43) with losses of extant individual's locations varying from 20% to 99% between the 1 m and 6 m scenarios due to only 6% of the island being above 6m asl. The indirect impacts of future sea level rise on all three islands could be very high, especially on Puerto Rico Island outside of existing protected areas.

Chapter 3: Phylogenetic placement of *Varronia rupicola*

This chapter focuses on the phylogenetic systematics of *Varronia rupicola* and specifically explores its relationship with other species of *Varronia* and *Cordia* native to the Puerto Rican Bank and insular Caribbean. There are no known studies of the phylogenetic placement of *V. rupicola* and in those phylogenetic studies undertaken that have included *Varronia* species *i.e.* Gottschling *et al.* (2001), very few were Caribbean samples.

Determining the placement of *V. rupicola* and its relationship with morphologically similar and often confused species is paramount for conservation planning. Also, to undertake a population genetics study (Chapter 4: Conservation genetics of *Varronia rupicola*), a phylogeny is needed to answer fundamental questions about the species placement. Using markers for ITS and *trnL-trnF* regions that have been successfully employed in previously published studies, this research will explore the phylogenetic placement of several *Varronia* and *Cordia* species native to the insular Caribbean for the first time.

- First, do new samples from the Caribbean support previous findings of *Varronia* as a monophyletic genus sister to *Cordia.*
- Second, is *V. rupicola* is a distinct taxon and, if so, what are its closest relatives?

Recommendations for further research will be made following the discussion of the results for species placements presented here for the first time.

3.1. Introduction

Varronia rupicola is one of nine species in the genus to occur in the PRB (Acevedo-Rodríguez and Strong, 2012). There has been historical confusion between three species native to the Caribbean region: *V. rupicola, V. lima* Desv. and *V. bahamensis* (Urb.) Millsp. (Figure 46).

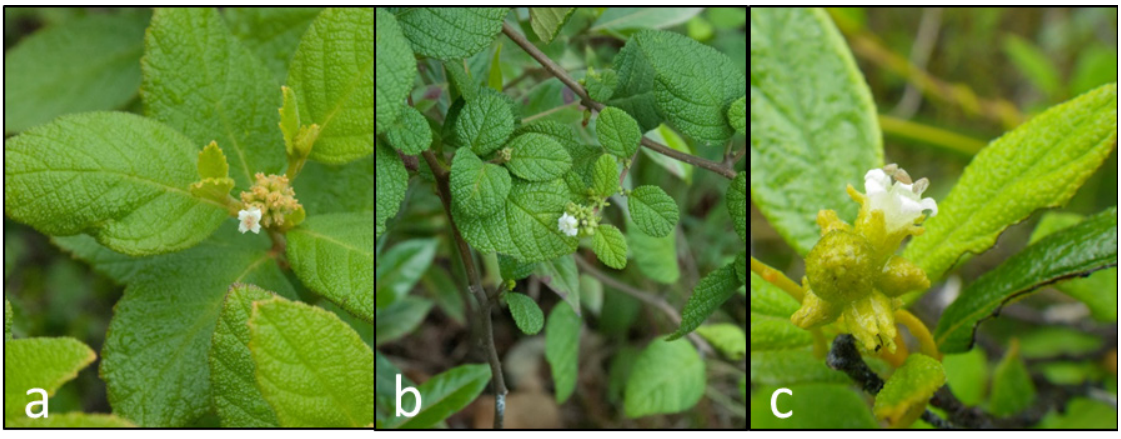

Figure 46: Three often confused Caribbean species of *Varronia*: (a) *V. rupicola*, (b) *V. lima* and (c) *V. bahamensis.* ©M.A. Hamilton.

Varronia lima was the first of these species to be described in 1808 from Hispaniola (Desvaux, 1808) and its current known range is Puerto Rico and Hispaniola (Acevedo-Rodríguez and Strong, 2012). Early collections in the Bahamas were recorded as *Cordia lima* by Grisebach (1859). These collections were later re-identified as a separate species with the publication of *Cordia bahamensis* Urban (1899). *Varronia bahamensis* is now known to occur throughout the Bahamas archipelago, including the Turks and Caicos Islands in the south (Acevedo-Rodríguez and Strong, 2012). Collections and publications up until the second work of D'Arcy (1975) recorded *V. bahamensis* as present on Anegada (Britton, 1916; D'Arcy, 1971). Curation and identification of Anegada material varies between herbaria; however, the specimens and historical records from the island are all now thought to be *V. rupicola* by the majority of workers in the PRB (Proctor, 1991; Clubbe *et al.*, 2004; U.S. Fish and Wildlife Service, 2010; Wenger *et al.*, 2010; Acevedo-Rodríguez and Strong, 2012). Resolving the phylogenetic placement of the aforementioned species is paramount for further population level analyses to be undertaken for *V. rupicola*.

3.1.1 Genomic information

Gottschling (2003) demonstrated the usefulness of ITS1 and *trnL* intron in resolving genus level relationships for *Cordia s.l.* and limited success at lower level resolution. Wikström *et al.* (1999) found a higher level of variation in the *trnF* intergenic spacer compared with the *trnL* intron and Eriksson *et al.* (2003) found the ITS region to be more variable than the *trnL-trnF* region. Cohen (2013) showed ITS and *trnL-trnF* to be useful for resolving relationships in subfamily Boraginoideae. Therefore, exploration of the entire region for ITS and *trnL-trnF* was chosen for this research in an attempt to provide a phylogenetic tree for selected *Cordia* and *Varronia* species from the Caribbean and confirm the placement of *V. rupicola*.

Due to the slow rate of evolution for mitochondrial DNA (mtDNA) in plants (Zhang and Hewitt, 2003), research has focused on identifying specific regions of nuclear or plastid DNA and producing protocols that enable replicable results across many taxa. Several genomic regions have been successfully identified and have become standards for sequenced-based molecular phylogenetic studies (White *et al.*, 1990; Baldwin, 1992; Baldwin *et al.*, 1995; Coleman and Mai, 1997; Small *et al.*, 1998; Álvarez and Wendel, 2003; Coleman, 2003; Shaw *et al.*, 2005, 2007; Borsch and Quandt, 2009). Increased use of these regions has provided great insight to the tree of life and offers opportunities for subsequent research to build upon. It has also highlighted many issues and the need for alternative methods for many groups (Álvarez and Wendel, 2003; Pirie *et al.*, 2007; Shaw *et al.*, 2007) and caution as discussed below.

Nucleotide sequences of nuclear ribosomal DNA (nrDNA) have been successfully used in comparative studies over a wide range of taxonomic levels (Medlin *et al.*, 1988; White *et al.*, 1990; Ainouche and Bayer, 1999; Schulenburg *et al.*, 1999; Calonje *et al.*, 2008). Regions of nrDNA like the bi-parentally inherited internal transcribed spacer (ITS) region (ITS1, 5.8s and ITS2) of 18-26s nrDNA can be very useful for phylogenetic studies as they are well conserved, easily examined and have high copy numbers (Baldwin, 1992; Baldwin *et al.*, 1995; Álvarez and Wendel, 2003; Coleman, 2003; Calonje *et al.*, 2008). Taxonomic relationships for closely related taxa (inter- or even intraspecific) can be explored using the rapidly evolving ITS region (White *et al.*, 1990; Calonje *et al.*, 2008). To make use of the ITS region and nearby regions, White *et al.* (1990) developed a series of universal primers to enable the sequencing of small amounts of DNA using polymerase chain reaction (PCR) and cycle sequencing (Figure 47). Making use of the 18S, 5.8S and 28S rRNA genes, the ITS primers amplify the non-coding regions, ITS1 and ITS2, between them (White *et al.*, 1990).

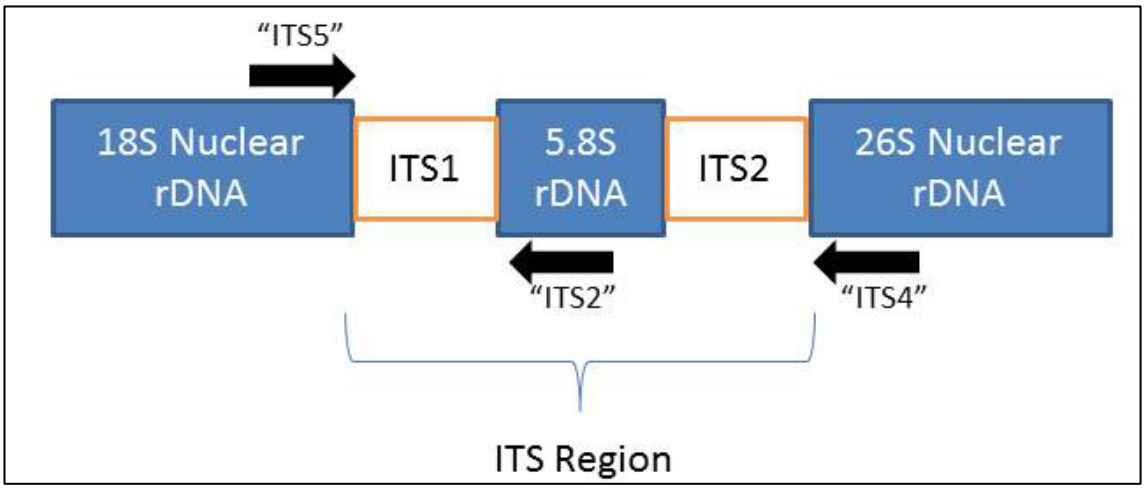

Figure 47: 18-26S repeat unit of nuclear ribosomal DNA (minus the intergenic spacer and much of the 26S subunit). Adapted from Baldwin (1992) showing the ITS region with approximate positions of primers (White *et al.*, 1990) **used to amplify single-stranded DNA for sequencing indicated by arrows.**

Interpretation of nrDNA can be complicated by sexual recombination and segregation. Different character states observed in nrDNA could represent phylogenetically relevant mutations that occurred after divergence from a common ancestor or be misleading if they existed in the common ancestor and were differentially transmitted or lost in daughter species (Baldwin, 1992; Álvarez and Wendel, 2003). The high copy number and rapid concerted evolution of nrDNA promotes intragenomic uniformity and may also promote uniformity in populations that interbreed thus minimising the importance of intrapopulational sampling for phylogenetic studies (Baldwin *et al.*, 1995). However, Álvarez and Wendel (2003) discussed three possible evolutionary fates of divergent rDNA copies after merging in a single genome - maintenance of repeat types, generation of new repeat types, and loss of repeat types via

concerted evolution – that are not mutually exclusive and have drastic implications on phylogenetic reconstruction. Feliner and Rosselló (2007) provided an in-depth overview for phylogenetic reconstruction using ITS and conclude that the region is, and will continue to be, valuable as long as proper considerations are made.

Maternally inherited plastid genomes (Corriveau and Coleman, 1988) contain non-coding DNA that has historically been considered "junk" DNA (Kelchner, 2002). Modern work has shown these non-coding DNA to have vastly varied sizes and important functions for protein coding (Willingham and Gingeras, 2006; Shaw *et al.*, 2007; Wang *et al.*, 2013). In their review of the most important non-coding plastid DNA (pDNA) markers, Borsch and Quandt (2009) discussed the well conserved secondary structure of introns, nucleotide sequences in genes that are removed by RNA splicing, that gives rise to a mosaic of extremely variable and highly conserved parts. These introns are classified by their conserved RNA folding patterns into three (*i.e*. I, II and III) groups (Michel and Dujon, 1983; Michel *et al.*, 1989; Kelchner, 2002). Spacer DNA is held between tandem repeat genes (*i.e.* rRNA) and can be partly, fully or not at all transcribed. The sequences of spacer DNA and introns are functionally less constrained (Quandt and Stech, 2004; Borsch and Quandt, 2009).

Plastid DNA, especially non-coding regions like the *trnL*^{UAA} intron and *trnL*^{UAA}-*trnF*^{GAA} intergenic spacer (Figure 48), has been used extensively for phylogenetic reconstructions as it is highly conserved across large geographic ranges and shows variation at the interspecific level (Borsch and Quandt, 2009; Haston *et al.*, 2009); however, polymorphisms in pDNA at the intraspecific level may result from normal variation or from interspecific pDNA transfer (Taberlet *et al.*, 1991). The *trnL* intron is the sole member of the group I intron group in the chloroplast genome (Kelchner, 2002).

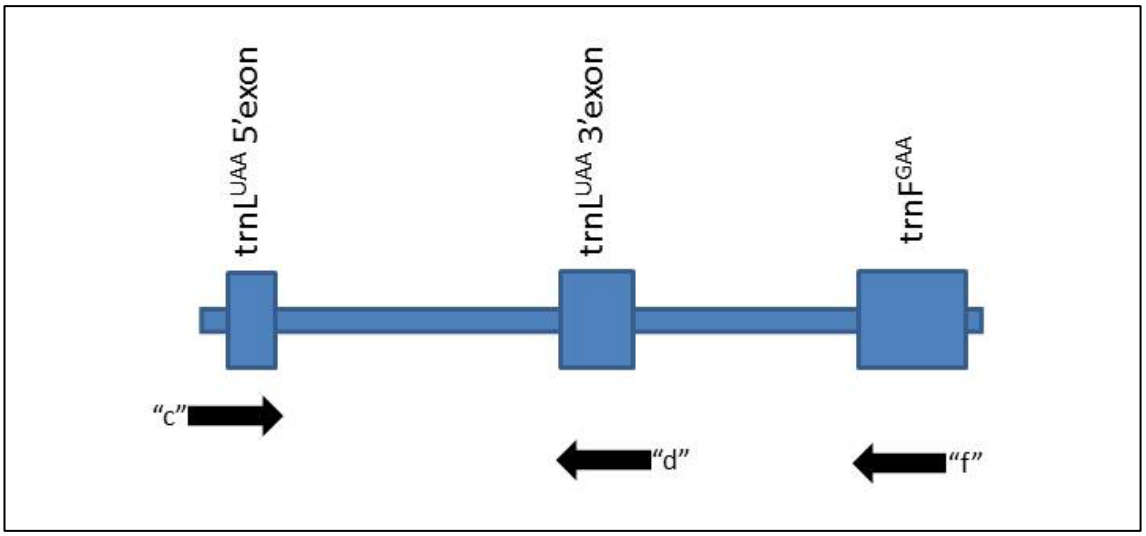

Figure 48: Directions and positions of universal primers for amplification of pDNA non-coding *trnL* and *trnL-trnF* regions. Figure adapted from Taberlet *et al.* (1991) with 3' ends of primers indicated by arrow tips.

Gielly and Taberlet (1994) reported intron evolution near that of the *trnF* intergenic spacer, making it slightly less variable and more suitable to higher level (e.g. intrafamilial) taxonomic studies (Taberlet *et al.*, 1991; Shaw *et al.*, 2005). Conversely, the *trnF* intergenic spacer shows a higher level of variation and may be suitable for lower level (e.g. interspecific) taxonomic studies (Wikström *et al.*, 1999; Shaw *et al.*, 2005).

There are several other frequently sequenced regions of pDNA (Small *et al.*, 1998; Shaw *et al.*, 2005, 2007). GenBank (Benson *et al.*, 2013) holds several *Varronia* and *Cordia* sequences for three pDNA regions: *rbcL, matK* and *ndhF*. The protein coding region, *rbcL,* codes for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO). The region has been sequenced from several plant taxa resulting in many phylogenetic studies (Olmstead *et al.*, 1992; Cuénoud *et al.*, 2002; Kocyan *et al.*, 2011) with several studies showing the region to be most suitable for studies at the intra- or interfamilial level and above (Chase *et al.*, 1993; Gielly and Taberlet, 1994; Fay *et al.*, 1997). Another protein coding region, *matK,* has been employed in many phylogenetic studies (Sang *et al.*, 1997; Cuénoud *et al.*, 2002) and has shown faster evolution times than *rbcL* (Gielly and Taberlet, 1994; Cohen, 2013) potentially making it useful for low level studies. *ndhF* is also a protein-encoding region (Cohen, 2013) that has been used for phylogenetic studies in other Boraginaceae *s.l*. and closely related families (Ferguson, 1998; Moore and Jansen, 2006).

3.1.2. Phylogenetic analysis techniques

Detecting evolutionary change requires the comparison of two or more homologous sequences from gene fragments or entire genes that share a common ancestor. Homologous sequences are aligned to form columns of homologous sites to identify the location of insertions and deletions. Indels in different lineages since divergence can result in varied length of homologous sequences. Alignment is undertaken to maximize sequence similarity and minimize gaps in complementary bases (Rodríguez–Trelles *et al.*, 2006). If sequences are from distantly related taxa, alignments can be very difficult to perform manually. In these situations, specialised software packages are used to implement algorithms to find the best alignment (Higgins and Lemey, 2009).

Phylogenetic trees constructed from alignments are used to illustrate evolutionary relationships for organisms, or genes, and depict which are most closely related. The branch tips of phylogenetic trees represent the extant taxa, often called operational taxonomic units (OTUs), and their genealogy (Barton *et al.*, 2007; Vandamme, 2009). Internal nodes of the tree represent hypothetical progenitors of OTUs. Phylogenetic trees can be rooted if an outgroup of

one or more OTUs is selected and is thought to be the most distantly related of the OTUs. The remaining OTUs form the ingroup.

Constructing phylogenetic trees from alignments is commonly undertaken using discrete character-state methods (Vandamme, 2009) with three regularly employed in the literature, listed here in order of rigour (Barton *et al.*, 2007): maximum parsimony (Fitch, 1971; Felsenstein, 1978), maximum likelihood (Felsenstein, 1981) and Bayesian inference (Dempster, 1968). The three are often employed concurrently by researchers to explore the variation in estimations as parsimony is a non-parametric technique while maximum likelihood and Bayesian techniques are parametric methods using explicit probabilistic models.

By seeking the tree that explains shared character states due to inheritance from common ancestors (Thornton, 2006), maximum parsimony (MP) leads to the tree that supposes the least evolutionary change to explain the data observed (Fitch, 1971). Alternatively, maximum likelihood (ML) selects values in a model to maximize the agreement between observed and selected model data (Felsenstein, 1981; Tateno *et al.*, 1994). Bayesian inference (BI) is based on the generation of posterior probabilities of a phylogenetic tree and a model of evolution that are based on priors and the data likelihood, generated by a multiple alignment (Dempster, 1968; Li, 1996; Larget and Simon, 1999; Mau *et al.*, 1999; Li *et al.*, 2000; Huelsenbeck *et al.*, 2014). Advances in computational power have made BI more popular as a method for assessing nodal confidence (Alfaro *et al.*, 2003), especially using the Markov chain Monte Carlo (MCMC) algorithms (Metropolis *et al.*, 1953; Hastings, 1970) and the Metropolis coupled MCMC (MC^3) variant (Altekar *et al.*, 2004). The latter requires more computational time but overcomes the possible entrapment in local optima that standard implementations of MCMC can suffer (Altekar *et al.*, 2004).

In order to estimate phylogeny using the above mentioned algorithms, a model of evolution is required to calculate the probabilities of change between nucleotides found along phylogenetic branches (Fitch and Margoliash, 1967; Posada, 2009). Given that the substitution model employed may change the phylogenetic analysis, selection of the most appropriate substitution model is an essential step for phylogeny estimation of DNA sequence alignments (Sullivan and Joyce, 2005; Posada, 2008; Johnson and Omland, 2014). Acknowledging the assertion of Box (1976) that all models are wrong and some are useful, identifying the correct model to suit those data being explored becomes even more important.

Models range in complexity from simple to overly complex. If the model is too simplistic, an underestimation of the number of substitutions two sequences have experienced since their last shared common ancestor is possible (Sullivan and Joyce, 2005). Models of evolution that

are overly complex can result in inflated variance (stochastic error), whereas those models that are too simplistic can ignore natural processes that are fundamentally important resulting in bias (systematic errors) in the results. Statistical selection of a nucleotide substitution model based on the data presented by implementing different selection strategies has become a common approach to assign a score to each candidate model enabling objective ranking (Posada and Buckley, 2004; Posada, 2008; Johnson and Omland, 2014). The models evaluated are members of the time-reversible nested family of models (Rodríguez *et al.*, 1990; Posada and Buckley, 2004; Posada, 2009).

Models frequently cited in the literature, from most to least complex, are: the General Time Reversible (GTR) (Tavare, 1986); the Hasegawa-Kishino-Yano (HKY85) (Hasegawa *et al.*, 1985); Tamura 3-parameter (T92) (Tamura, 1992); Kimura 2-parameter (K80) (Kimura, 1980) and Jukes–Cantor (JC69) (Jukes and Cantor, 1969). The complexity of these models depends on the way base frequencies and substitutions are handled (Table 6) where equal frequencies are the most simplistic. Additionally, there are models to describe rate variation among the sites in a sequence. The most commonly used models are gamma distribution (G), rate variation among sites, and proportion of static, or invariable (I), sites (Posada and Buckley, 2004).

Table 6: Nucleotide substitution models frequently cited in the literature.

Model	Acronym	Reference	Base frequencies	Substitutions
Jukes–Cantor	JC69	Jukes and Cantor, 1969	Equal	All substitutions equally likely
Kimura 2-parameter	K80	Kimura, 1980	Equal	One transition rate and one transversion rate
Tamura 3-parameter	T92	Tamura, 1992	Equal	One transition rate and one transversion rate
Hasegawa-Kishino-Yano	HKY85	Hasegawa et al., 1985	Variable	One transition rate and one transversion rate
General Time Reversible	GTR	Tavare, 1986	Variable	Symmetrical substitution matrix

Identifying trees, or parts thereof, that are well supported, adequate for inferring evolutionary systems and provide a conceptual framework for trait evolution requires confidence measures to be employed (Felsenstein, 1985; Efron *et al.*, 1996; Huelsenbeck *et al.*, 2000; Alfaro *et al.*, 2003). The most commonly used confidence measures are non-parametric bootstrap proportion and posterior probabilities. Bootstrap analysis is a statistical method that allows testing of the reliability of a given dataset through the creation of pseudo-replicates by resampling and was first applied to phylogenetics by Felsenstein (1985). Bootstrapping provides an estimation of error and, thus, the reliability of a tree. Posterior probability provides weighting of character analysis for each tree that it is correct. This enables

comparative analysis of all trees by sampling based on their posterior probability (Huelsenbeck *et al.*, 2000).

Modern phylogenetic analyses are an attempt at reconstructing evolutionary history (Huelsenbeck *et al.*, 2014). There is a plethora of software available to work with and interpret DNA sequences for constructing phylogenetic trees with statistical methods and computational demands varying widely. The purpose of employing these software is to identify the evolutionary tree that explains the observed data requiring the fewest evolutionary events (Felsenstein, 1978). Many challenges can be encountered when undertaking phylogenetic analysis and understanding the pitfalls and potential solutions is paramount before beginning any study. Sanderson and Shaffer (2002) reviewed the most common issues encountered (e.g. weak tree support, data conflict, model selection, computation time) and provide possible solutions. This research has tried to address these common issues through the software programmes chosen and analyses undertaken as presented in the following sections.

3.2. Materials and Methods

ITS and *trnL-trnF* regions were chosen for this study as they previously proved successful in determining generic limitation and provided insight into species limitations in Boraginaceae (Gottschling *et al.*, 2001; Gottschling, 2003; Gottschling *et al.*, 2005; Miller and Gottschling, 2007; Cohen, 2013). ITS has been shown to be easily amplified from herbarium specimens (Baldwin *et al.*, 1995) and with *trnL-trnF* offers the opportunity to include dead plants in the current study (Särkinen *et al.*, 2012). All DNA samples were extracted and sequenced in the Jodrell Laboratory, Kew.

3.2.1. Sampling

Dried plant material of various ages was used for DNA extraction. Samples were sourced from herbarium specimens held in the herbaria at Kew (K) or Fairchild Tropical Botanical Garden (FTG) as well as silica dried samples (Chase and Hills, 1991; Särkinen *et al.*, 2012) from cultivated and wild plants, both living and dead (Appendix 4: New samples used for phylogenetic analysis). All vouchers collected from cultivated and wild plants are deposited at K with duplicates lodged at other herbaria.

DNA extraction

Approximately 50 mg of dried plant material was used for genomic DNA extraction and followed a modified 2×CTAB protocol (Saghai-Maroof *et al.*, 1984; Doyle and Doyle, 1987; Csiba and Powell, 2006). Sterilised sand, a stainless steel bead and small pieces of leaf material were combined in a 2 ml Eppendorf® tube for each sample, and then ground into a fine powder using a Retsch Mixer Mill MM301 (Retsch, Haan, Germany, a Verder Group company).

Immediately after grinding, 750 µl of 65 °C pre-heated 2×CTAB buffer [100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB (hexadecyl trimethyl ammonium bromide)] and β-mercaptoethanol (1 ml:4 µl) was added to the samples. The samples were incubated for 20 minutes in a 65 °C water bath before adding 750 µl of SEVAG (24:1 chloroform:isoamyl alcohol). The sample was extracted for one hour at 20 °C by rocking before spinning down at 9000 rpm for ten minutes at 20 °C. The aqueous top layer containing genomic DNA was pipetted into new 2 ml Eppendorf® tubes with 500 µl of -20 °C isopropanol and gently mixed to precipitate DNA before storing for 24 hours at -20 °C. To collect the precipitate, the sample was spun at 4000 rpm for five minutes before pouring off the liquid. The remaining pellet was washed in 750 µl of 70% ethanol for five minutes on a rocker before spinning down DNA at 4000 rpm for five minutes. All remaining liquid was poured off and the sample left in a fume cupboard for 24 hours to allow complete evaporation. Genomic DNA was re-suspended in 125 µl of TE 0.1 buffer (10 mM Tris-HCl pH 8, 0.25 mM EDTA) and left to dissolve for 24 hours at 3 °C. Samples were rocked for five minutes to dissolve genomic DNA before it was purified using Nucleospin DNA purification columns following the protocol supplied by the manufacturer (QIAquick; Qiagen Ltd, Crawley, UK). To ensure a suitable quantity of genomic DNA was available for analysis (>10 ng/µl), a Nanodrop (ThermoScientific, Denver, CO) was used to measure each sample. A polymerase chain reaction (PCR) was performed for each region as described below.

ITS PCR

Genomic DNA samples were initially run using the primers ITS5 and ITS4 (White *et al.*, 1990) to amplify the entire ITS region (ITS1, 5.8s and ITS2). Any samples that failed to amplify were repeated using the primers ITS5 and ITS2 (White *et al.*, 1990) to only amplify the ITS1 region. ITS amplifications were performed in 25 µL reactions, containing 22.5 µL PCR Abgene mastermix (1.5 mM mg), 5 µL TBT-PAR [trehalose, bovine serum albumin (BSA), and polysorbate-20 (Tween-20) (Samarakoon *et al.*, 2013)], 0.5 µL each primer, 1.2 µL dimethylsulphoxide (DMSO) and 1.0 µL template DNA (~10 ng). DMSO was added to improve PCR amplification of the GC-rich ITS template (Kang *et al.*, 2005). The PCR profile for both primer pairs was as follows: initial denaturation of 94 °C for 2 min, followed by 32 cycles of denaturation at 96 °C for 1 min, annealing at 48 °C for 1 min, extension at 72 °C for 50 sec, followed by a final extension of 7 min at 72 °C. All PCR experiments included a positive control (known working sample) and a negative control [DNA replaced with sterile deionised water (sdH_2O)].

trnL^{UAA} intron – trnL^{UAA}-trnF^{GAA} spacer PCR

Genomic DNA samples were initially run using the primers c and f of Taberlet *et al.* (1991) to amplify the entire *trnL-trnF* region. Any samples that failed to amplify were repeated using the primers c and d (Taberlet *et al.*, 1991) to only amplify the *trnL^{UAA}* intron. Amplifications were performed in 25 µL reactions, containing 22.5 µL PCR Abgene mastermix (1.5 mM mg), 5 µL TBT-PAR (Samarakoon *et al.*, 2013), 0.5 µL each primer and 1.0 µL template DNA (~10 ng). The PCR profile for both primer pairs was as follows: initial denaturation of 96 °C for 2min, followed by 32 cycles of denaturation at 96 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2min, followed by a final extension of 7 min at 72 °C. All PCR experiments included a positive and a negative control.

PCR products from the ITS and *trnL-trnF* regions were purified using Nucleospin DNA purification columns following the protocol supplied by the manufacturer (QIAquick; Qiagen Ltd, Crawley, UK). Dideoxy cycle sequencing was performed next with an ABI Prism Big Dye version 3.1 reaction kit as per the manufacturers' protocol (Applied Biosystems Inc., Warrington, UK) using the chain termination method. Dideoxy cycle sequencing products were run on an ABI 3730 Genetic Analyser, according to the manufacturers' protocol (Applied Biosystems Inc., Warrington, UK).

3.2.2. Molecular analysis

Molecular analyses were undertaken using a range of software programmes selected for their ease of use, availability of documentation and easily interpretable outputs (Table 7). Complementary strands from the ABI 3730 sequencer were imported, aligned, combined into matrices and used to generate informative phylogenetic trees using Geneious software (Geneious version 7.1 created by Biomatters. Available from http://www.geneious.com/) on a PC running Windows 7.

Table 7: Software packages employed in this research for phylogenetic analyses showing software version, operating system of personal computer and use for the software.

Software package	Version	Operating system	Use
Geneious	7.1.7	Windows 7	Base calling, Sequence alignment, MegaBLAST, Matrix building, Tree building, NEXUS generation
MrBayes plug-in for Geneious	7.1	Windows 7	Bayesian analysis
MrBayes	3.2.2	Windows 8.1	Bayesian analysis
FigTree	1.4	Windows 8.1	Interpret MrBayes outputs, Tree building
MEGA6	6.0.5	Windows 8.1	Nucleotide substitution model selection, ML & MP analysis, Tree building

Two versions of MrBayes were used in this study. Initially, the MrBayes 3.2.1 plugin for Geneious software (developed by Marc Suchard and the Geneious team) was selected as it worked directly with the software package used to align DNA sequences on a PC running Windows 7. Initial analyses allowed the exploration of different models and check sequences applicability for this research. Subsequently, MrBayes 3.2.2 software (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) was used on a PC running Windows 8.1 to undertake the final Bayesian analyses that are presented here. FigTree (version 1.4 developed by Andrew Rambaut; Available from http://tree.bio.ed.ac.uk/) was used to interpret MrBayes outputs and generate trees on a PC running Windows 8.1.

Molecular Evolutionary Genetics Analysis (MEGA6) software (version 6.0.5; Available from http://www.megasoftware.net/) (Tamura *et al.*, 2013) was used for multiple analyses including: identification of the best-fit model of DNA evolution for ML and BI analyses; MP and ML analyses; and building all trees presented here on a PC running Windows 8.1.

ITS and trnL-trnF *regions*

DNA sequences were imported into Geneious software for assembly using the default settings. Following assembly of complementary strands, sequences were manually checked to verify software base-calling and edit where necessary. Assembly failed for many of the herbarium specimens sampled.

Assembled sequences for each region (ITS1, ITS, *trnL* and *trnL-trnF*) were aligned, respectively, in Geneious software using the default settings. Following alignment, the consensus sequence for ITS and *trnL-trnF* regions were used to perform two separate megaBLAST (Morgulis *et al.*, 2008) searches of GenBank (Benson *et al.*, 2013) from within Geneious returning a maximum of 1000 sequences per search. The sequences returned were sorted by species and all *Cordia* and *Varronia* sequences were selected for each region, respectively, and added to Geneious workspace for further analyses.

Separate visual assessments were undertaken for aligned sequences generated by this study and combined alignments of sequences from this study and GenBank for each region to compare base calling and length of sequences. Sequences from either source that were found to be largely incomplete (missing >75 bp) or had a high proportion of ambiguous bases were excluded from subsequent analysis. The latter was specifically undertaken for GenBank sequences as the source sequences are not visible and it is therefore not known if the base calling/editing was poor or the bases did not agree due to variation in copies of the region. *Heliotropium angiospermum* Murray was selected as outgroup for all analyses using GenBank samples HQ286121 (ITS) and HQ286151 (*trnL-trnF*).

3.2.3. Statistical analyses

Initial analyses of ITS and *trnL-trnF* regions were undertaken using the MrBayes plugin developed for Geneious software. Trees for each region were built using the GTR substitution model and default settings. The initial trees were used to assess sequence quality and taxon identification, particularly for GenBank sequences. Multiple GenBank sequences representing the same taxa that passed both visual alignment and initial tree inspection were excluded leaving a single representative sequence for the analyses. Initial analyses of OTU placements showed some conflict between nrDNA and pDNA markers; therefore, four separate analyses, A to D, were undertaken. Analysis A used ITS1 data for 121 sequences with an aligned sequence length of 287 bp. Analysis B combined all ITS data (ITS1, 5.8s and ITS2) from 92 sequences with an aligned sequence length of 705 bp. Analysis C used *trnL* data from 47 sequences with an aligned sequence length of 486 bp. Analysis D used all *trnL-trnF* data from 28 sequences with an aligned sequence length of 920 bp.

Bayesian analysis for the first four analyses, A-D, using MrBayes plugin for Geneious was performed separately with the GTR model, priors of unconstrained branch lengths of 10 and a random starting tree. Two simultaneous four chain (three heated, one cold) MC^3 analyses were run with 0.02 chain temperature for 900,000 generations sampling one tree each 200 generations. Burn-in was set at 100,000 and Gamma distributed (+G) rates. The resulting trees were consulted within Geneious (Figure 49) before further analyses were undertaken.

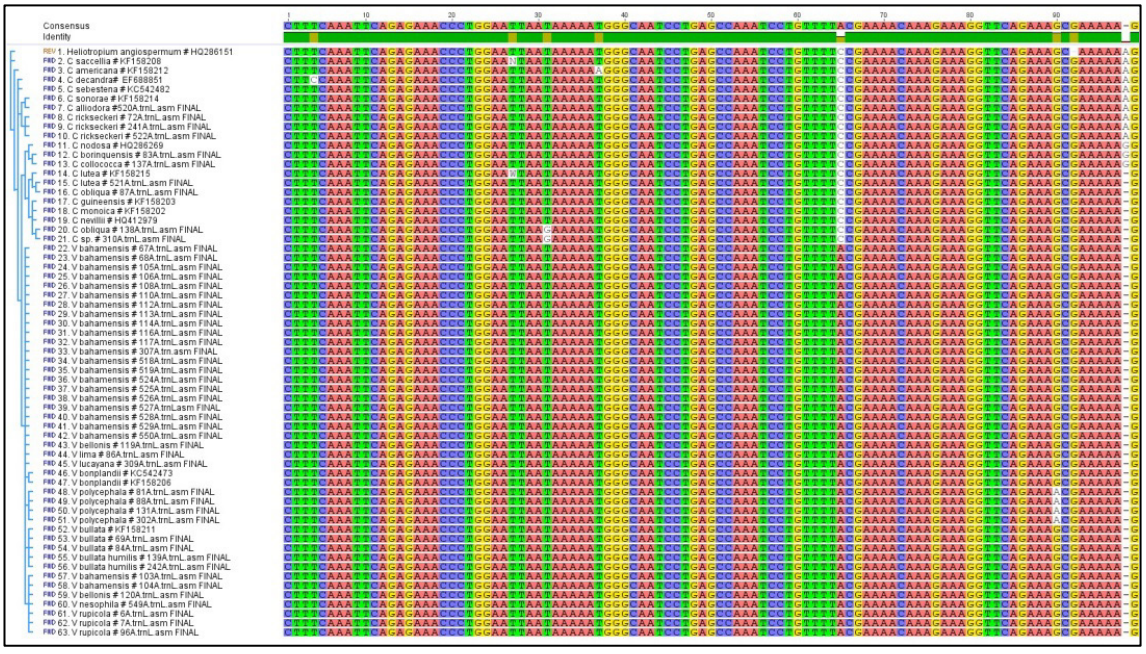

Figure 49: Nucleotide alignment generated in Geneious following initial Bayesian analysis showing minimal variation in bases across the *trnL* region.

Following the initial four analyses, A-D, it was determined that a fifth analysis, E, should also be undertaken with the combined ITS and *trnL-trnF* data generated by this research. Twenty-five

sequences for both regions were available along with GenBank outgroup, *Heliotropium angiospermum,* which resulted in a combined alignment of 26 taxa totalling 1625bp. Base change calculations were undertaken using MEGA6 for analyses A-E to explore the differences between sequences per taxa. All positions containing gaps and missing data were eliminated.

Programme specific, formatted files (*i.e.* .nex, .meg) for the five analyses, A-E, were exported from Geneious to enable MP, ML and BI analyses in external software packages. Nucleotide substitution model selection for ML and BI analyses was undertaken to identify best-fit models for the five separate analyses (Table 8). Using MEGA6, three different best-fit models were selected K80, HKY85 and T92 for the ML analyses A/B, C/D and E in MEGA6 software, respectively. The same models were used for BI analyses in MrBayes software except for analysis E where the partitioned regions of ITS and *trnL-trnF* were run using separate models, K80 and HKY85, respectively. Models for all analyses were selected based on low statistical modelling scores and the ability to implement the model in software packages used for phylogenetic estimations.

Table 8: DNA substitution models used in this research per model type and analysis.

Analysis	ML models	BI models
A	K80, +G	K80, +G
B	K80, +G	K80, +G
C	HKY85, +G	HKY85, +G
D	HKY85, +G	HKY85, +G
E	T92, +G	K80, +G; HKY85, +G

Maximum parsimony (MP) analysis and bootstrapping (n = 1000) was undertaken for the five separate analyses, A-E, using MEGA6. MP was performed with complete deletion of gaps/missing data and tree inference was undertaken using ten initial trees made randomly and default search level settings.

MEGA6 was also used for maximum likelihood (ML) analysis and bootstrapping (n = 1000) for the five separate analyses, A-E. Analyses A and B used the Kimura 2-parameter (K80) model, Gamma distributed (+G) with five discrete categories and complete deletion of gaps/missing data. The initial tree for ML was made automatically using default settings with very strong branch swap filter. Analyses C/D and E only differed in model selection with HKY85 and T92, respectively.

Bayesian Inference (BI) analysis for the five separate analyses using MrBayes was performed separately with uniform priors and a random starting tree. Two simultaneous five chain (four heated, one cold) MC^3 analyses were run with 0.04 chain temperature for 20,000,000 generations sampling one tree each 1000 generations. Burn-in was set at 25,000 and Gamma distributed (+G) rates. To verify convergence on the same likelihood, Bayesian analyses were repeated twice. Analyses A and B used the K80 model. Analyses C and D used HKY85. Analysis E contained partitioned data for ITS and *trnL-trnF*; therefore, the models K80 and HKY85 were implemented on the partitioned regions, respectively.

Consensus trees from BI analyses were first viewed in FigTree software and then exported in Newick format for viewing in MEGA6. Consensus trees from MP and ML were consulted directly in MEGA6. Graphics showing bootstrap (MP and ML) and posterior probability (BI) percentages shown here were produced in MEGA6.

3.3. Results

Assembly failed for many of the herbarium specimens sampled due to poor amplification of degraded DNA, most likely caused by preservation and storage techniques (Golenberg *et al.*, 1996). Extracted DNA from type specimens of *V. rupicola* and *V. bahamensis* was successfully re-run to sequence only the *trnL* and ITS1 regions. Thirty-three new sequences were generated for both the ITS1 and *trnL* regions (Appendix 4: New samples used for phylogenetic analysis). For the entire ITS and *trnL-trnF* regions, 28 and 27 new sequences were generated, respectively. In total, 168 sequences were selected for analysis with 102 of those coming from GenBank (Appendix 5: GenBank sequences used in phylogenetic analysis). Those from GenBank included 88 ITS1, with 64 of those also covering the entire ITS region, 14 *trnL* sequences and only one covering the entire *trnL-trnF* region.

Following Chase *et al.* (2000), bootstrap support is provided for MP and ML analyses undertaken with the following category descriptions: weak (50-74%); moderate (75-84%); strong (>85%). The category descriptions of posterior probabilities for BI analyses follow Duangjai *et al.* (2009): high support (>94%); moderate support (50–94%); weak support (<50%). Numbers of differences in the following text refers to base change calculations used to estimate evolutionary divergence between sequences of *V. rupicola* and all others.

3.3.1. Geneious analyses

Initial analyses of the ITS and *trnL-trnF* regions using MrBayes plugin for Geneious showed a well-supported (100%PP), monophyletic *Varronia*. However, there appeared to be conflict between nrDNA and pDNA markers for the relative placement and closest relatives of *Varronia rupicola.* This was probably due to limited divergence, especially in pDNA, and is probably due

to recent hybridisation events. This potential conflict and difference in sequence length and number of available samples in each of the nrDNA and pDNA regions resulted in five separate analyses, A-E (Table 9).

Table 9: Statistical analyses of sequence data by analysis and data source. "Combined" refers to sequences from GenBank with new sequences generated by this research; "This study" refers to new sequences generated by this research alone. Calculations of identical sites, pairwise identity and GC percentage were not performed for Analysis E as this would not be informative.

Analysis	Source	# sequences	Length	Identical sites	% Identical	Pairwise % identity	GC% of non-gaps
A	Combined	121	287	83	28.9	83.1	53.4
	This study	33	274	147	53.6	89.5	54.0
B	Combined	92	705	286	40.6	84.1	55.7
	This study	28	683	411	60.2	88.7	55.0
C	Combined	47	486	406	83.5	96.6	33.6
	This study	33	477	429	89.9	97.8	34.1
D	Combined	28	920	772	83.9	96.9	34.3
	This study	27	905	801	88.5	97.4	34.8
E	Combined	26	1625	–	–	–	–
	This study	25	1588	–	–	–	–

Four Bayesian analyses using the MrBayes plugin for Geneious were attempted. Due to computational limitations (lack of available memory on ageing hardware) posterior probabilities files were not generated for analysis B or D even though consensus trees were generated. ML and MP analysis using Geneious was not possible due to limited functionality/lacking additional software. For these reasons, final analyses were undertaken outside of the Geneious platform and are reported in the next section.

3.3.2. ITS and *trnL-trnF* final analyses

After deleting gaps and missing data, analyses A through E involved 178, 511, 453, 837 and 1415 positions in the final dataset, respectively. Limited divergence in Caribbean *Varronia* species, especially in pDNA, and specific variation is discussed below for each analysis. MP, ML and BI analyses were undertaken for all five datasets and are reported below by analysis.

Analysis A - ITS1

Strongly supported clades using analysis group A (Figure 50) are shown for *Varronia*, MP (94%), ML (97%) and BI (100%), as well as *Cordia*, MP (81%), ML (86%) and BI (100%), across all analyses. There is minimal variation in the OTUs between MP, ML and BI analyses. Type specimen sequences for *V. rupicola* (samples 3B2 and 4B2) and *V. bahamensis* (sample 74B2) resolved in the same clade as sequences of living and recently dead plants curated under the same names. *Varronia rupicola* and *V. bahamensis* sequences showed zero differences, and using all three analyses (MP, ML and BI), were sister to *V. bellonis* (one difference). The

positions of *V. lima* (six differences) and *V. nesophila* (two differences) changed considerably between the three analyses.

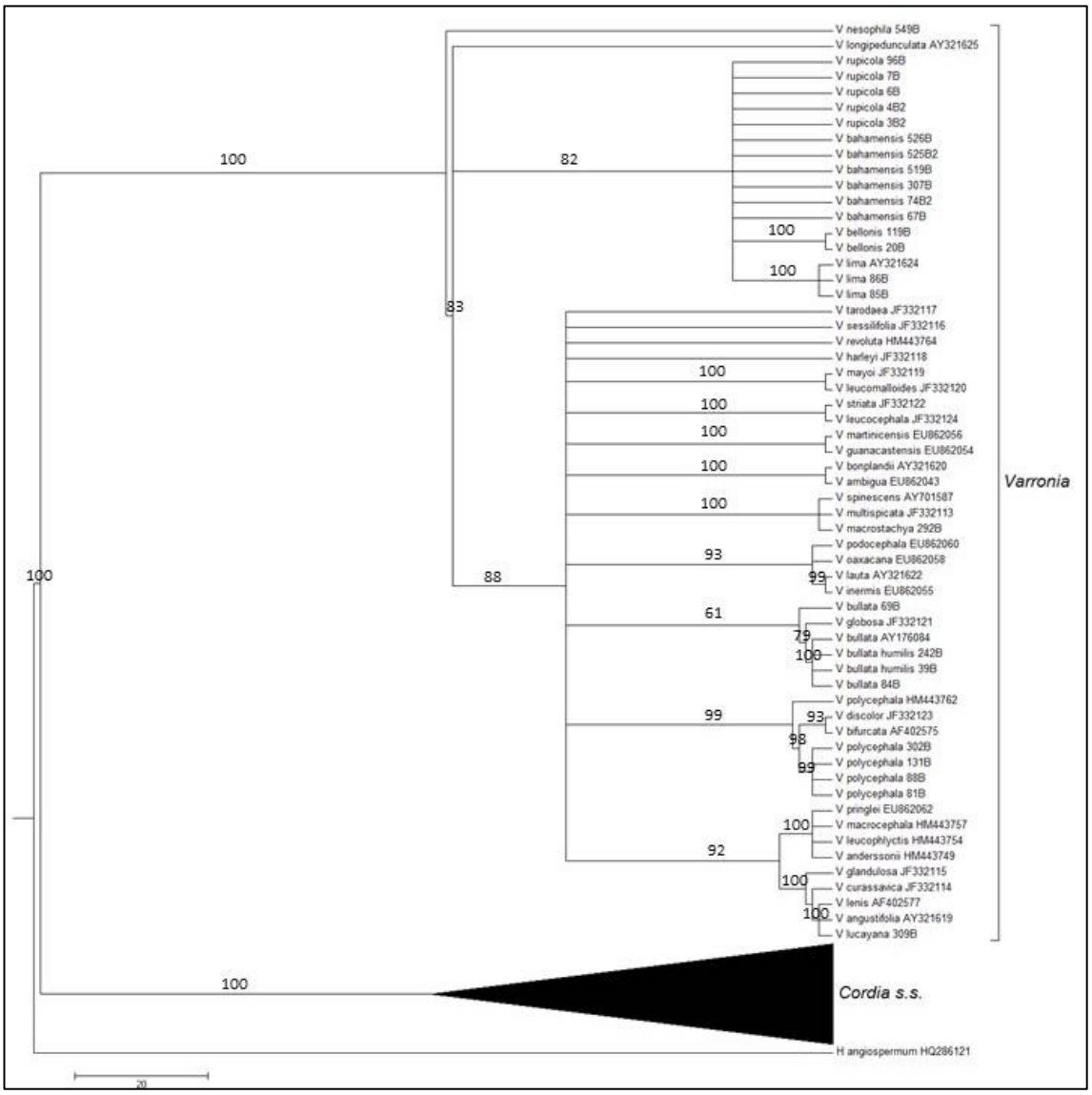

Figure 50: Bayesian analysis A consensus tree showing the *Varronia* clade with branch numbers as posterior probability (PP) percentages and PP values <50% not shown. *Cordia s.s.* has been compressed to show detail of the *Varronia* clade with *H. angiospermum* as outgroup. Numbers/letters following specific epithets are either sample numbers for this research or GenBank numbers and correspond to Appendix 4 or 5, respectively.

Analysis B - entire ITS region

Analysis group B also showed strongly supported clades (Figure 51) across the analyses for *Varronia,* MP (99%), ML (99%) and BI (100%), as well as for *Cordia,* MP (81%), ML (61%) and BI (95%). There is minimal OTU variation between MP, ML and BI analyses. *Varronia rupicola* and *V. bahamensis* sequences showed one difference and were shown in a well-supported clade in all three analyses. The positions of *V. bellonis* (one difference), *V. lima* (17 differences) and *V. nesophila* (13 differences) changed considerably between the three analyses.

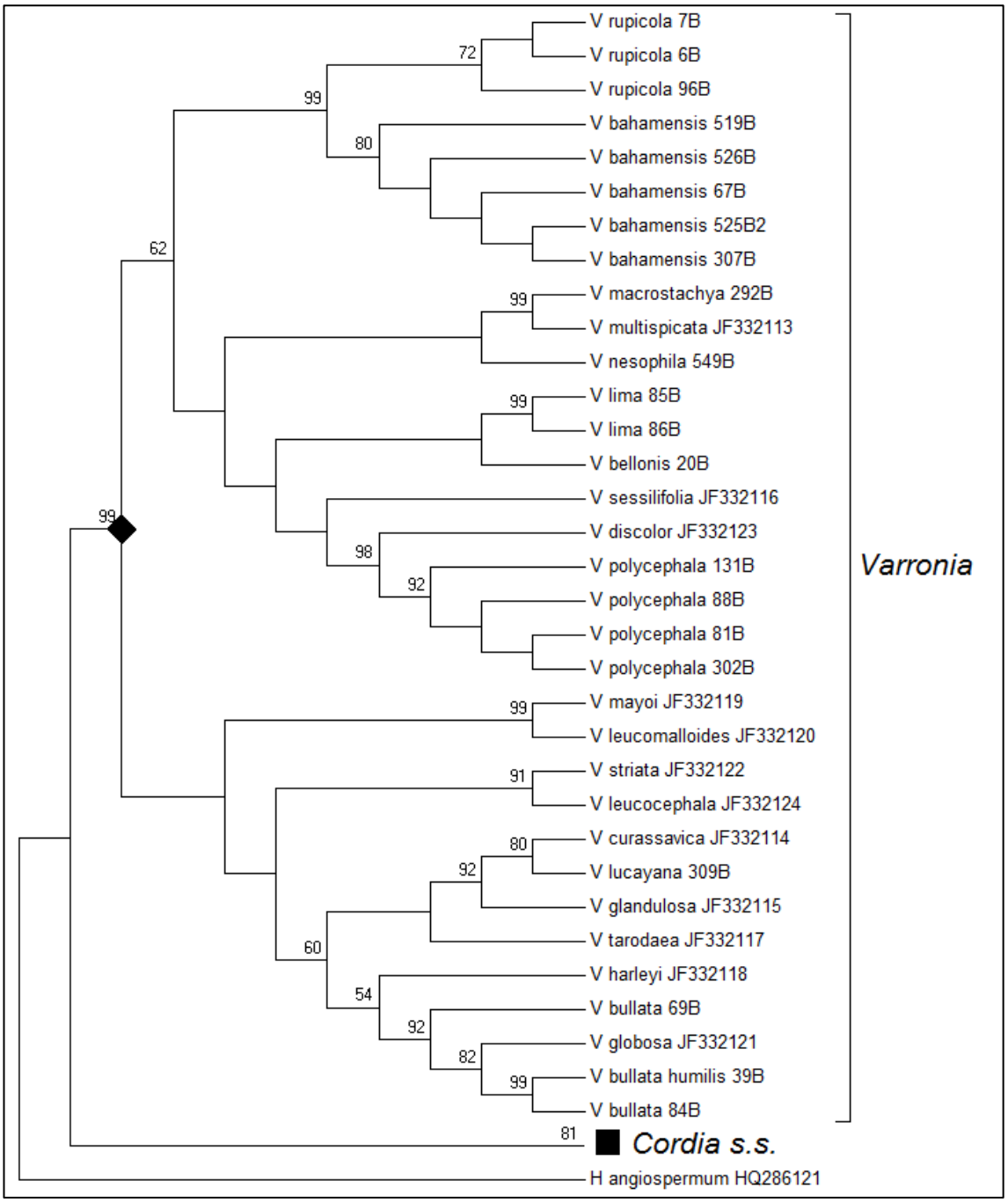

Figure 51: Maximum parsimony analysis B consensus tree showing the *Varronia* clade with branch numbers as bootstrap percentages and BS values <50% not shown. *Cordia s.s.* has been compressed to show detail of the *Varronia* clade with *H. angiospermum* as outgroup. Numbers/letters following specific epithets are either sample numbers for this research or GenBank numbers and correspond to Appendix 4 or 5, respectively.

Analysis C - trnL

Strongly supported *Varronia* clades (Figure 52) were shown for analysis C across MP (99%), ML (98%) and BI (100%); however, *Cordia* is not resolved. There is minimal variation in the OTUs between the three analyses with *V. rupicola* sequences showing two differences with *V. bahamensis* and *V. lima* and not resolved in a clade in any of the analyses. *Varronia rupicola* was resolved in a well-supported clade with *V. bellonis* and *V. nesophila* (both with zero differences) in all analyses.

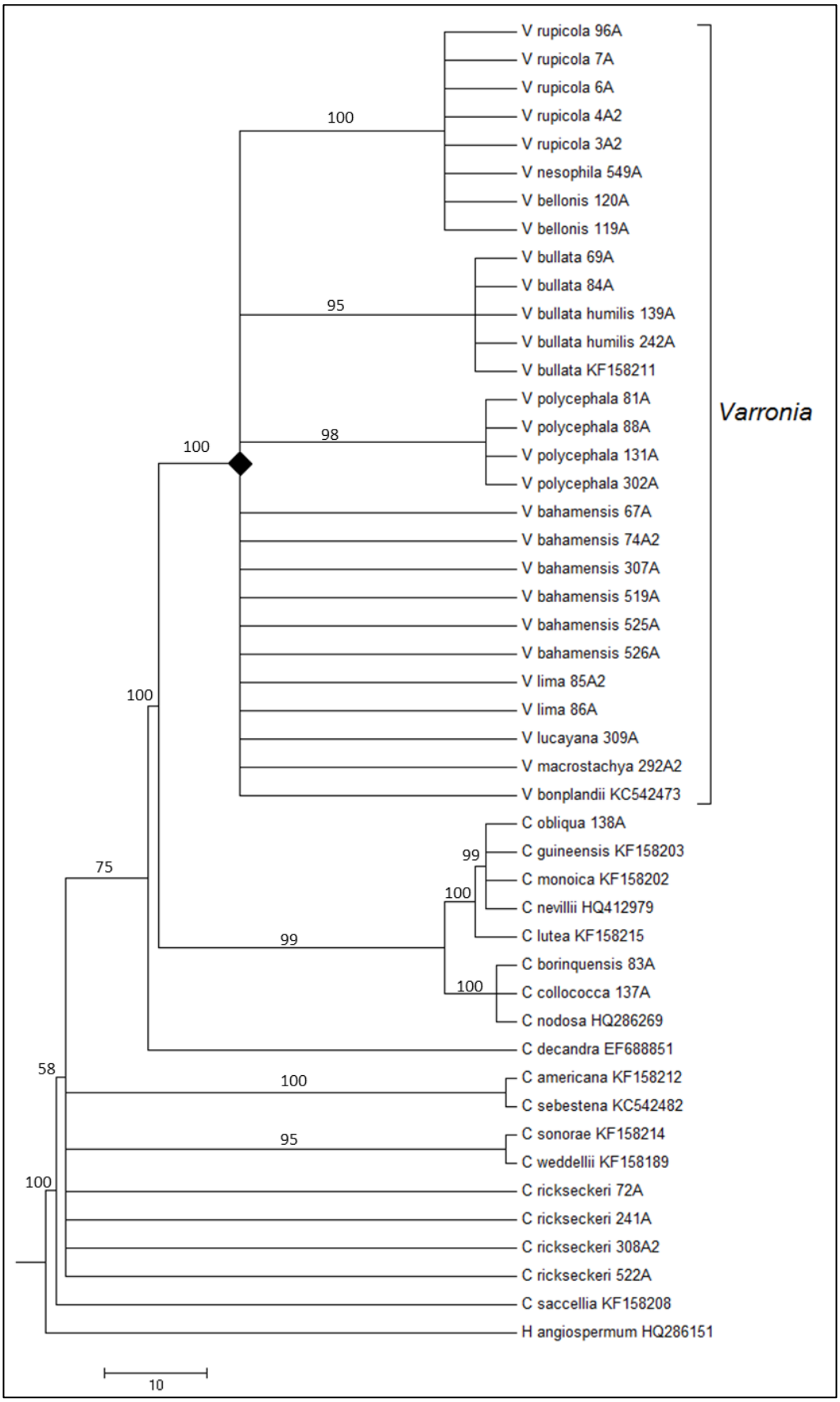

Figure 52: Bayesian analysis C consensus tree showing all clades with *H. angiospermum* as outgroup with branch numbers as posterior probability (PP) percentages and PP values <50% not shown. Numbers/letters following specific epithets are either sample numbers for this research or GenBank numbers and correspond to Appendix 4 or 5, respectively.

Analysis D – entire trnL-trnF *region*

Similar to Analysis group C, *Cordia* was not resolved in any analysis of group D, whereas all analyses showed a strongly supported (Figure 53) *Varronia* clade (100% for MP/ML/BI). There is minimal variation in the OTUs between MP, ML and BI analyses. *Varronia rupicola* and *V. bahamensis* sequences showed two differences and were not resolved in a clade in any of the three analyses. *Varronia rupicola* was resolved in a well-supported clade in the three methods, sister to *V. bellonis* (zero differences) and *V. nesophila* (zero differences). *Varronia lima* (three differences) was resolved near *V. bahamensis* in all analyses.

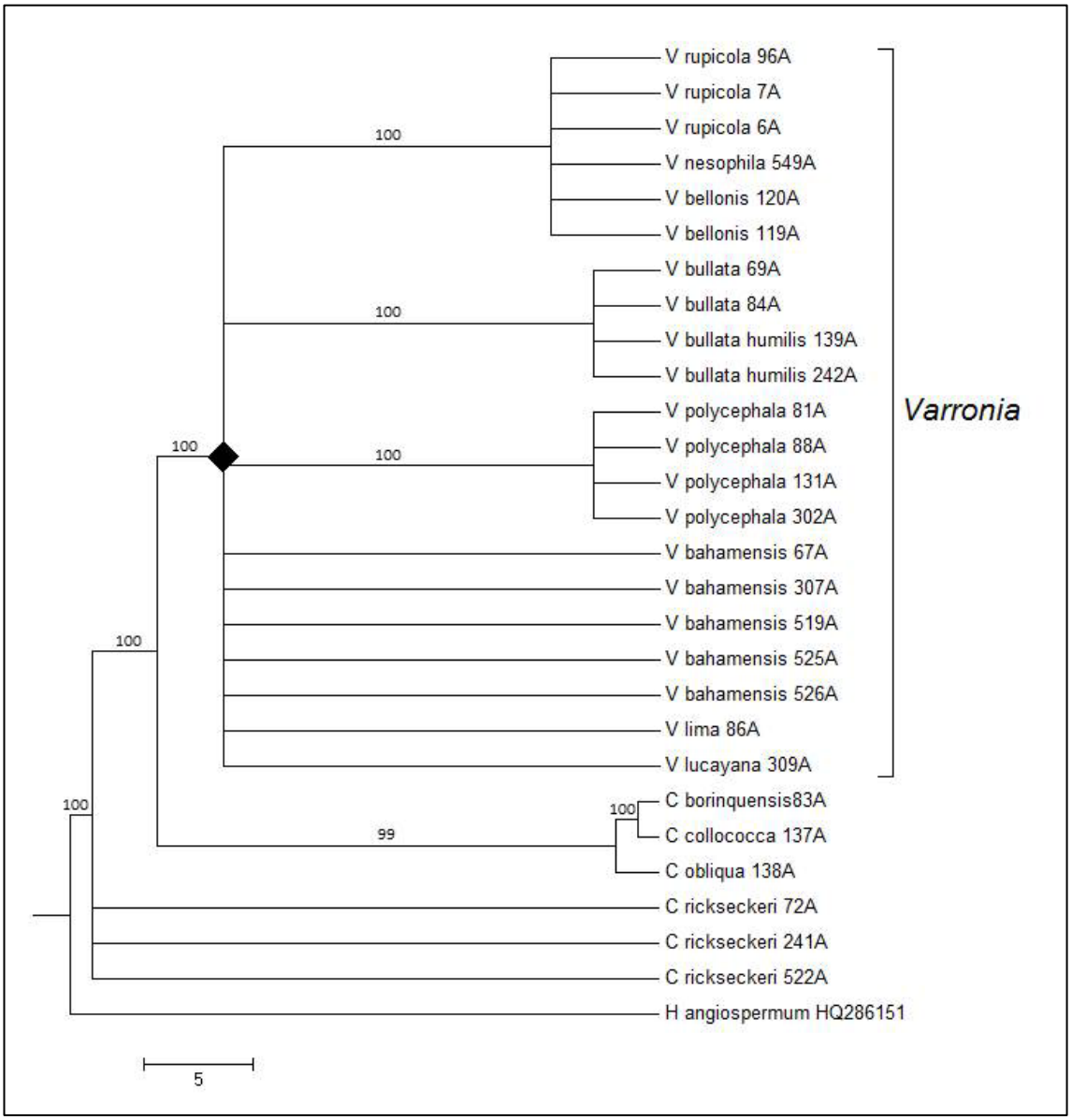

Figure 53: Bayesian analysis D consensus tree showing all clades with *H. angiospermum* as outgroup with branch numbers as posterior probability (PP) percentages and PP values <50% not shown. Numbers/letters following specific epithets are either sample numbers for this research or GenBank numbers and correspond to Appendix 4 or 5, respectively.

Analysis E - combined ITS and trnL-trnF

Strongly supported clades (Figure 54) across all three analyses for *Varronia* (100% for MP/ML/BI) and *Cordia* (MP (80%) ML (53%) and BI (57%)) were shown for analysis group E. There is negligible variation in the OTUs between MP, ML and BI analyses. *Varronia rupicola* and *V. bahamensis* sequences showed four differences and were shown in a well-supported (MP (96%), ML (95%) and BI (100%)) clade in all analyses. The positions of *V. bellonis* (14 differences), *V. lima* (21 differences) and *V. nesophila* (18 differences) changed minimally between the three methods.

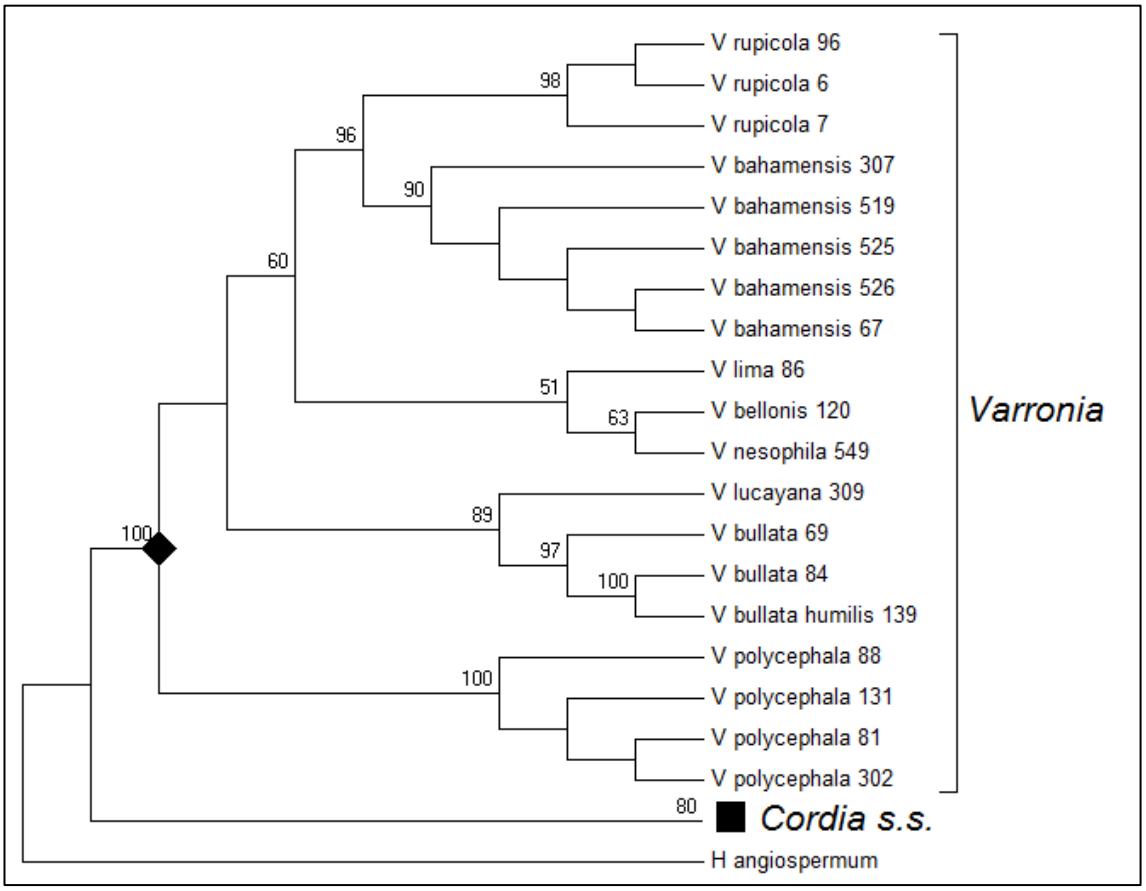

Figure 54: Maximum parsimony analysis E consensus tree showing the *Varronia* clade with branch numbers as bootstrap percentages and BS values <50% not shown. *Cordia s.s.* has been compressed to show detail of the *Varronia* clade with a GenBank selected outgroup, *H. angiospermum*. Numbers/letters following specific epithets are either sample numbers for this research or GenBank numbers and correspond to Appendix 4 or 5, respectively.

3.4. Discussion and conclusions

Varronia rupicola has been traditionally placed in Boraginaceae *s.l.* subfamily Cordioideae based on morphological characters. Sixty-seven *Varronia* species were listed by Acevedo-Rodríguez and Strong (2012) as native to the Caribbean, nine of which are native to the Puerto Rican Bank. Sequences were not available from three of these species for any analyses: *V. linnaei* (Stearn) J.S.Mill., *V. portoricensis* (Spreng.) Feuillet and *V. wagnerorum* (R.A.Howard)

Borhidi. Sequences of one other species, *V. curassavica* Jacq., were not available for analyses C, D and E. Inclusion of sequences from these species in future research is recommended.

The initial conflict observed between nrDNA and pDNA regions for the OTUs led to separate analysis for ITS and *trnL-trnF* regions. Weeks *et al.* (2010) found similar conflict for Galapagean *Varronia* species and suggested that this conflict was probably due to hybridisation events and the differences in inheritance seen between the maternally inherited *trnL-trnF* pDNA region and bi-parentally inherited ITS nrDNA region. Subsequent interpretation of phylogenetic trees and the number of base differences per sequence generated by this research suggested that combined analyses of nrDNA and pDNA markers should be undertaken as most of the issues with OTUs were due to excessive branch swapping as a result of very low divergence. Similarly low levels of infrageneric pairwise sequence variation for both regions have been seen in Mediterranean Cistaceae (Guzmán and Vargas, 2005) and Resedaceae globally (Martín-Bravo *et al.*, 2007) as well as specifically for the *trnL-trnF* region in Korean *Citrus* (Jung *et al.*, 2005).

3.4.1. Phylogenetic relationships

The outgroup, *Heliotropium angiospermum*, was chosen as previous studies have shown *Heliotropium* sister to *Cordia s.l.* (Gottschling *et al.*, 2001). All phylogenetic trees presented here using ITS and *trnL-trnF* regions confirm that *V. rupicola* is a part of the monophyletic genus *Varronia* with >94%BS or 100%PP, depending on analysis group and method (Table 10). Given the data generated, *V. rupicola* and several closely related species appear to be of relatively recent hybrid origin. Determining if *V. rupicola* is a distinct taxon and its placement was complicated by low divergence, especially in the *trnL-trnF* region of pDNA. Similar results have been explored in other groups (e.g. *Pinus*) using multiple low-copy nuclear loci (Syring *et al.*, 2005) without fully resolving the issue.

Table 10: Support values for monophyletic *Cordia s.s.* and *Varronia* by analysis method with dashes (–) in analysis C and D denoting *Cordia s.s.* was not resolved as monophyletic.

Character-state method	Analysis A		Analysis B		Analysis C		Analysis D		Analysis E	
	Cordia s.s.	*Varronia*	*Cordia s.s.*	*Varronia*	*Cordia s.s.*	*Varronia*	*Cordia s.s.*	*Varronia*	*Cordia s.s.*	*Varronia*
MP (BS%)	81	94	81	99	–	99	–	100	80	100
ML (BS%)	86	97	61	99	–	98	–	100	53	100
BI (PP%)	100	100	95	100	–	100	–	100	57	100

ITS region (ITS1, 5.8s and ITS2)

Based on ITS, nrDNA shows highly supported clades for *Varronia* and *Cordia*. Type specimen sequences for *V. rupicola* and *V. bahamensis* resolved in the same clade as sequences of living and recently dead plants of the same names in analysis A. Variation in the OTUs between MP, ML and BI analyses, especially evident for ITS1, appears to be the result of excessive branch swapping due to low divergence. This suggests the region is useful for intergeneric studies while it has a limited ability to be used for inter- and intraspecific studies. Additional bases from 5.8s and ITS2 resulted in better resolution demonstrating good interspecies, but still limited intraspecific, usefulness. For *V. rupicola* and *V. bahamensis*, well-supported clades were resolved in all three analyses (MP, ML and BI) for the two species with placement support (BS/PP) ranging widely (Table 11).

Table 11: Support values for monophyletic *V. rupicola* and *V. bahamensis* by analysis method with asterisks (*) denoting *V. rupicola* and *V. bahamensis* are in a supported (>50% BS) clade. Analyses A, C and D are not shown as BS/PP% values were <50% and/or *V. rupicola* and *V. bahamensis* were not resolved as monophyletic.

Character-state method	Analysis B		Analysis E	
	V. rupicola	*V. bahamensis*	*V. rupicola*	*V. bahamensis*
MP (BS%)	72*	80*	98*	90*
ML (BS%)	57*	64*	97*	95*
BI (PP%)	96*	58*	99*	75*

Sequences of several other species of *Varronia* and *Cordia* are presented here for the first time. The positions of OTUs often changed considerably between the three analyses methods (MP, ML and BI); however, sequences curated under the same name resolved in the same clade in each analysis suggesting that naming of the material is accurate. GenBank sequences also held under the same name, or synonyms (e.g. *V. globosa* is a synonym of *V. bullata*), resolved in the same clade as sequences generated by this research.

$trnL^{UAA}$ intron – $trnF^{GAA}$ spacer

Cordia is not resolved using pDNA in any of the analyses (MP, ML and BI). This is probably due to the small sample size but it may point to a bigger systematic query. Either way, further analyses are recommended using more sequences and other regions as discussed later. Conversely, a strongly supported *Varronia* clade is resolved in all three methods. Type specimen sequences in analysis C for *V. rupicola* and *V. bahamensis* resolved in the same clade as sequences of living and recently dead plants curated under the same names. Zero differences between *V. rupicola, V. bellonis* and *V. nesophila* resulted in a well-supported clade in the three methods. Unlike nrDNA, *V. rupicola* and *V. bahamensis* sequences were not

resolved as sisters in a clade in any of the three methods. *Varronia lima* resolved near *V. bahamensis* in all three methods of both analyses C and D.

As with nrDNA analyses, pDNA sequences of several other species of *Varronia* and *Cordia*, presented here for the first time, showed considerable variation in OTU positions; however, resolution in the same clade for sequences curated under the same name was seen across all analyses. This also held true for GenBank sequences also held under the same name, or synonyms.

Combined analysis of ITS and trnL-trnF *data*

All sequences used in analysis E were generated in this research. With few exceptions and only for more widespread taxa, sequences of these species were not already available in GenBank. Those available did not cover all of the ITS and *trnL-trnF* regions; therefore, sequences generated in this research are new contributions for these species that will be made available to the scientific community through GenBank.

Most *Varronia* sequences used for the analyses in this research form clades with low levels of sequence variation, a potential indicator of the recent diversification of this group. Many clades have low bootstrap proportion (BP), especially for analyses A-D, and the OTU positions vary between the three analysis methods. Rogue taxa with an unstable position in the clade due to an elevated substitution rate causing homoplasy or extremely low rates inside and outside the clade can all cause low BP (Sanderson and Shaffer, 2002). There was no obvious evidence of ribosomal paralogy (Feliner and Rosselló, 2007) observed in this research; therefore, ITS sequence data have been interpreted as orthologous.

As discussed by Baldwin (1992), comparison of phylogenetic trees generated from pDNA and nrDNA can help identify problems associated with cytoplasmically inherited DNA through introgression and hybridisation events. All three analysis methods (MP, ML and BI) show strongly supported clades (illustrated in Figure 55 for convenience) for *Varronia* and *Cordia* and negligible variation is observed in the resolution of OTUs in analysis E when compared to other analyses, A to D. *Varronia rupicola* and *V. bahamensis* were resolved in a well-supported clade (see Table 11) using all three methods (MP, ML and BI) with positions of *V. bellonis*, *V. lima* and *V. nesophila* changing minimally and apparently as a result of swapping due to low divergence. This suggests that the reconstructed phylogenetic tree is a fair estimate of the species relationships (Feliner and Rosselló, 2007).

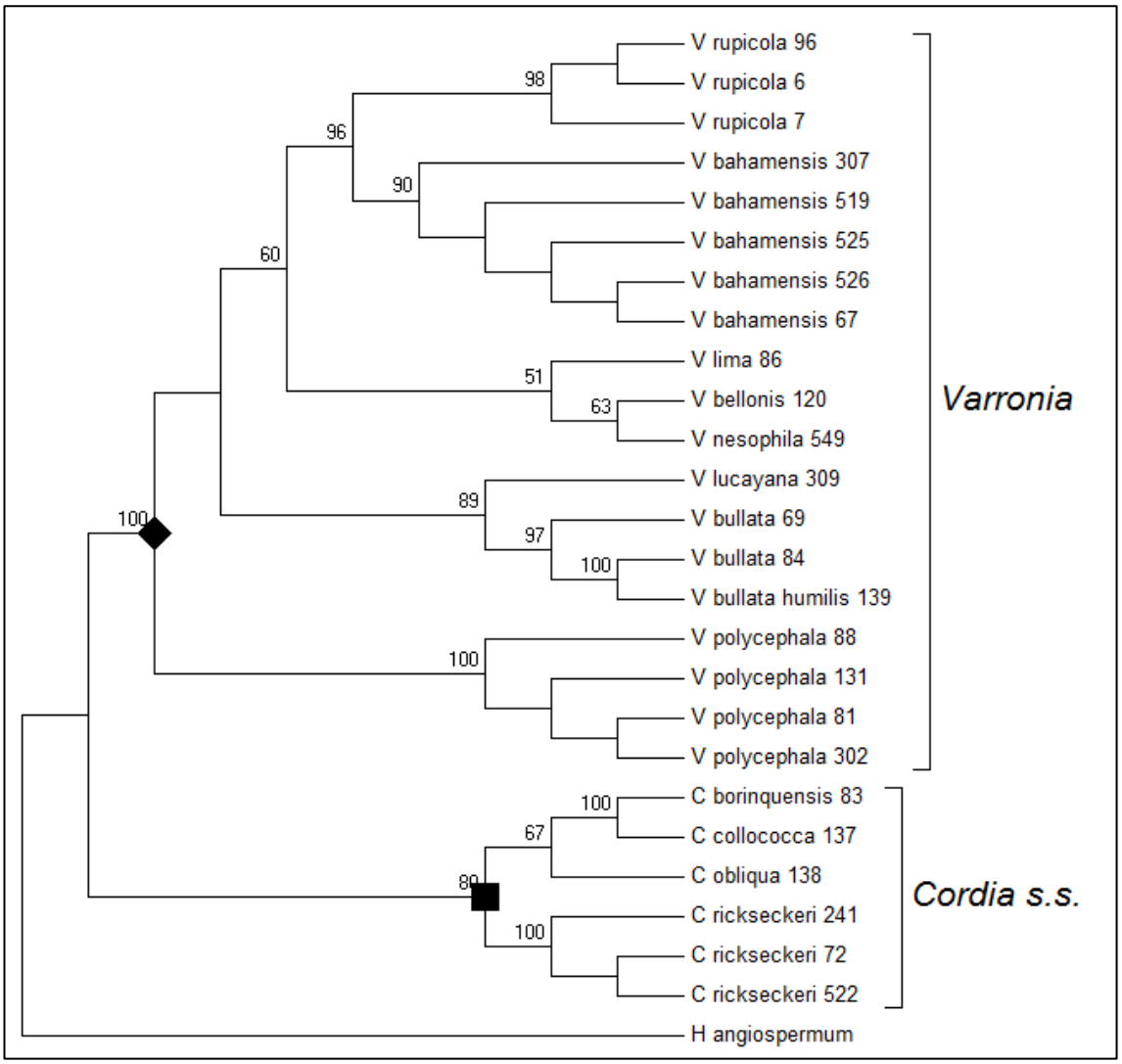

Figure 55: Maximum parsimony consensus tree for analysis E showing 25 sequences generated by this research with a GenBank selected outgroup, *H. angiospermum,* branch numbers as bootstrap percentages and BS values <50% not shown. Numbers/letters following specific epithets are either sample numbers for this research or GenBank numbers and correspond to Appendix 4 or 5, respectively.

3.4.2. Recommendations for further research

Minimal base change differences observed between sequences of *V. rupicola, V. bahamensis, V. bellonis*, *V. lima* and *V. nesophila* and their placement in the trees resolved in this research suggest that further analyses incorporating new markers should be undertaken using a broader group of taxa, particularly insular Caribbean species of *Varronia*. This would provide the opportunity to explore the relationships of these species with more resolution and identify potential parents for hybrid species. Of particular interest is the resolution of *V. nesophila,* endemic to the Lesser Antilles and sampled on the island of Montserrat, and the Puerto Rican endemic, *V. bellonis*, as sister species. A similarly intriguing situation is observed for the widespread (Caribbean, Central and South America) species, *Cordia collococca*, and the Puerto Rican endemic, *C. borinquensis*. Sequence variation was observed between samples of the PRB endemic, *C. rickseckeri*, collected in the countries of Puerto Rico and the British Virgin Islands.

Further analyses are recommended for all of the aforementioned species using samples from across their range.

Infrageneric groups were distinguishable from the data provided; however, further sampling of a broader group of taxa would be required to resolve *Cordia* based solely on pDNA. Further work is required to fully resolve the interspecific relationships in the focal group of Caribbean *Varronia* and a total evidence approach is recommended. Other pDNA regions (e.g. *matK*, *trnS-trnG* spacer and the group II *rpl16* intron) should be explored. The *rpl*16 intron could be especially useful as group II introns can be excellent phylogenetic tools due to their inability to move, high rate of evolution and ease of amplification (Small *et al.*, 1998; Kelchner, 2002). Shaw *et al.* (2005) found the *trnS-trnG* spacer to have the highest number of potentially informative characters (PIC) across 21 non-coding pDNA regions sampled. In *Heliotropium*, *matK* has proven to be more informative than *rbcL* for interspecific studies (Frohlich, pers. comm. 2014).

There are also other non-coding, low-copy (e.g. ETS) or single-copy nrDNA genes (e.g. chalcone synthase (CHS)) that could be explored. However, there are several considerations to take into account, especially the additional time required for sequencing most nrDNA other than ITS (Small *et al.*, 1998; Calonje *et al.*, 2008). Non-concerted evolution in ETS has been well documented and requires additional investigation prior to tree building to determine orthology or paralogy (Bailey *et al.*, 2003). Nuclear granule-bound starch synthase gene (GBSSI, or *waxy*) has been used is several studies and provided phylogenetic resolution in Poaceae and Rosaceae (Mason-Gamer *et al.*, 1998; Evans *et al.*, 2000) as has the nuclear-encoded alcohol dehydrogenase loci (*Adh*); however, additional effort is required to demonstrate orthology and caution is required due to possible concerted evolution for these regions of nrDNA (Small *et al.*, 1998; Feliner and Rosselló, 2007; Calonje *et al.*, 2008).

Given the above factors influencing nrDNA analysis and the results of this research, the next recommended steps are to undertake further pDNA analysis prior to exploring any nrDNA regions.

3.4.3. Main conclusions

Miller and Gottschling (2007) resurrected *Varronia* using morphological, geographical and molecular data from ITS1 only. The findings of this study (illustrated in Figure 56 for convenience) using new samples support a monophyletic *Varronia* using sequence data from ITS, *trnL-trnF* and combined ITS/*trnL-trnF* regions. This research also supports a monophyletic *Cordia* using sequence data from ITS and combined ITS/*trnL-trnF* regions only as the *trnL-trnF* region was not able to resolve the genus. The inability to resolve *Cordia* using the *trnL-trnF*

region solely may be due to hybridisation events that give rise to polymorphisms that are not a result of normal variation (Taberlet *et al.*, 1991). Based on ITS and combined ITS/*trnL-trnF* regions data, *Varronia rupicola* is a distinct species endemic to the Puerto Rican Bank. The closest relative of *V. rupicola* sampled is *V. bahamensis,* an endemic to the Bahaman Archipelago.

Further species sampling should be incorporated into the existing ITS and *trnL-trnF* matrices to produce new phylogenetic trees from across the range of the species. New and existing samples should be used to undertake further pDNA analyses using new regions (*i.e*. *matK*, *trnS-trnG* spacer and the group II *rpl16* intron) to produce informative phylogenetic trees.

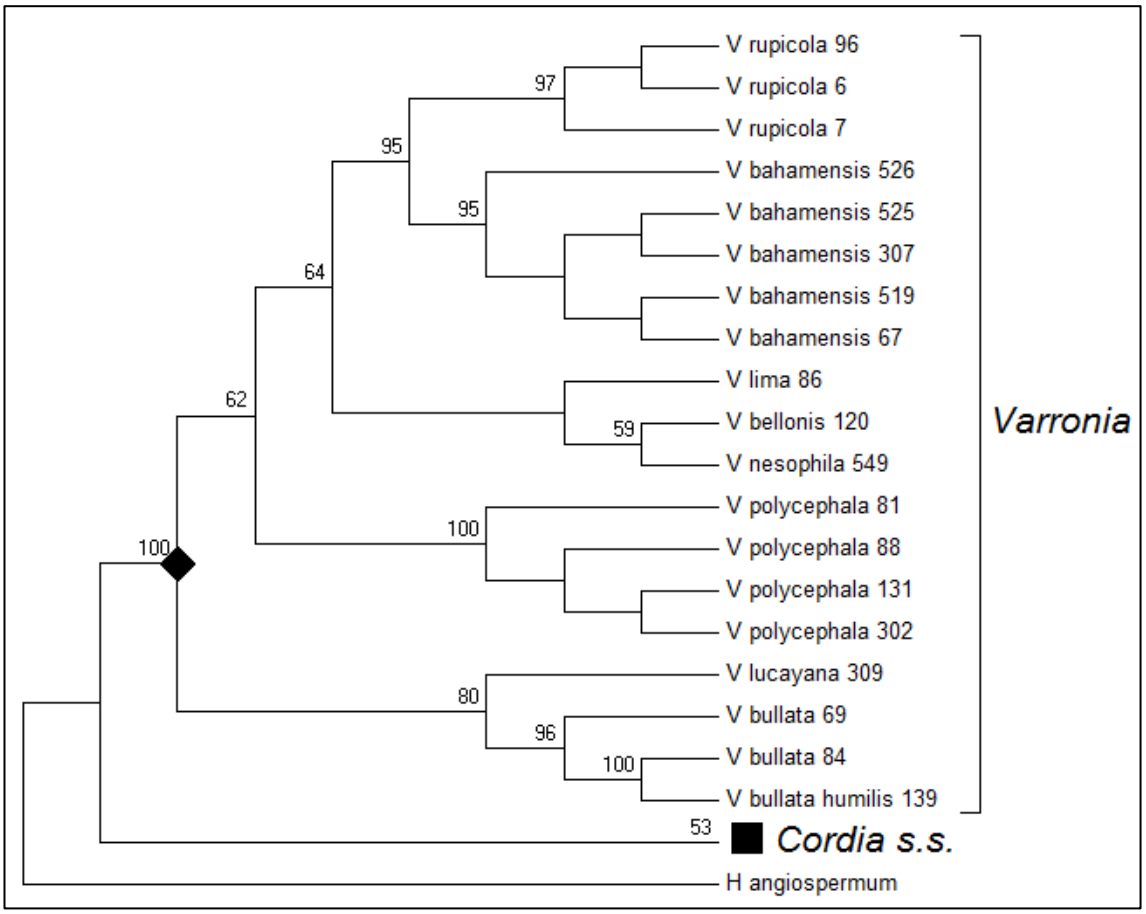

Figure 56: Maximum likelihood analysis E consensus tree showing the *Varronia* clade with branch numbers as bootstrap percentages and BS values <50% not shown. *Cordia s.s.* has been compressed to show detail of the *Varronia* sequences generated by this research with a GenBank selected outgroup, *H. angiospermum*. Numbers/letters following specific epithets are either sample numbers for this research or GenBank numbers and correspond to Appendix 4 or 5, respectively.

Chapter 4: Conservation genetics of *Varronia rupicola*

This chapter aims to provide answers for a series of important questions for the conservation and management of *V. rupicola* genetic diversity.

- First, are populations on the islands of Anegada, Vieques and Puerto Rico genetically distinct from one another?
- Second, are there genetically distinct populations found within any of the islands?
- Third, has genetic diversity been impacted by a reduction in population size?
- Fourth, do existing *ex-situ* collections adequately represent the extant genetic diversity of wild populations?
- Fifth, do existing *ex-situ* collections or samples from historical specimens reveal a loss of wild genetic diversity?

4.1. Introduction

Research presented here is the first step toward a better understanding of the genetic diversity and demography of *V. rupicola* for developing a scientifically underpinned species conservation and management strategy. Developing comprehensive *ex-situ* collections, effective *in-situ* conservation areas and appropriate management options for the future requires a fundamental understanding of the genetic diversity for a species of conservation concern. Recent work by Sanchez *et al.* (2014) for the Caribbean pine demonstrates the importance of establishing conservation collections to capture genetic diversity in the face of current and future threats to the species in the wild. Lacking *in-situ* conservation areas and effective management in areas that do exist are known to be adversely impacting *V. rupicola* on the islands of Puerto Rico and Vieques (U.S. Fish and Wildlife Service, 2014b).

There are no known studies of the genetic variation or structure of wild populations or *ex-situ* collections of *V. rupicola*. Very few genetic studies have been previously carried out on species of *Varronia* or *Cordia s.s.* in the Neotropics. Those studies particularly focusing on population genetics methods (Boshier *et al.*, 1995a, 1995b; Chase *et al.*, 1995; Spoon and Kesseli, 2008) provide very little insight for Caribbean species of *Varronia* or data for comparative analysis. This is particularly highlighted by studies focusing on large trees with wide distributions that are useful for agroforestry (Boshier *et al.*, 1995a, 1995b; Chase *et al.*, 1995) and the lack of studies reporting the use of microsatellites beyond development and cross-species amplification (Spoon and Kesseli, 2008).

Using simple sequence repeats (SSRs), or microsatellites, population genetics of wild populations of *V. rupicola* were investigated to determine demographic patterns as well as

gene flow and diversity. Microsatellites were also used to evaluate genetic diversity of *ex-situ* collections and compare with wild populations. This enables an assessment of the representativeness of existing conservation collections held for the species globally.

Microsatellites are used for the study of populations as they tend to have little or no influence on phenotypic expression thus making them selectively neutral. This means that SSRs can generally provide genetic estimates that are not influenced by the process of natural selection (Silvertown and Charlesworth, 2001). The highly polymorphic nature of SSR regions (Zhang and Hewitt, 2003) is due to mutations that mainly result from recombination or misalignment during DNA replication (Schlötterer, 2000; Navascués and Emerson, 2005) and is what enables the detection of within population genetic drift and gene flow between populations (Fischer *et al.*, 2000; Ouborg *et al.*, 2010).

Microsatellite regions are amplified with polymerase chain reactions (PCR) using specific oligonucleotide primers. The primers are developed in pairs to guide microsatellite loci amplification by binding to the conserved regions flanking the microsatellite. Following amplification, microsatellites can be analysed using capillary electrophoresis. Important considerations for evaluating microsatellite variation data are geographical factors such as barriers between sampling locations, historical factors like sea-level fluctuations and anthropogenic factors like land-use. These can play important roles in limiting gene flow in a metapopulation leading to fragmentation and isolation which can, in turn, lead to differences among populations and increase with time. Population fragments show a decline in heterozygosity and allelic frequencies. This is exacerbated in species occurring on islands that are already limited by physical environmental factors and increases the risk of extinction (Frankham *et al.*, 2002).

Many research fields have successfully employed the use of microsatellites and shown that the small length, abundance, multiple alleles and co-dominant inheritance are useful for demonstrating genetic structure and diversity across populations (Powell *et al.*, 1996; Chistiakov *et al.*, 2006; Fernandez-Silva *et al.*, 2013). Species-specific microsatellites were chosen for this study as they are much less susceptible to cross-contamination issues that can occur when using universal primer techniques like AFLP (Selkoe and Toonen, 2006). This coupled with the advances in next-generation sequencing that have brought the costs associated with developing bespoke oligonucleotide primers down and reducing the time investment necessary to select polymorphic primers (Rothberg and Leamon, 2008; Wheeler *et al.*, 2008; Fernandez-Silva *et al.*, 2013) supported this decision. All DNA samples were extracted and genotyped by the author in the Jodrell Laboratory, Kew as described below.

To explore population differentiation, Wright (1943, 1965) developed the now widely used *F*-statistics to calculate the fixation indices F_{IT}, F_{ST} and F_{IS}. *F*-statistics infer population structure (F_{ST}) based on the equation, $F_{ST} = (F_{IT}-F_{IS})/(1-F_{IS})$, as well as mating system and inbreeding (F_{IS} and F_{IT}). Another important methodological development was made by Slatkin (1993) to test the hypothesis of isolation by distance (IBD). This is undertaken using regression analysis of matrices containing geographical distances (km) between paired populations and pairwise population genetic differences (F_{ST}).

Several software packages are available for exploring genetic diversity through estimation of allelic frequencies and population structure through clusters. Excoffier and Heckel (2006) provide a comprehensive review of these packages highlighting each program's assumptions and common problems encountered. A major limitation of many packages is the fact that most can only process haploid or diploid data with a maximum of two alleles per sample. According to Guillot (2005a) the popular packages that undertake cluster analyses for estimating population structure assume there is the idealised state of Hardy-Weinberg equilibrium (HWE) and linkage equilibrium (HWLE). In wild populations, the HWLE principle of genetic variation remaining constant might not be realised due to drift, inbreeding, migration and selection. Another important factor to consider when estimating populations is the effect of IBD. For these reasons, estimations of IBD, inbreeding and overall genetic variation need to be undertaken to make informed estimations of the population structure of a species.

An idea of the ploidy of the species being studied is needed to interpret results from population level analysis (*i.e.* alleles observed); however, this can be complicated by genome restructuring and gene silencing with the latter resulting in diploidisation of a polyploid (Soltis and Soltis, 1999). As discussed in 1.4.3. The genus *Varronia*, the tendency of *Cordia s.l.* chromosomes to stick together has resulted in sparse karyological data for the group (Britton, 1951; Heubl *et al.*, 1990). Opler *et al.* (1975) stated that the ancestor of extant *Cordia s.l.* was probably adapted to lepidopteran pollination and heterostylous. Three main evolutionary lines for *Cordia s.l.*, *x* = 7, 8 and 9, as well as a derived complex, *x* = 15, were described by Heubl *et al.* (1990) who found all *Varronia* studied to be diploid or tetraploid. These *Varronia* were derived from *x* = 9 and although diploid taxa are present within the group, Heubl *et al.* (1990) considered *Varronia* a relatively young and advanced group.

4.2. Materials and Methods

4.2.1. Karyology

In order to interpret genetic data from microsatellites, the ploidy of *V. rupicola* had to be first explored. To determine if the species was a diploid or polyploid and if more than two alleles

could be expected per nuclear microsatellite marker, different methods were employed. First, traditional karyotyping and then modern flow cytometry methods were undertaken as described below.

Karyotyping

Fresh root samples from cultivated plants of *V. rupicola* growing at the Royal Botanic Gardens, Kew were used to undertake Feulgen staining techniques described in Kynast *et al.* (2014). Root tips, 1.5 to 2 mm in length, were collected from nursery grown plants between 08:00 and 10:00, to increase the number of cells at metaphase, into vials containing 2.5 ml sterile deionised water (sdH_2O) for transport to laboratory facilities where they were processed immediately. Root tips were carefully cleaned with a fine tip brush to remove soil/debris before being transferred into vials containing 4 ml of pre-treatment solution #12, consisting of 1:1 mix of 4xpre-treatment solution #1 (5 mM colchicine): Pre-treatment solution #5 (para-dichlorobenzene saturated solution). Vials with root tips were stored at 3 °C for seven hours as a pre-treatment. Fixation was undertaken using a Modified Farmer's Fixative, consisting of a 3:1 mix of three volume parts of $96\%_{v/v}$ ethanol: one volume part of glacial acetic acid. Fixed material was stored at 4 °C until processed using the Feulgen staining technique to selectively stain DNA.

Fixed roots were rinsed in ~15 ml SDH_2O at 20 °C for 20 minutes to remove fixative from the tissues. Next, roots were hydrolyzed in ~10 ml of 1 M HCl at 60 °C for seven minutes to remove purine bases from DNA and macerate the root tissues. Roots were then stained in ~2 ml of Schiff's reagent at 20 °C for 20 minutes. Following staining, the root tip was dissected to remove the root tip shield cells (not stained) and the stained meristem before squashing the meristem in 1% lacto-propiono-orcein (LPO) for contrast enhancement. The squashed meristem was viewed using microscopy to locate chromosomes at mitotic metaphase for counting.

Flow cytometry

Fresh leaf samples from cultivated plants of *V. rupicola* and *V. bahamensis* growing at the Royal Botanic Gardens, Kew (Kew) were used to undertake flow cytometry techniques as described in Dolezel *et al.* (2007). Leaves were collected from nursery grown plants into sealed plastic bags for transport to laboratory facilities where they were stored at 3 °C until processed. Following initial trials, *Allium sativum* L. was selected as the standard using the General Plant Buffer (GPB) adjusted to pH 7.0, consisting of 0.5 mM spermine 4HCl, 30 mM sodium citrate, 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 80 mM KCl, 20 mM NaCl, 0.5% (v/v) Triton X-100, on a Partec PA cytometer (Partec, Münster, Germany). Leaf

material processing followed a general protocol consisting of ~1cm^2 of leaf material cut into a sterile Petri dish using scissors. Using a pipette, 1000 ul GPB was added to the sterile Petri dish over the leaf material. A new razor blade was used to finely chop leaf material in the sterile Petri dish before adding a further 1000 ul GPB. Next, the pipette tip was cut off at 45° angle, 0.5 cm from end to enable the transfer of the buffer and chopped leaf material into a clean filter where it was allowed to drain into a 3.5 ml tube. Next, 100 ul of propidium iodide was pipetted into the tube with the GPB and leaf material before vortexing for 30 seconds. The mixture was allowed to set for 15 minutes before vortexing for a further 30 seconds. The tube with solution was then visually inspected to ensure no plant debris remained before loading the tube in the flow cytometer using the protocol supplied by the manufacturer (Partec, Münster, Germany) to measure the genome size using liberated nuclei.

4.2.2. Next-generation sequencing

Eurofins Genomics (Ebersberg, Germany) performed 454 pyrosequencing for development of oligonucleotide primers (Margulies *et al.*, 2005) using a *V. rupicola* sample supplied by the author from the *ex-situ* collections of the Royal Botanic Gardens, Kew (accession 2005-1557) grown from a seed of Anegada, BVI origin. Following commercial sequencing of genomic DNA extracted by the author, raw data returned was processed using QDD software (Meglécz *et al.*, 2010). Primer design was undertaken within QDD using Primer3 (Rozen and Skaletsky, 2000; Koressaar and Remm, 2007; Untergasser *et al.*, 2012) as described below.

DNA Extraction

Fresh leaves were collected and immediately processed using a modified 2×CTAB protocol (Saghai-Maroof *et al.*, 1984; Doyle and Doyle, 1987; Csiba and Powell, 2006). The amount of 15 ml of 2×CTAB isolation buffer [100mM Tris-HCl pH 8.0, 1.4M NaCl, 20mM EDTA, 2% CTAB (hexadecyl trimethyl ammonium bromide)] containing 60 µl ß-mercaptoethanol was pipetted into a Falcon™ (Fisher Scientific UK Ltd, Leicestershire, UK) 50 ml conical centrifuge tube and heated in a 65 °C water bath. Liquid nitrogen was used to freeze 150 mg of leaf tissue before grinding in ~2 ml of the pre-heated CTAB buffer using a mortar and pestle. The ground sample in buffer was added to the tube containing the remaining ~13 ml pre-heated 2×CTAB buffer. The sample was incubated for 20 minutes in a 65 °C water bath before adding 15 ml of SEVAG (24:1 chloroform:isoamyl alcohol). It was then rocked for one hour at 20 °C before spinning down at 8000 rpm for ten minutes at 20 °C. The top clear layer containing genomic DNA was pipetted into a 50 ml conical centrifuge tube with 5 ml of -20 °C isopropanol, gently mixed and left to precipitate DNA for three hours at -20 °C. To collect the precipitate, the sample was spun at 3000 rpm for ten minutes before pouring off the liquid. The remaining DNA pellet was washed in 3 ml of 70% ethanol for five minutes before spinning down at 3200 rpm for three

minutes. All remaining liquid was poured off and the sample left in a fume cupboard for 24 hours to allow complete evaporation.

Genomic DNA was re-suspended in 3 ml of TE buffer (10 mM Tris-HCl pH 8, 0.25 mM EDTA) and left to dissolve for 24 hours. Nucleospin DNA purification columns were used to purify extracted genomic DNA following the protocol supplied by the manufacturer (QIAquick, Qiagen Ltd, Crawley, UK). A Nanodrop (ThermoScientific, Denver, CO) was used to quantify the amount of genomic DNA in the sample and ensure a suitable quantity (>50 ng/µl) was available for 454 pyrosequencing.

454 pyrosequencing and primer development

Approximately 64 ng/µl of genomic DNA was sent for sequencing on a Titanium GS-FLX 454 sequencer platform (454 Life Sciences Corporation, a Roche company, Branford, CT, USA). The raw data, with tags and barcodes already removed, returned from Eurofins Genomics was quality filtered using the pipeline software package QDD (version 2.1 by Emese Meglecz, Aix-Marseille University, Marseille, France) on a Linux computer to screen sequences with three or more di-, tri-, tetra-, penta- or hexabase repeats. Primers were designed using Primer3 (version 1.0) within QDD following removal of redundant sequences. Default settings were maintained for Primer3 except for product length which was set to 90-300 bp; maximum, optimum and minimum oligonucleotide sizes set as 27, 20 and 18, respectively; maximum, optimum and minimum mixture specific melting temperatures (*Tm*) set at 63.0 °C, 60.6 °C, and 57.0 °C, respectively; maximum difference in *Tm* for the primers of 10.0 °C; maximum, optimum, and minimum GC content set at 80%, 50% and 20%, respectively; and no GC clamp.

Following design and removal of all but the highest ranked primers for each region, primers were ordered from Eurofins Genomics with the M13 tail at the 5' end of forward primers for cost savings. Primer testing was undertaken using genomic DNA extracted from silica dried leaves as described in 3.2.1. Sampling - DNA extraction. Three samples were selected, one originating from each of the islands of Anegada, Vieques and Puerto Rico, for primer testing using PCR and genotyping methods described below.

4.2.3. Sampling for population analyses

Dried plant material of various ages was used for population analyses. The vast majority of samples (374 of 380) came from silica dried leaves (Chase and Hills, 1991; Särkinen *et al.*, 2012) collected by the author to help ensure high quality DNA was used, minimising amplification error (Selkoe and Toonen, 2006). Vouchers collected by the author to accompany silica dried leaves from cultivated and wild plants are deposited at the Kew Herbarium (K) with duplicates lodged at other herbaria. Vouchers were not collected for every silica sample due to

time constraints and the potential negative impact collecting from wild plants may have caused. All vouchers are listed in Appendix 1: *Varronia rupicola* records.

Silica dried DNA samples from 260 wild (Figure 57) and 114 cultivated plants were collected. A summary of samples is provided in Table 12 and a full list of the samples by location is provided in Appendix 6: Population genetics samples. Samples from six air dried herbarium specimens held at the Department of Natural and Environmental Resources herbarium (SJ) in San Juan, Puerto Rico were collected to test for genetic diversity loss. These specimens ranged in date collected from most recently in 1995 to oldest in 1978.

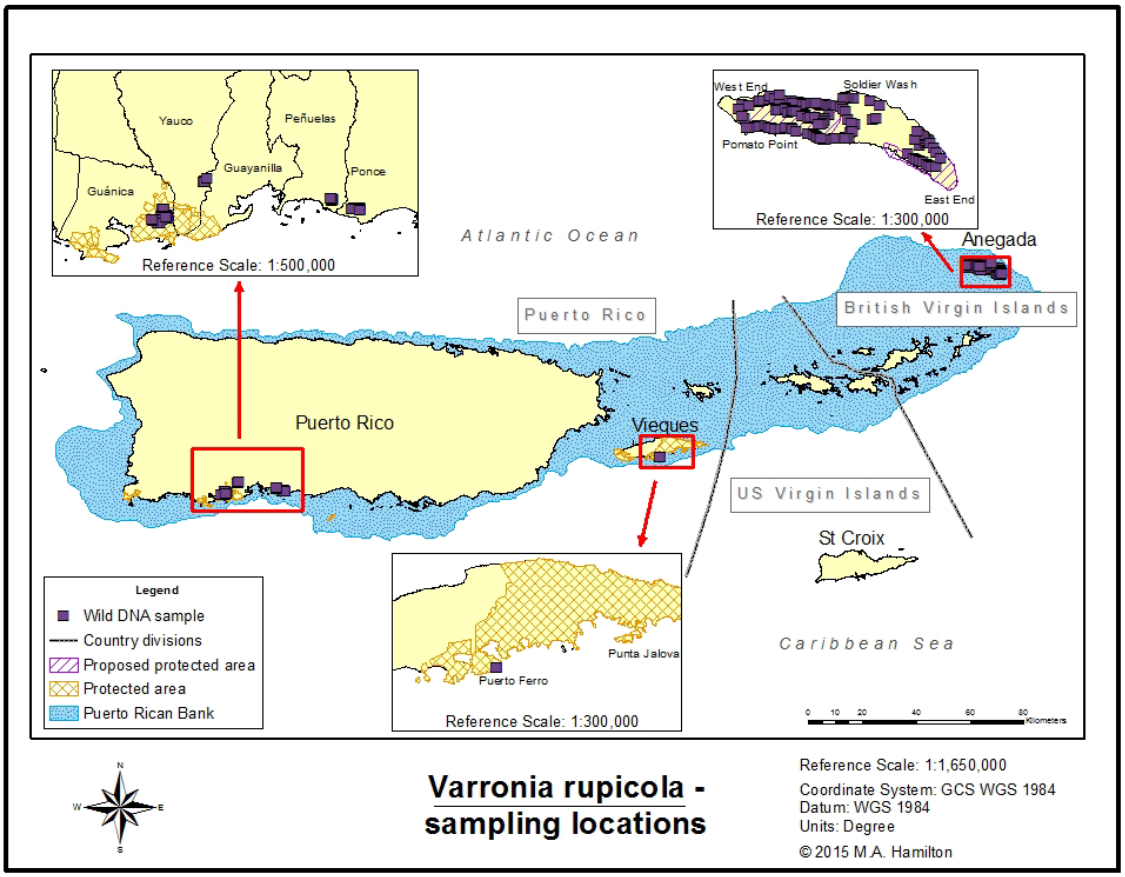

Figure 57: Map showing *Varronia rupicola* DNA sample collecting locations (purple square) for samples made across the species native range in the Puerto Rican Bank including within proposed and existing protected areas in Anegada (inset top right), Vieques (inset bottom) and south-western Puerto Rico (inset top left).

Ex-situ collections

Through discussion with Puerto Rican Bank colleagues and contacting major botanical institutions in Europe and the USA, *ex-situ* collections were identified within the Puerto Rican Bank, the UK and the USA. Anegada source material has been used to develop collections at Kew, UK; the Fairchild Tropical Botanical Garden, Miami, Florida, USA; and the J.R. O'Neal Botanic Garden, Road Town, Tortola, BVI. Source material from the island of Puerto Rico has been used to develop collections at Kew, UK as well as the Cabo Rojo NWR and the Guánica

State Forest, both in Puerto Rico. Every plant held in these *ex-situ* collections (n = 114) was sampled (Table 12) unless it was felt that the sampling would be detrimental to the survival of the plant (e.g. seedlings and unhealthy saplings with few leaves were not sampled in the Guánica State Forest nursery).

Table 12: Summary of samples used for analyses showing the number of samples by origin of material (Number of samples by origin) per collecting location (Source island) broken-down by origin of material from extant wild plants (Extant wild), extant *ex-situ* plants (Extant cultivated) or herbarium specimens of dead plants (Dried herbarium). All wild and *ex-situ* plant material originated from silica dried leaf samples. Herbarium specimen material originated from air-dried herbarium vouchers.

Source island	Number of samples by origin		
	Extant wild	Extant cultivated	Dried herbarium
Puerto Rico	110	66	3
Vieques	6	0	1
Anegada	144	48	2
Total number of samples	**260**	**114**	**6**

Wild populations

Sampling of the wild populations (Table 12) varied between the British Virgin Islands and Puerto Rico due to the numbers of extant individuals remaining in each country. The sampling strategy employed is described in 2.2.1. Survey and sampling of wild populations.

4.2.4. Population sample analysis

DNA sample extraction, amplification and genotyping were undertaken by the author in the Jodrell Laboratory, Kew. Samples used are reported in Appendix 6: Population genetics samples. DNA extraction is described in 3.2.1. Sampling - DNA extraction.

Polymerase Chain Reaction (PCR)

To guide amplification of microsatellite loci, 44 primer pairs were selected for testing (Appendix 7: Oligonucleotide testing). Selections were made after removing all primers produced by QDD with a target length less than six bases, ranking other than one for "best primer for target region" and sequences with long strands of repeated bases (e.g. GTTTTT) to increase the chances of successful amplification. Primers were ordered from Eurofins Genomics and initially tested against three *V. rupicola* samples, one from each of the islands harbouring extant populations, to determine if the loci were polymorphic. Based on melting temperature, primers were divided into three groups of different annealing temperatures, Group 1 = 51 °C, Group 2 = 52 °C and Group 3 = 56 °C. As forward primers had the M13 tail at the 5' end, primers were also divided for use of either the JOE- (6-carboxy 4', 5'-dichloro-2', 7'-dimethoxy fluorescein) or FAM- (6-carboxy-fluorescein) labelled M13 primer.

A Perkin-Elmer GeneAmp 9700 thermo cycler was used for PCR amplifications. A three minute pre-melt at 94 °C was followed by 40 cycles consisting of 20 seconds denaturing at 94 °C; annealing for 40 seconds (the annealing temperature varied according to the primer group, as described above); and a 20 second extension at 72 °C. A further eight cycles were added to the programme that included denaturing at 94 °C for 60 seconds, annealing at 50 °C for 60 seconds and a 60 second extension at 72 °C. The programme had a final extension of 30 minutes at 72 °C before cooling to 16 °C. The 10 µl standard reaction contained: 1 µl DNA (10 ng/µl), 5 µl 2×DreamTaq Master Mix (DreamTaq™ DNA Polymerase, Thermo Scientific™), 0.4 µl forward primer (10 pmol/µl), 0.8 µl reverse primer (10 pmol/µl), 2.24 µl sterile deionised water and 0.16 µl M13 primer (10 pmol/µl), JOE or FAM (Applied Biosystems®, a Thermo Scientific™ company) depending on the group, as described above.

Following initial testing, polymorphic microsatellite loci were amplified as described above using selected *V. rupicola* samples (n = 380). The selected loci were also tested, as described above, for cross-species transferability and amplification in six samples of *V. bahamensis*, three samples of *V. bullata*, two samples of *V. lima* and three samples of *V. polycephala*. These 14 samples (Table 13) were extracted as previously described for *V. rupicola* population samples.

Table 13: Samples of 14 *Varronia* species used for cross-species amplification testing of ten microsatellite loci showing the species binomial (Species), study identification number (Sample ID), voucher number (Voucher) and origin of source material (Origin). Source material abbreviations: Wild = Silica dried leaf material from wild source; Cultivated = Silica dried leaf material from *ex-situ* material; HS = leaf material originated from air-dried herbarium vouchers collected from wild plants that are now dead.

Species	Sample ID	Voucher	Origin
V. bahamensis	67	MAH863	Wild
V. bahamensis	74	K000583257	HS
V. bahamensis	307	K000297743	HS
V. bahamensis	519	FTG2009-0504A	Cultivated
V. bahamensis	525	MAH861	Wild
V. bahamensis	526	FTG00151995	HS
V. bullata	69	MAH780	Wild
V. bullata	84	MAH925	Wild
V. bullata	139	MAH950	Wild
V. lima	85	MAH922	Wild
V. lima	86	MAH923	Wild
V. polycephala	81	MAH927	Wild
V. polycephala	88	MAH920	Wild
V. polycephala	131	MAH941	Wild

Two positive and one negative control were run with every PCR performed in this study to help ensure results were consistent across different PCR machines and sequencer runs. A 2%

agarose gel stained with ethidium bromide was used to visualise PCR products prior to genotyping.

Genotyping

PCR products (1 µl of each) were added to wells of a 96-well plate each containing 10 µl Hi-Di™ formamide injection solvent and 0.12 µl GeneScan™ 500 ROX™ dye size standard (both from Applied Biosystems®, a Thermo Scientific™ company). Adhesive plate seals were used to cover plates prior to six seconds centrifugation. This was followed by heating for four minutes at 95 °C on a dry bath heater. Plates were immediately put on ice for ten minutes before a final six second centrifugation. Sequencing was performed on an ABI 3730 Genetic Analyser (Applied Biosystems®, a Thermo Scientific™ company), according to the manufacturers' protocol.

GeneMapper® Software (version 4.1, Applied Biosystems®, a Thermo Scientific™ company) was used for microsatellite genotyping analyses following the methodology of Papadopulos *et al.* (2014). Projects were developed and bins defined by eye for each locus. Sequences of *V. rupicola* were added to the projects first to analyse peaks and determine alleles. Automatic scoring of alleles for each locus performed by GeneMapper was followed by manual confirmation and any required editing (*i.e.* due to stuttering). To minimise peak scoring bias, blind processing was employed using extraction codes. Each sequencer run contained two positive and one negative control. The control replicates were compared in GeneMapper for each locus to help ensure results were consistent across different PCR machines and sequencer runs. Any samples that failed to amplify or had a low signal for a locus were repeated. Sequences of other *Varronia* species were added separately to projects for each locus to assess cross-species amplification without confounding the process of automatic allele scoring for each locus performed by GeneMapper.

4.2.5. Statistical analyses

To determine if populations between and within the islands were variable and look for evidence of lost and missing diversity across wild populations and *ex-situ* collections, statistical analyses were undertaken within and across the loci. The majority of samples were scored for all loci. Alleles that only occur in one population, so-called private alleles, are an important genetic diversity parameter to measure (Kalinowski, 2004). For this reason, those samples missing data for some of the loci and found to include private alleles for other loci were retained in the dataset for statistical analyses. The total percentage of missing data for all samples varied to 6% for locus 'VRgr3_2' and 9% of locus 'VRBREYV'. All other loci had less than 5% missing data and most of these samples were only missing data for one locus.

Samples were categorised based on the source of the material and divided into three separate groups, A-C (Table 14), for undertaking analyses. Origin and source of samples is provided in Appendix 6: Population genetics samples. Analyses undertaken were subdivided hierarchically to explore allelic diversity and genetic structure at the regional (*i.e.* taxon) level as well as the country and population levels for the analysis groups, A-C. The regional level analyses encompassed the Puerto Rican Bank including the countries of Puerto Rico and the British Virgin Islands. Inter-population level analyses initially considered the three islands of Puerto Rico, Vieques and Anegada due to distance between the islands (>125 km) and current sea separation (see Chapter 2: Biogeography of *Varronia rupicola*). Puerto Rico and Anegada were subsequently further divided to include two populations each (see Appendix 6: Population genetics samples). These divisions were based on preliminary analyses that inferred population separation and showed genetic distance (see 4.3. Results).

Table 14: Number of samples included in three analysis groups, A-C, by source of material (extant wild plants, extant *ex-situ* plants and dead plants from herbarium specimens) where all wild and *ex-situ* plant material originated from silica dried leaf samples and herbarium specimen material originated from air-dried herbarium vouchers.

Analysis group	Description	# samples
A	Extant wild plants	260
B	Extant wild & *ex-situ* plants	374
C	Extant wild, extant *ex-situ* plants & herbarium specimen samples	380

Genotype data from ten GeneMapper software projects, one per locus, was exported for analyses and further formatting. Initially, GeneMapper project exports were combined into a master genotypes table in Microsoft® Excel® 2010 (© 2010 Microsoft Corporation) by sample number to enable the addition of data fields for sample source (extant wild plants, extant *ex-situ* plants or dead plants from herbarium specimens), geographical coordinate data and locality information. The loci were classified based on the number of alleles per locus. Loci containing a maximum of two alleles were classed as "diploid-acting" and those with more than two alleles as "polyploid-acting". This classification is due to the majority of software packages only having the ability to analyse data from loci with a maximum of two alleles. The 'genotypes master table' of analysis group C enabled data to be further formatted for use with selected software programmes and software add-ins as described below. All data formatting and analyses were performed on PCs running Windows® 7 or Windows® 8.1 with 64-bit operating systems.

The Microsoft Excel 2010 plug-in, Genetic Analysis in Excel 'GenAlEx' version 6.5.01 (Peakall and Smouse, 2006, 2012a), was used to identify allelic diversity. Initially analysis group C

samples were used in a regional analysis of the two countries. The region was subsequently divided by the three islands to explore inter-island diversity. Prior to cluster analyses described below, GenAlEx enabled the exploration of expected versus observed Hardy-Weinberg equilibrium (HWE) using analysis group A samples.

CREATE (Coombs *et al.*, 2008) software (version 1.3.7) was used to convert raw data from eight diploid-acting loci for analysis group A in the genotypes master table into formatted files for use with FSTAT (Goudet, 1995), Arlequin (Excoffier and Lischer, 2010) and Genetix (Belkhir *et al.*, 2004) software packages. PGDSpider (Lischer and Excoffier, 2012) software (version 2.0.7.3) was used to further convert files exported from CREATE for use with Geneland (Guillot *et al.*, 2005a, 2005b, 2008; Guillot, 2008; Guillot and Santos, 2009, 2010; Guedj and Guillot, 2011; Guillot *et al.*, 2012) and Structure (Pritchard *et al.*, 2000; Falush *et al.*, 2003, 2007; Hubisz *et al.*, 2009) software packages.

PAST (Hammer *et al.*, 2001) software (version 1.0) was used for principal coordinates analyses (PCoA) based on the algorithm of Davis (1986). PCoA is a multivariate analysis that considers the entire dataset, regardless of ploidy, and enables the visualisation of among individual genetic distances (Dufresne *et al.*, 2014). For this research, PCoA enabled the two polyploid-acting loci to be included in a multivariate analysis along with the eight diploid-acting loci. Subsequently, the contribution of the two polyploid-acting loci was explored to identify populations through genetic structure inference. To enable PCoA analyses, genotypic data from all ten loci for analysis group C was converted into a '0,1' matrix per locus showing presence/absence for each allele by sample. Geographic coordinate data was added to the matrix for each sample prior to undertaking PCoA. Sample number and collection locality information was added to facilitate visualisation of results. Each locus was analysed separately to check input data formatting before combining data for the ten loci to perform a final analysis of all extant wild samples (analysis group A). All PCoA analyses were undertaken using the Jaccard (1901) similarity measure to account for absence data (*i.e.* multiple occurrences of zero) within the matrix. The transformation exponent (c = 4) was used for the analysis as initial trials using higher or lower values (*i.e.* two or six) showed less resolution.

Clustering analyses were undertaken using Bayesian techniques implementing Markov chain Monte Carlo (MCMC) methods to detect population boundaries using two software packages, Geneland and Structure. According to Guillot *et al.* (2009), these packages share assumptions about the presence of cluster-specific minimum allele frequencies and cluster members being at HWLE. Both packages infer the number of populations by identifying genetic discontinuities and locating the portions of space with the highest likelihood using several different models

that are selected by the user. The packages differ in their inference abilities due to the underlying models. Structure lacks a spatial model and thus the ability to include geographic coordinates for each sample in the analysis; however, the package does include the functionality to define collecting locations as *a priori* (Hubisz *et al.*, 2009). The packages also differ in their ancestry models. Structure is able to model with or without genetic admixture (Pritchard *et al.*, 2000), whereas Geneland is only able to model without admixture (Guillot *et al.*, 2005b). The no admixture model assumes that individuals come purely from a single population while the admixture model allows for mixed ancestry of individuals. Both packages are able to model allele frequencies as correlated or uncorrelated (Pritchard *et al.*, 2000; Guillot *et al.*, 2005b). The uncorrelated model assumes allele frequencies are independent in populations (Pritchard *et al.*, 2000). Conversely, correlated allele frequencies use the F-model which assumes that allele frequencies are quite similar due to shared ancestry or migration (Falush *et al.*, 2003). This can assist in detecting clusters where divergence is low; however, it can also lead to overestimation of clusters in some datasets (Falush *et al.*, 2003; Guillot, 2008).

Geneland (version 4.0) and Structure (version 2.3.4) software were used to estimate the number of populations (*K*) and population membership of samples for analysis group A. Several preliminary runs were undertaken to determine the most appropriate model parameters to use before final runs were undertaken. Analysis group A samples were analysed using the eight diploid-acting loci combined as neither package has the capability to analyse data with more than two alleles per loci or mixed ploidy. Four separate analyses (Table 15) were run using different parameters for each software package as described below.

Table 15: Parameters used for four separate analyses using genotypic data from eight diploid-acting loci for analysis group A samples using Geneland and Structure software packages.

	Geneland Parameters		Structure Parameters	
Analysis number	**Spatial model**	**Allele frequencies**	**Admixture**	**Allele frequencies**
Analysis 1	No	Correlated	Yes	Correlated
Analysis 2	No	Uncorrelated	Yes	Uncorrelated
Analysis 3	Yes	Correlated	No	Correlated
Analysis 4	Yes	Uncorrelated	No	Uncorrelated

Geneland software analyses one and two used either correlated or uncorrelated allele frequencies, respectively, without the spatial model. Analyses three and four used the spatial model (geographic coordinates for each sample) and either correlated or uncorrelated allele frequencies, respectively. All analyses included ten independent runs using 10^5 iterations with thinning of 100, maximum of 600 nuclei, minimum of one population and maximum of seven populations. All other settings were left at default values. Post-processing of the model with

the highest probability was undertaken using 10^5 iterations with a burn-in of 50 and pixel values in the spatial domain of 200 for X and 100 for Y. A final run was performed using the same settings except the maximum of populations was set to five to explore changes in posterior probability and convergence.

Structure software analyses one and two used the admixture model and either correlated or uncorrelated allele frequencies, respectively. Analyses three and four used the no-admixture model and either correlated or uncorrelated allele frequencies, respectively. All analyses included *a priori* information on collecting location (Falush *et al.*, 2003; Hubisz *et al.*, 2009). Ten replicates were run for each value of *K* from 1-10 using 10^6 iterations with thinning of 10^3. All other settings were left at default values. Post-processing of the Structure results were performed using the CLUMPAK (Kopelman *et al.*, 2015) pipeline (http://clumpak.tau.ac.il/index.html) and included separate analyses for the best *K* described by Pritchard *et al.* (2000) and Evanno *et al.* (2005).

Following cluster analyses and assignment of populations, GenAlEx was used to test assignments using the frequency method of Paetkau *et al.* (Paetkau *et al.*, 1995, 2004). The method assumes HWLE and starts with the calculation of population-wide allelic frequencies followed by derivation of a log-likelihood value. Missing alleles are handled through assignment of non-zero numbers and the individual being assigned is left out of calculations during assignment to a reference population (Efron, 1983; Paetkau *et al.*, 1995). Default settings, leave one out option and zero frequencies = 0.01, were used to undertake population assignment assessments.

The software packages Arlequin (version 3.5), GenAlEx, FSTAT (version 2.9.3.2) and Genetix (version 4.05.2) were used to perform hierarchical analyses using analysis group A samples. Due to conflicting views in the research community about the best approach to use for calculating *F*-statistics (Meirmans, 2006; Song *et al.*, 2006), a range of approaches were taken for these estimations to compare results. The fixation indices are presented here, unless otherwise noted, using Wight's (1965) original symbols (F_{ST}, F_{IS} and F_{IT}) as there are many analogues, particularly for F_{ST} (e.g. Nei's G_{ST} (1986), Slatkin's R_{ST} (1995), Weir and Cockerham's θ (1984) and Hedrick's standardized G_{ST} (2005)). In general terms, the indices or their analogues measure different aspects of heterozygote deficiencies: global deficit, F_{IT}; among population deficit, F_{ST}; or within population deficit, F_{IS} (Goudet, 1995). FSTAT and GenAlEx can make use of both Weir & Cockerham's (1984) variance estimation based on allelic number as well as Nei's (Nei, 1973, 1977; Nei and Chesser, 1983) genotypic number based calculations. The latter weighs all samples equally, whereas the former weighs frequencies of alleles

according to sample size. This can lead to large variations in the two estimators when sample sizes vary significantly, as in this study where Vieques only has six samples whereas Anegada has well over 100 samples. GenAlEx can also perform Hedrick's (2005) standardized *G*-statistics (*i.e.* G_{ST} *and* G''_{ST}). The former index is an F_{ST} analogue adjusted for bias (G_{ST} = (*cHt-cHs*)/*cHt*) and the latter index, G''_{ST}, is further corrected for bias when the number of populations is small. Approaches employed for calculating these indices are shown below. Null hypotheses for genetic data and statistical tests used to accept or reject them are shown in Table 16.

Table 16: Null hypotheses and statistical tests used to accept or reject the null hypotheses for genetic data

Null hypothesis	Genetic parameters	Test (s) undertaken	Software
No genetic difference between pairs of populations	Pairwise F_{ST}	Non-parametric permutation test with 26000 permutations of haplotypes among populations	Arlequin 3.5
No inbreeding in the population (F_{IS}=0)	F_{IS} per population	Exact tests with 10000 permutations among individuals within each population	Genetix 4.05.2
Populations are at Hardy-Weinberg Equilibrium (random mating in populations)	HWE_{DEV} = *Ho* - *He* (Observed - Expected heterozygosity)	Exact tests using a modified Markov chain with length of 10^7 and 10^5 dememorization steps	Arlequin 3.5
No linkage disequilibrium between pairs of loci	LD	Exact tests using contingency tables and Markov chain with length of 10^5 and 10^4 dememorization steps	Arlequin 3.5
No correlation between genetic differences and geographical distance (isolation by distance)	Pairwise geographical distance (km) and pairwise F_{ST}	Regression analysis using matrix correspondence[1]; Mantel tests with 9999 permutations[2]	1) Arlequin 3.15 2) GenAlEx 6.5.01

FSTAT, using Weir & Cockerham's as well as Nei's estimations, was used for calculations of all indices over each locus in analysis group A samples as well as calculating F_{IS} over all loci per population to estimate inbreeding. To test the null hypothesis of no inbreeding, F_{IS} was calculated using 10^3 permutations and a 95% confidence limit was chosen resulting in P<0.05 will reject the hypothesis. Calculations for all *F*-statistics indices over each locus, between loci and between populations using analysis group A samples was undertaken with default settings in GenAlEx using Weir & Cockerham's estimations, Nei's estimations and Hedrick's standardized estimations. Arlequin, using Weir & Cockerham's (1984) variance estimation for *F*-statistics, was used to calculate all indices over each locus for analysis group A samples with 26,000 permutations. A confidence limit of 95% was chosen and the null hypothesis of no inbreeding rejected with P<0.05.

Genetix 4.05.2 was used to calculate *F*-statistics for the entire population based on Weir and Cockerham's estimations. Total indices were estimated per locus over all samples. F_{IS} was also estimated per population over all loci, and used as a parameter for estimation of inbreeding in

each population. The maximum number of permutations allowed by the software (10,000) was used to check significance level and accept or reject the null hypothesis.

The hypothesis of isolation by distance (IBD) based on the method developed by Slatkin (1993) through regression analyses using matrix correspondence was tested to determine if there was significant gene flow between the populations. This is of particular interest as the pollinators and dispersers of the species are not known and a lack of gene flow will suggest that the pollen and/or seeds are unable to move between populations requiring further study. Geographical distances in kilometres (km) were derived from the median of samples geographical coordinates by population. According to Veness (2010), geographical distance calculations are based on a spherical Earth and can, therefore, contain errors up to 0.3%. This amount of error is acceptable for inference of IBD as geographical coordinates derived from GPS units are only accurate to 10m and the mean value per population is used. Movable Type Scripts (MTS© 2002-2015 Chris Veness) available online (http://www.movable-type.co.uk/scripts/latlong.html) was used to calculate the shortest distance between the median values of analysis group A collecting coordinates using the haversine formula for each pair of populations. These calculations enabled the population of a square-matrix of pairwise population geographical distances (km) for IBD testing. Arlequin, making use of Slatkin's (1995) linearised distances, was used to generate a square-matrix of pairwise population Slatkin (1993) linearised F_{ST} values ((t/M= F_{ST}/(1-F_{ST}) with M=2N for diploid data). Analysis group A samples were analysed with Arlequin set to run 26,000 permutations. To check the significance of correlation between Slatkin's linearised F_{ST} and geographical distances, Mantel tests of 9^4 permutations were performed in GenAlEx on the two matrices across the five populations to calculate the squared correlation coefficient between observed and predicted values parameter (R^2). This parameter, according to Norusis (2008), infers the observed variability in genetic differences attributed to geographical distance.

Arlequin software was also used to undertake AMOVA (analysis of molecular variance) analyses to calculate genetic variability within populations, among populations within regions and among regions (Excoffier *et al.*, 1992). The method calculates sums of squared deviations by partitioning total variance into components of covariance across the hierarchy. The correlation of uniting gametes is used as a function of variance, and instead of gene frequencies variation, AMOVA uses the unique allele combinations of the diploid-acting loci (Weir and Cockerham, 1984; Excoffier *et al.*, 1992; Weir, 1996). AMOVA was used to calculate pairwise population F_{ST} values to estimate the population origin of samples following Meirmans (2006). To test the null hypothesis of population pairs lacking genetic

differentiation, F_{ST} was calculated via AMOVA in Arlequin using the default value of 26000 permutations and a 95% confidence limit (P<0.05 rejects the null hypothesis) was chosen.

Calculations of Nei's unbiased genetic distance (G_{ST}'), Nei's gene diversity (*uHs*) and Nei's estimate of genetic diversity (H_T) (Nei, 1973, 1977; Nei and Chesser, 1983) were undertaken between pairs of populations using default settings in GenAlEx and FSTAT. By adjusting for sample size, G_{ST}' and *uHs* overcome some of the issues associated with indices that do not correct for variations across samples. Nei's G_{ST}' accounts for levels of heterozygosity and allelic variation enabling a measure of genetic distance based on frequency of alleles (Nei, 1978). Nei's *uHs* is a representation, within subpopulations, of the expected heterozygosity under HWE and is therefore useful for diversity comparisons between regions or across populations. To explore genetic diversity across the entire population and within each locus, H_T was also calculated over all loci and over all populations for each locus.

The indices used to infer heterozygosity range in value for each index. The F_{ST} index (including G_{ST}' and G''_{ST}) varies between 0 and +1, where zero indicates a lack of genetic distance between paired populations and one being the highest population subdivision and maximum between population differentiation (Wright, 1965; Nei, 1978). For *F*-statistics indices used for inbreeding and mating system inference, the inbreeding coefficient (F_{IS}) and the total inbreeding coefficient (F_{IT}), values vary from +1 to -1 with inbreeding and excess homozygosity indicated by positive values. Negative F_{IS} values indicate outbreeding and if F_{IT} and F_{ST} values are equal, mating is random.

According to Wright's formula (1965), calculation of genetic flux *Nm* between populations was performed in GenAlEx. This method's appropriateness has been called into question in the literature (Whitlock and McCauley, 1999; Frankham *et al.*, 2002, p.330); however, it was undertaken and reported here to explore whether it is a useful measure in relation to *V. rupicola* samples for determining restrictions in gene movement between populations.

In order to assess Hardy-Weinberg equilibrium (HWE) as well as linkage equilibrium (HWLE) and subsequently interpret the results from cluster analyses using programmes assuming HWE and HWLE, additional population level analyses were undertaken using Arlequin software. To compare expected versus observed heterozygosity and HWE deviations, allelic frequencies were calculated per population over all loci to determine observed heterozygosity using the null hypothesis (no deviations from HWE and mating is not random) based on Levene (1949) and Guo and Thompson (1992). As the data contained two alleles, expected heterozygosity was calculated using the equation $\sum_i p_i=1$ described in Silvertown and Charlesworth (2001, p.54) where p_i is the frequency of the *i*-th allele. AMOVA analysis employing a Markov chain

consisting of 10^7 steps was started with 10^5 dememorisation steps using analysis group A sample data for the eight diploid-acting loci to test HWE.

To test for linkage disequilibrium (LD), exact tests of sample differentiation were undertaken among samples based on genotype frequencies following the contingency tables method described by Slatkin (1994). Detecting population demographic histories and allelic recombination can be achieved through LD testing. According to Flint-García *et al.* (2003), LD relates to inbreeding, population size and bottlenecking and is correlated to recombination or mutation. The former correlation can decrease allele associations while the later correlation can increase allele associations. Arlequin was used to test the null hypothesis of loci having no association through exact tests implementing a Markov chain consisting of 10^5 steps was started with 10^4 dememorisation steps using analysis group A sample data for the eight diploid-acting loci. A 95% confidence limit was chosen with $P<0.05$ rejecting the null hypothesis of no association.

4.3. Results

4.3.1. Karyology

Karyotyping

Chromosomes sticking together during mitotic metaphase only allowed estimations of chromosome numbers to be made. Based on a relatively few observations (n = 4), *V. rupicola* is an octoploid with 2*n* = 72 (Figure 58) assuming *x* = 9 (Heubl *et al.*, 1990).

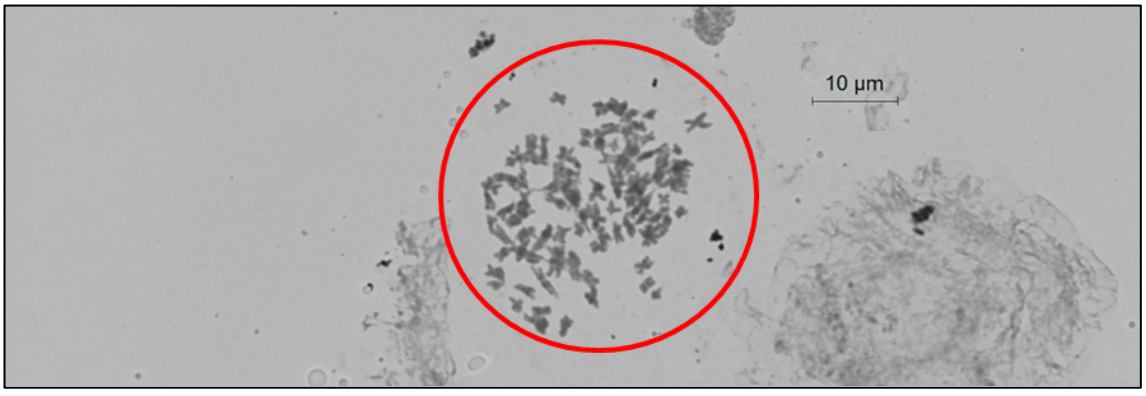

Figure 58: *Varronia rupicola* chromosomes (inside red circle) at mitotic metaphase showing clumping and a possible 2*n* = 72 giving an octoploid if *x* = 9 as described by Heubl *et al.* (1990). ©M.A. Hamilton.

Flow cytometry

Fresh leaves collected from *V. rupicola* and *V. bahamensis* plants were used to establish a flow cytometry protocol for these species using *Allium sativum* (garlic) as a standard. *Varronia rupicola* samples (n = 3) showed the same genome size (2C-value of 2.6 pg = 2542.8 Mbp) when compared to the garlic standard (Figure 59 (a)); however, the single *V. bahamensis*

sample available had a smaller genome size (2C-value of 2.1 pg = 2053.8 Mbp) than *V. rupicola* (Figure 59 (b)).

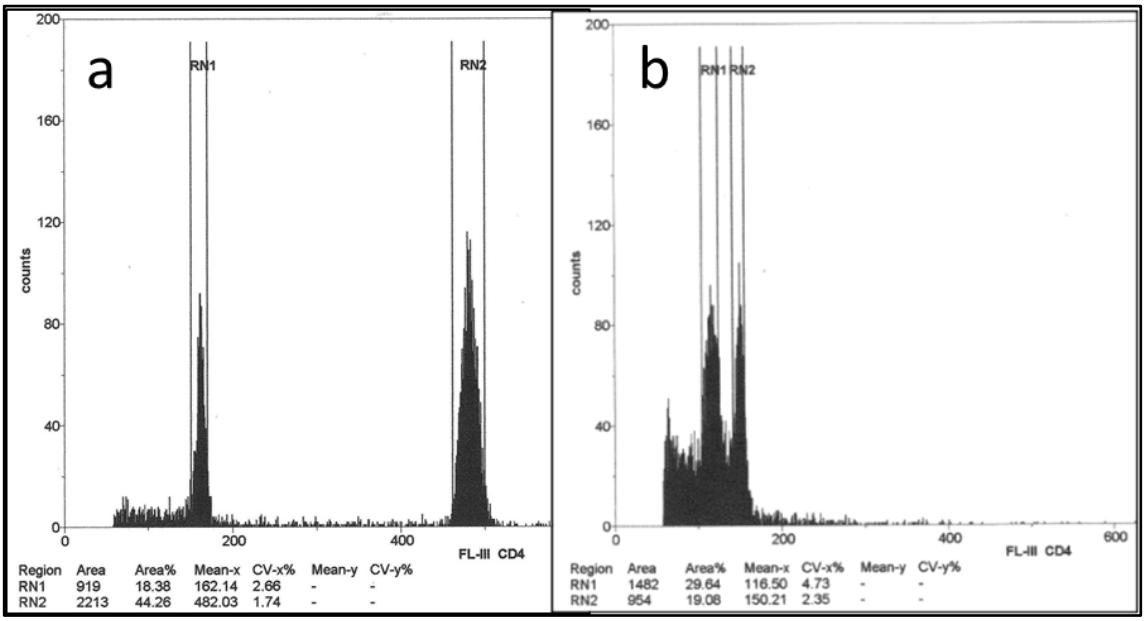

Figure 59: (a) Histogram of relative fluorescence intensities of G_1 nuclei (corresponding to 2C gDNA content) of *V. rupicola* (RN1) and *Allium sativum* (RN2); (b) Histogram of relative fluorescence intensities of G_1 nuclei (corresponding to 2C gDNA content) of *V. bahamensis* (RN1) and *V. rupicola* (RN2). ©M.A. Hamilton.

4.3.2. Next-generation sequencing

The extracted *V. rupicola* genomic DNA sample sent for sequencing produced a total of 12,130,785bp with a mean fragment size of 553bp and 39.3% GC content. In total, 21,907 individual sequences were produced with 20,875 of those having at least 80bp. Following QDD pipeline 1 processing, 2,507 sequences (11.4% of total sequences) contained at least one microsatellite. QDD pipeline 2 processing found 871 consensus sequences with 713 unique sequences (28.4% of the 2,507 sequences containing at least one microsatellite). A total of 865 sequences (3.9% of total sequences) contained target microsatellites and sequences with primers totalled 642 (2.9% of total sequences) following QDD pipeline 3 processing. A BLAST® search was performed (QDD pipeline 4) that returned very few potential matches, none of which were plastid regions or strict matches.

Further screening of the data following primer development to select best primers for target regions, as suggested by Gardner *et al.* (2011), resulted in 381 sequences (1.7% of total returned) including target microsatellites with six or more di-, tri-, tetra-, penta- or hexabase repeats that were used for selecting 44 primer pairs for testing (Appendix 7: Oligonucleotide testing). Of the primer pairs tested, 27 loci (61.4%) provided interpretable sequence data; however, only 13 (29.5%) were polymorphic for the trial samples (one from each island with extant populations).

Of the polymorphic loci, eight (18.2% of total tested) were diploid-acting (Figure 60) and five (11.4% of total tested) were polyploid-acting. The latter were often difficult to interpret; therefore, only two (4.5% of total tested) polyploid-acting loci were selected for further analyses. In total, ten polymorphic loci (22.7% of total tested) were analysed; however, analyses for the two polyploid-acting loci was limited due to most software programmes only being able to process haploid or diploid data. This is a major obstacle for interpreting polyploid species data, particularly if all loci are polyploid-acting. This research was able to discover eight diploid-acting loci for analysis in the most commonly used software packages (*i.e.* Geneland and Structure). Subsequently, data from the diploid- and polyploid-acting loci were combined to compare population clustering results only for diploid-acting loci. This was undertaken using PCoA to ensure that clustering results did not change significantly when introducing polyploid-acting loci data.

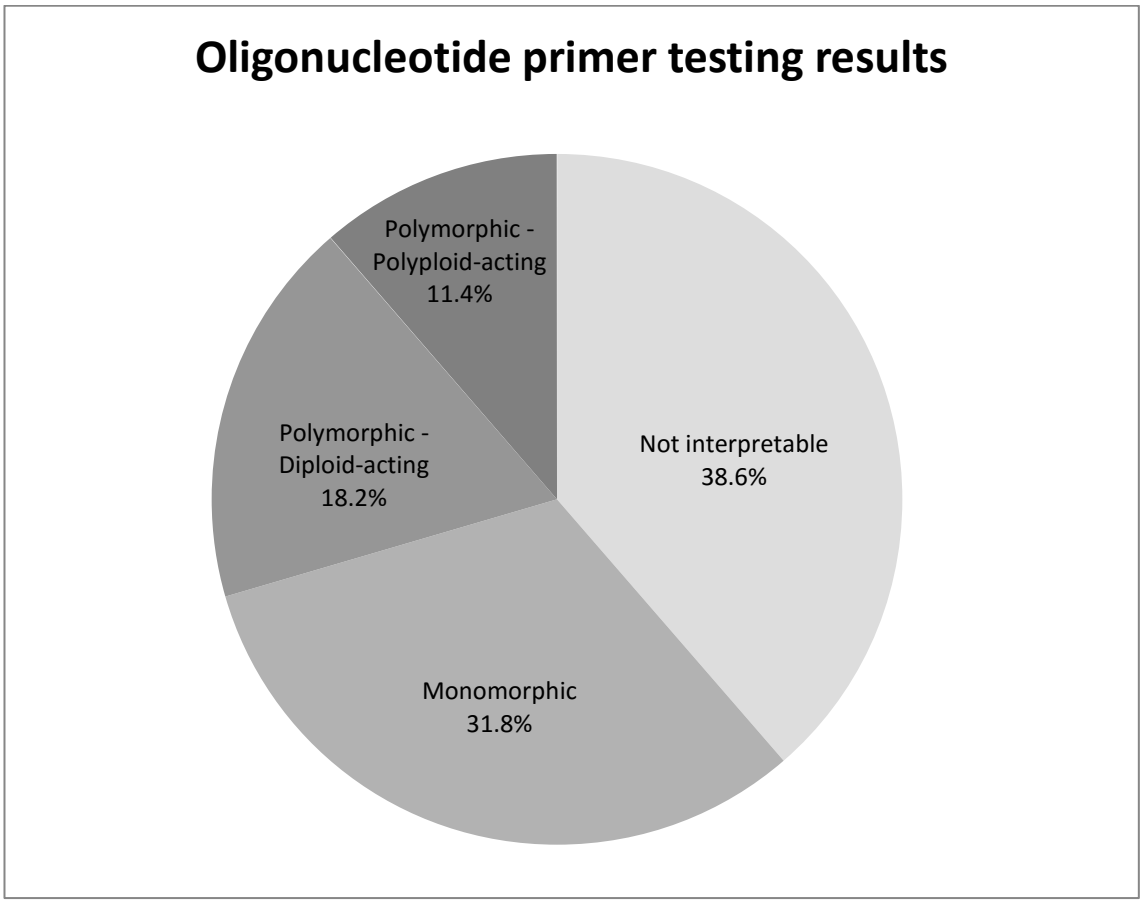

Figure 60: Testing results of 44 oligonucleotide primers for population level analyses of *Varronia rupicola* samples.

Samples that failed to amplify or gave weak peaks were repeated. Those still not scorable were excluded from final analyses. Of 443 extracted samples, 380 samples were selected for analyses. Of these, 260 samples were from extant wild plants, 114 were from extant *ex-situ* collections and six were from herbarium specimens collected between 1978 and 1995

(Appendix 6: Population genetics samples). The samples excluded (n = 63) were predominantly from *ex-situ* collections (n = 54) that were not from wild seed sources. The remaining nine excluded samples were from Anegada (Figure 61) leaving 144 samples from that island. Six samples originated from Vieques at a single location (Figure 62) and 110 samples originated from the island of Puerto Rico (Figure 63).

Following repeats and final sample selections, three analysis groups were defined. Group A (n= 260) contained only samples of extant wild plants. Group B (n = 374) consisted of extant wild and *ex-situ* plants. Group C (n = 380) included the same samples as group B as well as a further six herbarium specimen samples.

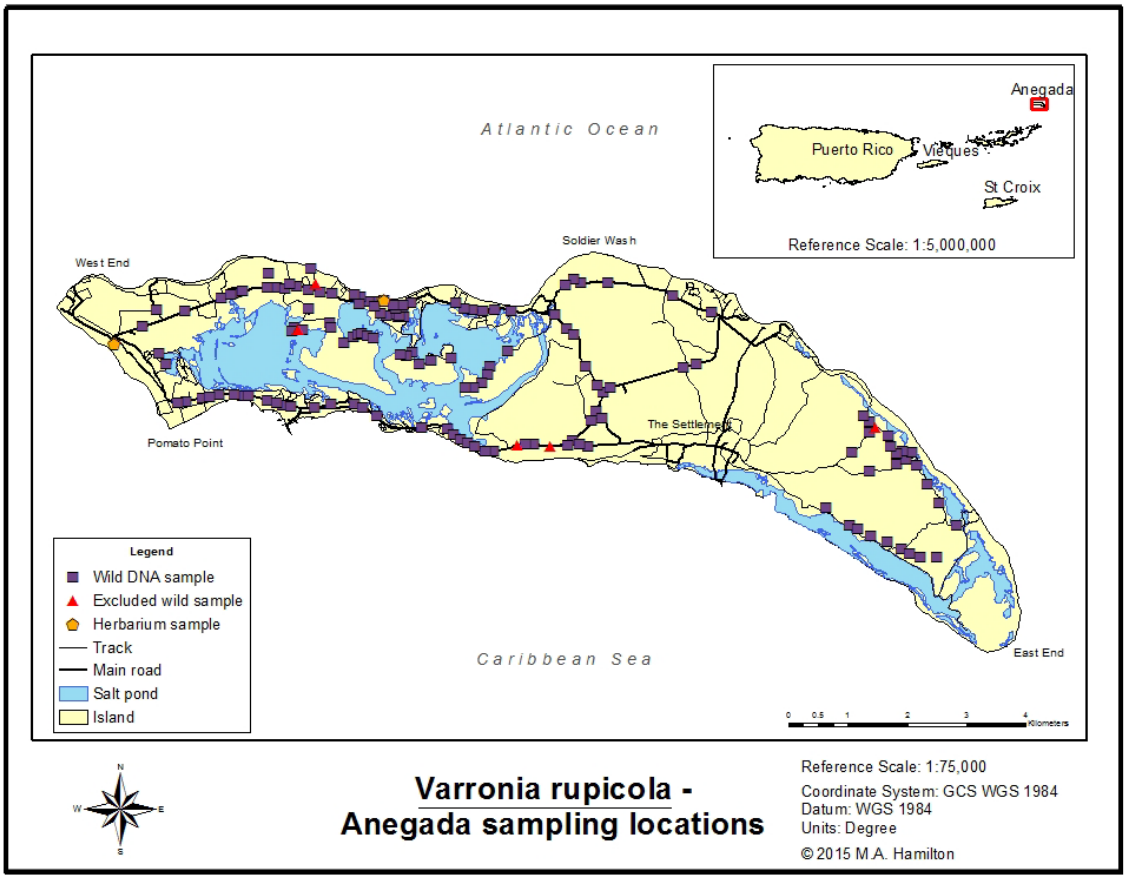

Figure 61: Map showing sampling of *Varronia rupicola* on Anegada for wild DNA samples collected and used during this research (purple squares), historical herbarium vouchers (orange pentagons) and wild DNA samples excluded from this study (red triangles).

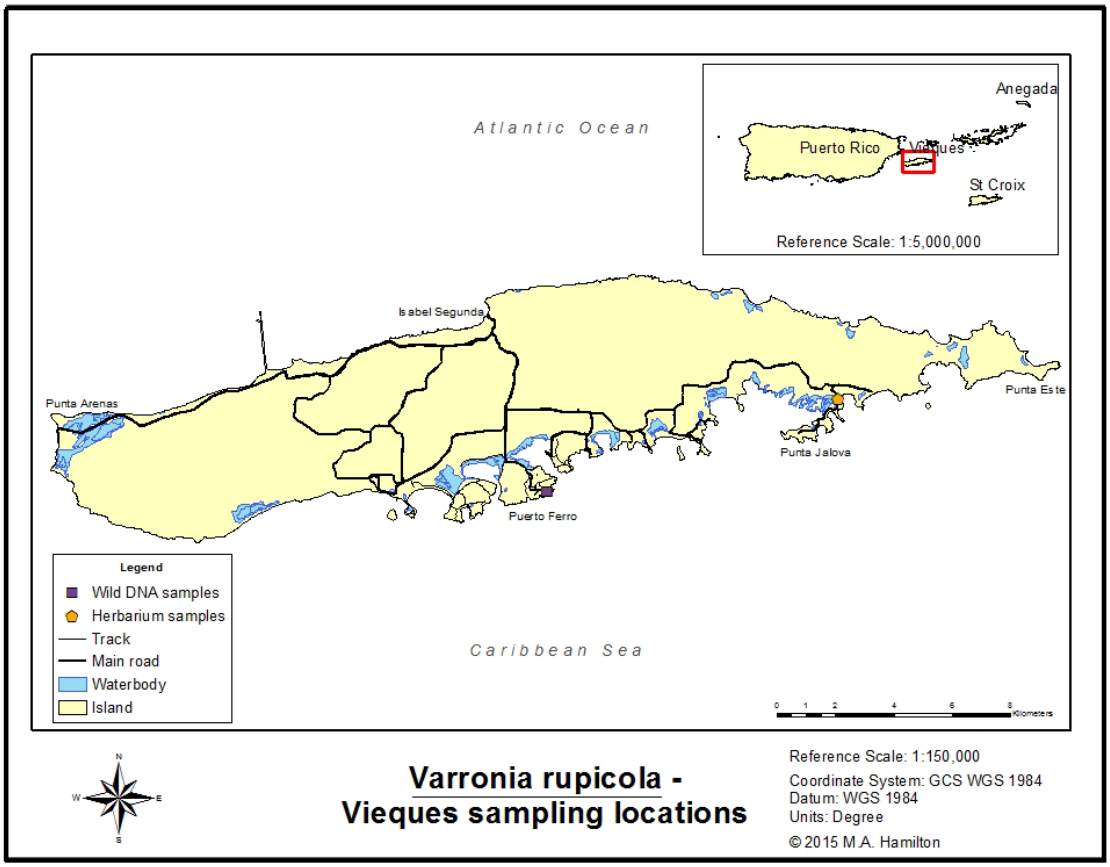

Figure 62: Map showing sampling of *Varronia rupicola* on Vieques for wild DNA samples collected during this research (purple squares) and historical herbarium vouchers (orange pentagons).

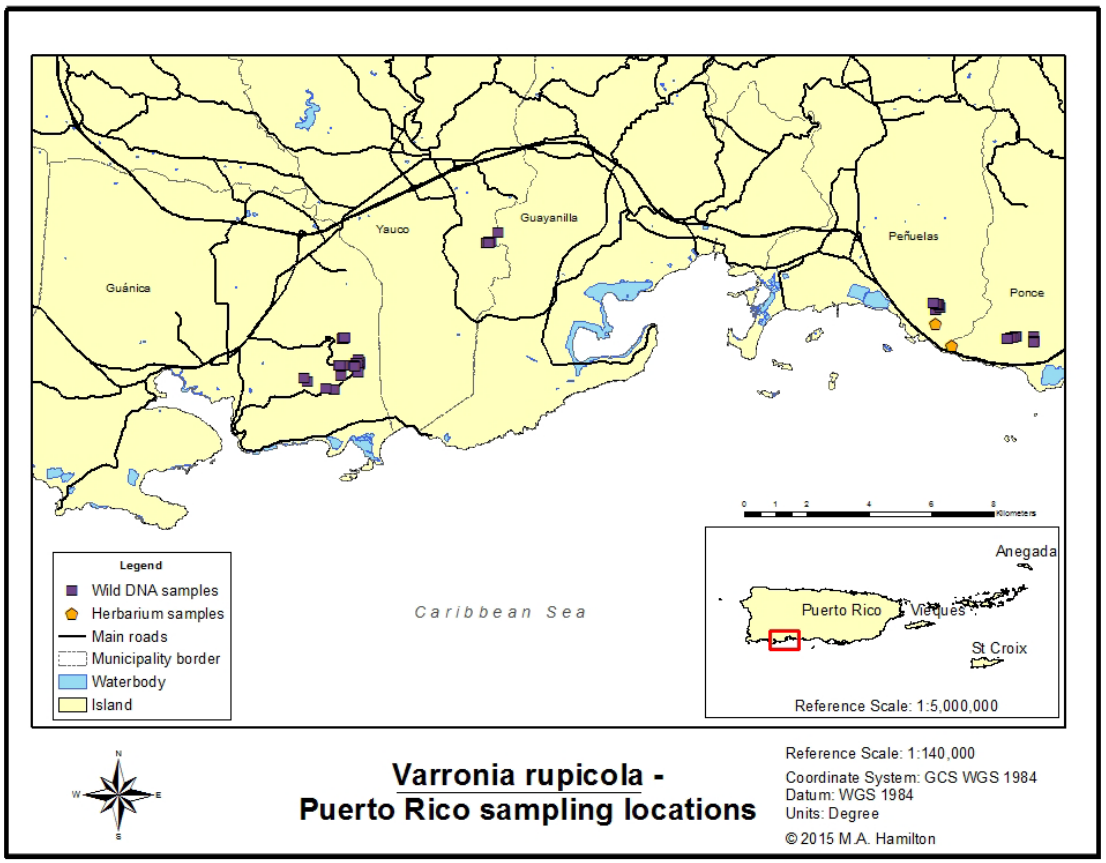

Figure 63: Map showing sampling of *Varronia rupicola* on Puerto Rico for wild DNA samples collected during this research (purple squares) and historical herbarium vouchers (orange pentagons).

Of the diploid-acting loci, all were polymorphic for wild samples (analysis group A) at the regional and country levels; however, two loci, VRBREYV and VRBL3FZ, were monomorphic for Vieques when undertaking population analysis (Table 17) resulting in a 95% mean value for polymorphic loci across the populations.

Table 17: Percentage of eight diploid-acting microsatellite loci showing polymorphism at regional, country and population levels for analysis group A samples of *Varronia rupicola*. Population descriptions and designations per sample are provided in Appendix 6: Population genetics samples.

Regional level		**Population level**	
Region	**% polymorphic**	**Population**	**% polymorphic**
Puerto Rican Bank	100%	Anegada East	100%
Country level		Anegada West	100%
Country	**% polymorphic**	Guánica	100%
British Virgin Islands	100%	Ponce	100%
Puerto Rico	100%	Vieques	75%

The only locus that had >5% missing data was the diploid-acting locus 'VRBREYV'. As all samples missing data for this locus originated from Anegada, this was magnified when undertaking population level analysis and will be discussed in 4.3.3. Allelic diversity. All other loci had less than 4% missing data for wild samples (Table 18). A single sample, number 432, from Anegada was missing data for three loci and was specifically retained due to the presence of rare private alleles.

Table 18: Missing data for nuclear microsatellites per locus; overall by ploidy group and over all loci for analysis groups A-C with the number of samples missing data per locus and per analysis group A-C (*A #, B #, C #*) and percentage of data missing per locus and per analysis group A-C (*A %, B %, C %*).

	Locus	***A #***	***A %***	***B #***	***B %***	***C #***	***C %***
Diploid-acting	VRgr3_2	9	3.5%	9	2.4%	9	2.4%
	VRBS43W	5	1.9%	7	1.9%	8	2.1%
	VRBREYV	18	6.9%	23	6.1%	24	6.3%
	VRBL3FZ	0	0.0%	0	0.0%	0	0.0%
	VRB5M1O	6	2.3%	8	2.1%	8	2.1%
	VR_gr271_2	2	0.8%	2	0.5%	2	0.5%
	VRA5NLR	5	1.9%	5	1.3%	6	1.6%
	VRE18LG	0	0.0%	0	0.0%	0	0.0%
	Overall	*45*	*2.2%*	*54*	*1.8%*	*57*	*1.9%*
Polyploid-acting	**Locus**	***A #***	***A %***	***B #***	***B %***	***C #***	***C %***
	VRD2DN4	10	3.8%	10	2.7%	10	2.6%
	VR_gr27_2	2	0.8%	3	0.8%	4	1.1%
	Overall	*12*	*2.3%*	*13*	*1.7%*	*14*	*1.8%*
All loci		***A #***	***A %***	***B #***	***B %***	***C #***	***C %***
		57	**2.2%**	**67**	**1.8%**	**71**	**1.9%**

Testing for cross-species transferability of the ten microsatellites (Table 19) selected for *V. rupicola* population analyses was successful for 50% of the loci across *V. bahamensis*, *V. bullata*, *V. lima* and *V. polycephala*. Of these five loci, only one, VRD2DN4, was successfully amplified for all four species. Results of the other four loci varied with VRA5NLR and VRE18LG only amplifying in one species each. Amplification was better for the loci VRBL3FZ and VR_gr27_2 with amplification in three species each.

Table 19: Amplification results for four *Varronia* species using ten microsatellite loci selected for *V. rupicola* population analyses with successful amplification of samples indicated by a plus (+) or no amplification indicated by a minus (-).

Locus	*V. bahamensis*	*V. bullata*	*V. lima*	*V. polycephala*
VRgr3_2	-	-	-	-
VRBS43W	-	-	-	-
VRBREYV	-	-	-	-
VRBL3FZ	**+**	-	**+**	**+**
VRD2DN4	**+**	**+**	**+**	**+**
VRB5M1O	-	-	-	-
VR_gr271_2	-	-	-	-
VRA5NLR	-	-	-	**+**
VR_gr27_2	**+**	**+**	-	**+**
VRE18LG	**+**	-	-	-

Below the results of allelic diversity and genetic structure are reported to compare genetic variability and explore population structure as well as diversity of extant plants.

4.3.3. Allelic diversity

Using analysis group C (all samples), alleles were summarised per locus, by ploidy group and overall (Table 20). The eight diploid-acting loci ranged in size from 145-350bp with 92 total alleles. The two polyploid-acting loci had 15 total alleles and ranged from 179-335bp. Two loci, VRD2DN4 and VRE18LG, failed to show any private alleles. All other loci had at least one private allele and the maximum observed was 13 private alleles for locus VRgr3_2.

The fixation indices (F_{IT}, F_{ST} and F_{IS}) calculated over all populations was lowest for VRE18LG which only had two alleles and was the only locus to show outbreeding. This locus showed the highest value (7.117) for estimation of migrants per generation (*Nm*) which ranged from 0.309 for locus VRBREYV and had a low, mean overall value of 1.863. Overall gene diversity (H_T) was 0.680 with the highest for VRBS43W (0.904) and the lowest value (0.453) observed for VRA5NLR which showed the only values similar for F_{IT} and F_{ST} which mean mating is random.

Table 20: Summary of microsatellite genetic parameters divided by loci groups and overall. Abbreviations and formulae: allelic range (*A-range*); number of alleles (*An*); number of private alleles (*Ap*); Weir & Cockerham's (1984) **estimations of F_{IT} and F_{IS} as well as Nei's estimate of gene diversity (H_T); Hedrick's (2005) G''_{ST} and migrants per generation (*Nm*). Significant *P*-values indicated by: "*" = 5% nominal level, "**" = 1% nominal level, and "***" = 0.1% nominal level. "^" denotes that values does not include polyploid-acting loci due to software limitations.**

	Locus	*A-range*	*An*	*Ap*	F_{IT}	G''_{ST}	F_{IS}	H_T	*Nm*
Diploid-acting	VRgr3_2	270-332	17	13	0.328 ***	0.703 ***	0.325 ***	0.760	0.620
	VRBS43W	250-350	15	5	0.338 ***	0.757 ***	0.334 ***	0.904	1.292
	VRBREYV	256-319	13	7	0.412 ***	0.920 ***	0.410 ***	0.828	0.309
	VRBL3FZ	200-230	6	1	0.380 ***	0.493 ***	0.381 ***	0.578	0.733
	VRB5M1O	210-300	25	10	0.297 ***	0.690 ***	0.296 ***	0.899	1.518
	VR_gr271_2	230-285	7	4	0.380 ***	0.187 *	0.369 ***	0.531	2.175
	VRA5NLR	180-230	7	4	0.308 ***	0.305 ***	0.312 ***	0.453	1.140
	VRE18LG	145-200	2	0	-0.693	0.071 **	-0.721 ***	0.490	7.117
Polyploid-acting	VRD2DN4	179-210	5	0	~	~	~	~	~
	VR_gr27_2	285-335	10	1	~	~	~	~	~
Overall*		**145-350**	**107**	**45**	**0.290^ *****	**0.510^ *****	**0.252^ *****	**0.680^**	**1.863^**

Forty-five (48.9%) of the 92 alleles for diploid-acting loci are private to one of the two countries, British Virgin Islands or Puerto Rico. Of these, one private allele appears to have been lost from Puerto Rico as it was only detected in historical herbarium specimens from that country and was found in extant wild material from Anegada (see Appendix 9: Private alleles detected for microsatellite loci).

Twenty-four (26.1%) of the 92 alleles for diploid acting loci are private to the British Virgin Islands. Of these, eight are shared between the two populations on Anegada defined as Anegada East (eastern half of Anegada, BVI including the localities of East End and Warner) and Anegada West (western half of Anegada, BVI including the localities of Bones Bight, Bones Low Point, Bumber Well Cay, Capt. Auguste George Airport, Citron Bush, Cow Wreck Bay, Flamingo Pond, Jack Bay, Keel Point, Low Cay, Middle Cay, North Raibin Slob, Nutmeg Point, Pearl Point, Pomato Point, Sambeal Slob, Setting Point, Soldier East Point, Soldier Point, Saltheap Point, The Settlement, Vagabond Pond, West End, Windlass Bight and Windlass Low Point); one is of unknown provenance (probably Anegada West origin) from *ex-situ* collections

held at Fairchild Tropical Botanical Garden; ten are only found in Anegada West samples; and five are only found in Anegada East samples.

Twenty (21.7%) of the 92 alleles for diploid-acting loci are private to the country of Puerto Rico with three of these only found on the island of Vieques. Of the remaining 17 private alleles from the country of Puerto Rico, four are only found in the population defined as Guánica (municipalities of Guánica and Yauco); seven are only found in the Ponce population (municipalities of Peñuelas and Ponce); four are shared between Guánica and Ponce; one is shared between Guánica and Vieques; and one is shared between Ponce and Vieques.

Of the 92 alleles for diploid-acting loci, 29 (31.5%) are private to one of the five populations. Of these, two private alleles (both from Anegada source material) were detected from *ex-situ* collections and not in wild populations. Only one (6.7%) of the 15 alleles for polyploid-acting loci is private to the British Virgin Islands where it is only found in the Anegada West population.

Levels of allelic richness per locus and population as well as across all samples per locus were estimated using FSTAT (Table 21). Standardising this parameter by rarefaction to the size of the smallest population (*i.e*. six for Vieques) enables allele frequency distributions to be compared between populations without bias (Leberg, 2002). Allelic richness per locus at the population level (*Rs*) for wild samples of *V. rupicola* varied from 1.0 for the two monomorphic loci, VRBREYV and VRBL3FZ, in Vieques samples to 7.5 for locus VRB5M1O in the Anegada East samples. The latter also showed the largest allelic richness (7.6) over all samples per locus (*Rt*) while the smallest (2.0) was observed for locus VRE18LG which did not vary across the populations due to only having two alleles.

Table 21: Estimations of allelic richness over wild populations performed using FSTAT software showing summary per population (*Rs*) and overall (*Rt*) allelic richness data for diploid-acting loci (Locus) with *Rs,* the estimate of allelic richness per locus and sample, and *Rt,* the estimate of allelic richness over all samples genotyped for each locus, based on the minimum sample size of six individuals.

Locus	Guánica (*Rs*)	Ponce (*Rs*)	Vieques (*Rs*)	Anegada East (*Rs*)	Anegada West (*Rs*)	Over all samples (*Rt*)
VRgr3_2	3.7	2.1	2.0	4.0	5.0	5.0
VRBS43W	5.4	4.3	4.0	5.1	6.0	7.0
VRB5M1O	6.8	6.9	3.0	7.5	5.9	7.6
VR_gr271_2	2.0	3.1	2.0	2.8	2.6	3.0
VRE18LG	2.0	2.0	2.0	2.0	2.0	2.0
VRBREYV	4.1	1.9	1.0	4.7	4.8	6.1
VRBL3FZ	3.2	2.4	1.0	2.5	3.3	3.8
VRA5NLR	1.2	2.1	2.0	3.0	3.0	2.6
Over all loci	**3.5**	**3.1**	**2.1**	**3.9**	**4.1**	**4.6**

Allelic richness observed over all loci ranged from 2.1 in Vieques, the smallest population, to 4.1 for the largest population, Anegada West (see Chapter 2: Biogeography of *Varronia rupicola*). This suggests that reduction of the Vieques population has had a detrimental effect on allelic richness. In fact, allelic richness appears to be linked to population size as observed in Figure 64. The number of private alleles (*Pa*) also follows this trend with the exception of the Ponce population that has slightly more *Pa* than the larger Guánica population.

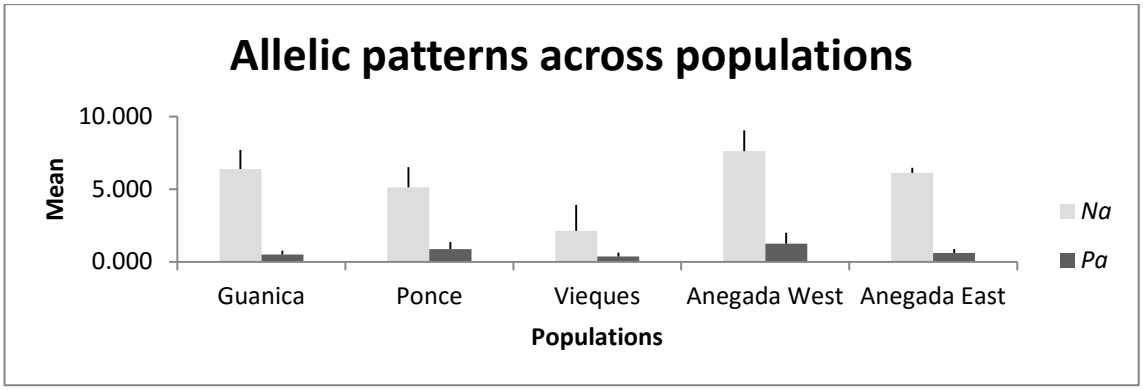

Figure 64: Summary graph of allelic patterns in wild populations (ordered west to east) showing the number of different alleles (*Na*) and number of private alleles (*Pa*) using analysis group A data and eight diploid-acting loci.

4.3.4. Genetic structure

Results of hierarchical analyses are reported below at regional (*i.e.* taxon), country and population levels. These analyses were based on analysis group A (extant wild samples) unless otherwise noted. Most software packages are unable to analyse microsatellite data with more than two alleles; therefore, the majority of results presented are for the eight diploid-acting loci and should be interpreted as such. Additional, however limited, analyses were undertaken to make use of the polyploid-acting loci. Where this is the case, the additional use of the two polyploid-acting loci in the analysis is explicitly defined.

Genetic variation within populations was the most significant source of variation (65%) found through global, locus by locus AMOVA analysis with results as a weighted average over all loci using R_{ST} (Slatkin, 1995) distance method (Figure 65). Variation among populations within countries (11%) was the smallest source of variation with regional variation between the two countries (25%) considerably higher.

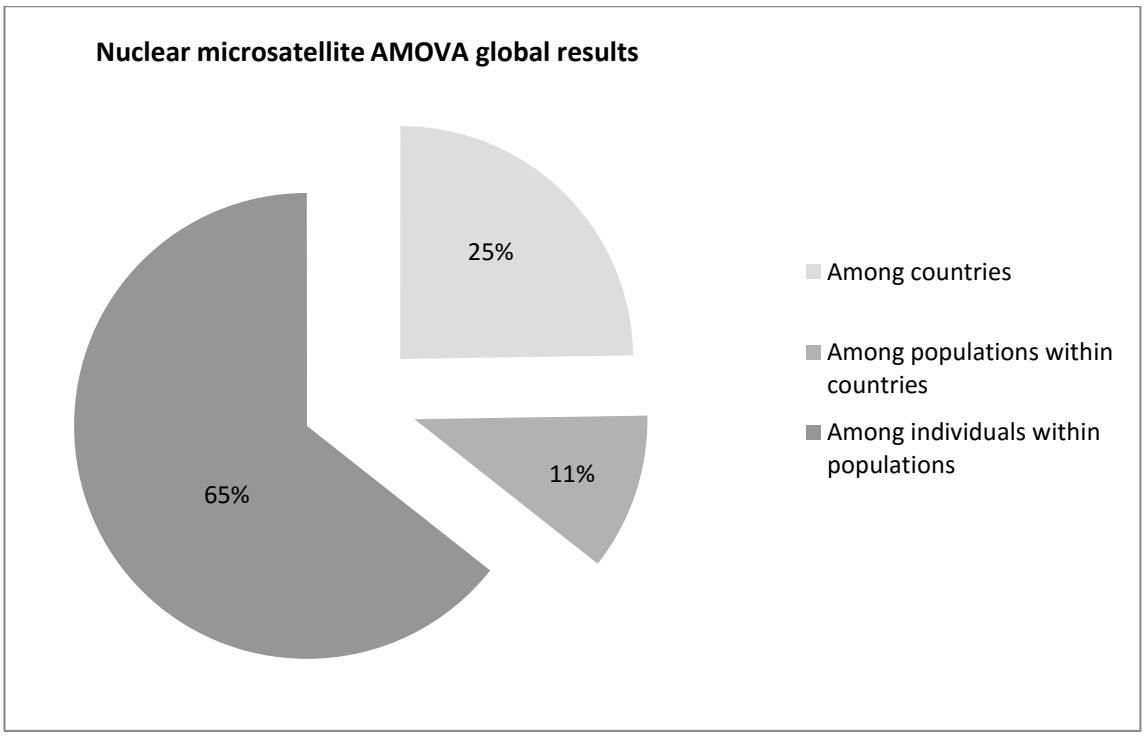

Figure 65: Analysis of molecular variance (AMOVA) regional variation (%) for analysis group A samples within populations, among populations within countries as well as among countries with analysis results as a weighted average over all loci using sum of squared size difference (R_{ST}) distance method (Slatkin, 1995).

Analyses of group A (extant wild) samples were undertaken at three hierarchical levels: regional (*i.e.* taxon), country (*i.e.* Puerto Rico and BVI) and population (*i.e.* Anegada west, Anegada east, Guánica; Ponce, and Vieques) levels (Table 22). Results of these analyses are presented in the following sections.

Table 22: Hierarchical analyses showing observed (*Ho*), expected (*He*) and unbiased (*uHe*) heterozygosity, within population deficit (F_{IS}) and HWE deviation (HWE_{DEV}) for diploid-acting loci of extant wild samples.
Abbreviations and formulae: Number of samples (*N*); number of alleles (*An*); number of private alleles (*Ap*); *Ho* = number of heterozygotes/*N*; *He* = 1-Sum *pi*^2; HWE_{DEV} = *Ho* - *He*; *uHe* = (2*N*/(2*N*-1))He*; F_{IS} = (Mean *He*-Mean *Ho*)/Mean *He*. Regional HWE_{DEV} *P*-values calculated in Arlequin. F_{IS} and F_{IS} *P*-values calculated in Genetix. All other values were calculated in GenAlEx. Significant *P*-values indicated by: "*" = 5% nominal level; "***" = 0.1% nominal level.**

Location	*N*	*An*	*Ap*	*Ho*	*He*	*uHe*	F_{IS}	HWE_{DEV}
PRB	260	92	44	0.501	0.631	0.634	**0.252** ***	-0.169 ***
BVI	144	70	24	0.572	0.668	0.671	**0.147** ***	-0.096 ***
Anegada East	25	49	5	0.535	0.633	0.647	**0.176** ***	-0.098 ***
Anegada West	119	61	10	0.580	0.656	0.659	**0.120** ***	-0.076 ***
Puerto Rico	116	67	20	0.430	0.594	0.597	**0.280** ***	-0.164 ***
Guánica	72	51	4	0.470	0.565	0.569	**0.175** ***	-0.095 ***
Ponce	38	41	7	0.379	0.464	0.470	**0.196** ***	-0.085 ***
Vieques	6	17	3	0.292	0.344	0.375	**0.239** *	-0.052 *

Regional and country level genetic structure

Heterozygosity and deviation from HWE was calculated at the regional (*i.e.* species) level (Table 22) over all loci to explore variation and test significance. Lower than expected mean observed heterozygosity at the taxon level (0.501) was observed across all loci and overall at the regional level. The overall positive value for F_{IS} of 0.252 showed a *P*-value ($P<0.001$) that was highly significant and the null hypothesis was rejected. Mating is not fully random as F_{IT} and F_{ST} values are not equal with F_{IT} being slightly larger.

The overall among population heterozygosity value for all loci of 0.214/0.510 (F_{ST} /G''_{ST}) was highly significant ($P<0.001$) and corresponds with AMOVA results (see Figure 65) showing low population subdivision and low between population differentiation. All among population heterozygosity values, F_{ST}, showed significant *P*-values ($P<0.05$) rejecting the null hypothesis of population pairs lacking genetic differentiation.

Country level analyses (see Table 22) were undertaken to compare with regional results and check for high levels of variation in heterozygosity between the two countries. Values of observed heterozygosity were slightly higher for BVI (0.572) and slightly lower for Puerto Rico (0.430) than regional values. Overall deviation from HWE was higher for Puerto Rico (-0.164) than BVI (-0.096) and significant *P*-values ($P<0.001$) were observed for all HWE deviations per country as at the regional level. The overall inbreeding coefficient (F_{IS}) for BVI was 0.147 and 0.280 for Puerto Rico indicating low levels of homozygosity and weak inbreeding are present. Both had significant *P*-values ($P<0.001$) and the null hypothesis was again rejected at the country level.

Following PCoA analysis a final graph (Figure 66) was produced showing individuals colour-coded by population and defined with convex hull outlines for each population. The first two axes explained most of the variation, 15% and 6% respectively, and suggests a high level of group distinction according to Peakall and Smouse (2012a). The PCoA analysis clearly separated the samples from the three islands; however, it was unable to make a distinction among the populations on the islands of Anegada and Puerto Rico. The broad grouping of Anegada and Vieques should be noted and will be discussed (see 4.4.3. Genetic Diversity) in relation to cluster analyses using Bayesian methods presented here.

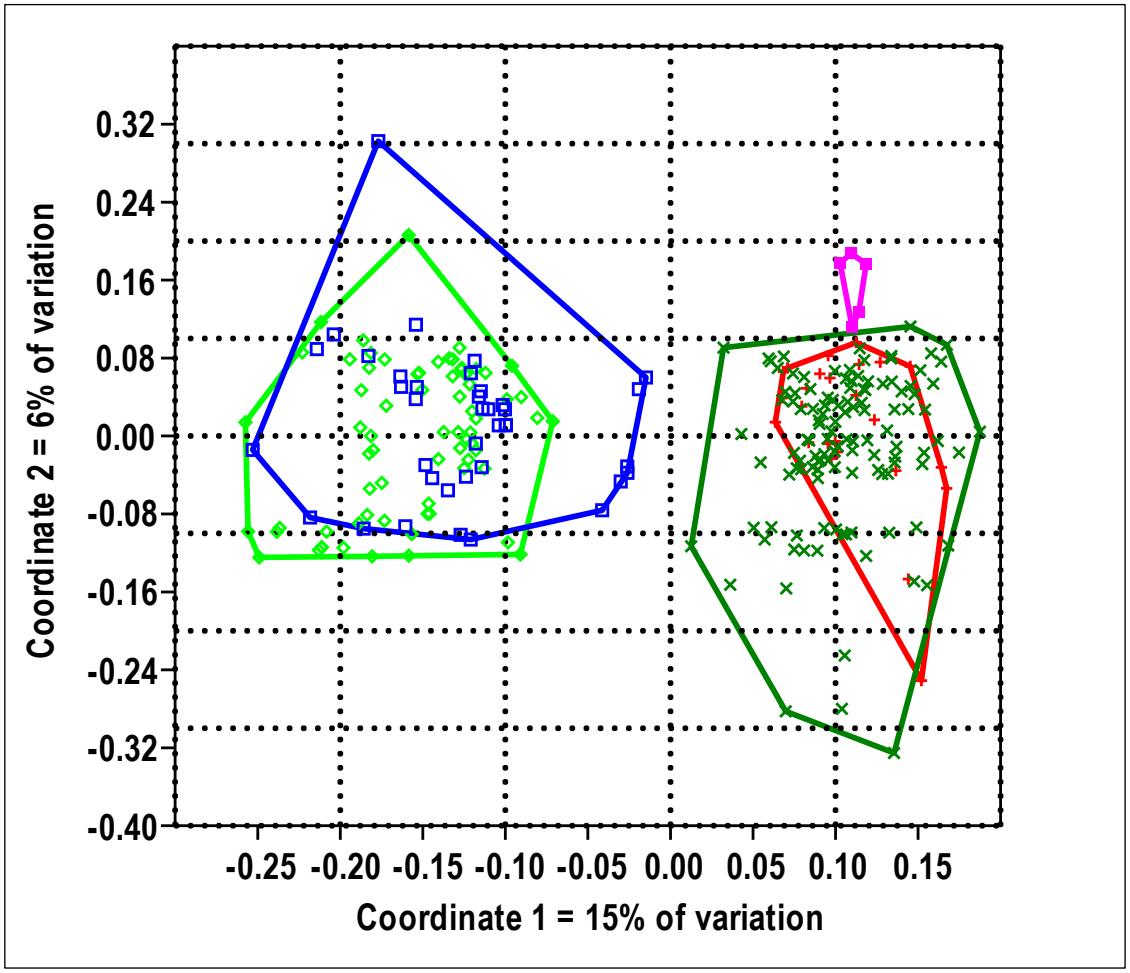

Figure 66: Principal coordinates analysis results of wild populations for ten polymorphic microsatellite loci with axis scales based on Eigen values using Jaccard similarity measure. Outlines are convex hulls: Pink = Vieques island samples with pink squares per sample; Red = Anegada East population samples with red pluses per sample; Dark green = Anegada West samples with green crosses per sample; Blue = Ponce population samples with blue squares per sample; Light green = Guánica population samples with green diamonds per sample.

Bayesian cluster analysis techniques implementing MCMC methods were undertaken in Geneland and Structure. Four analyses were undertaken in each programme to estimate population membership and the number of populations (*K*) for analysis group A (extant wild) samples using eight diploid-acting microsatellite loci (Table 23). Both packages gave similar results using the different models. Without either the spatial model or admixture (admix), Geneland failed to detect populations using uncorrelated allele frequencies and only detected two populations using correlated allele frequencies. Implementing the spatial model increased Geneland's ability to detect populations with seven detected using correlated allele frequencies and five using uncorrelated allele frequencies. The latter value of $K = 5$ had the highest posterior probability (89%) between the two analyses using the spatial model when run with maximum $K = 5$. All runs converged at the same value of *K*.

Table 23: Results for number of populations (*K*) analyses estimated from Geneland using the run with the highest posterior probability and Structure using best *K* default calculation (Pritchard *et al.*, 2000) **and Delta *K* "^"** (Evanno *et al.*, 2005) **on the CLUMPAK website. "*" denotes use of sampling locations as *a prior*; "~" denotes calculation not possible due to software limitations.**

Software	Geneland				Structure			
Spatial model	without spatial		with spatial		without spatial		with spatial	
Allele frequencies	Correlated	Uncorrelated	Correlated	Uncorrelated	Correlated*	Uncorrelated*	Correlated	Uncorrelated
Without admix	2	1	7	5	7 (2^)	5 (2^)	~	~
With admix	~	~	~	~	7 (2^)	5 (2^)	~	~

Although Structure lacks a spatial model, the package allowed collecting locations to be defined as *a priori* (Hubisz *et al.*, 2009) for the analyses and also has the ability to use two different ancestry models, with or without genetic admixture (Pritchard *et al.*, 2000). Structure analyses estimations of *K* were the same with or without admixture; however, results varied between correlated and uncorrelated allele frequencies when using the original best *K* analysis (Pritchard *et al.*, 2000). All estimations following Evanno *et al.* (2005) were *K* = 2, whereas those following Pritchard *et al.* (2000) matched Geneland analyses using the spatial model where *K* = 7 for correlated allele frequencies and *K* = 5 for uncorrelated allele frequencies. The correlated allele frequencies has been shown to overestimate clusters in some datasets (Falush *et al.*, 2003; Guillot, 2008) and the Evanno *et al.* (2005) estimations of *K* have been shown to underestimate when differentiation is low (Waples and Gaggiotti, 2006); therefore, *K* = 5 was selected (Figure 67) for these analyses and will be further discussed in 4.4.3. Genetic Diversity.

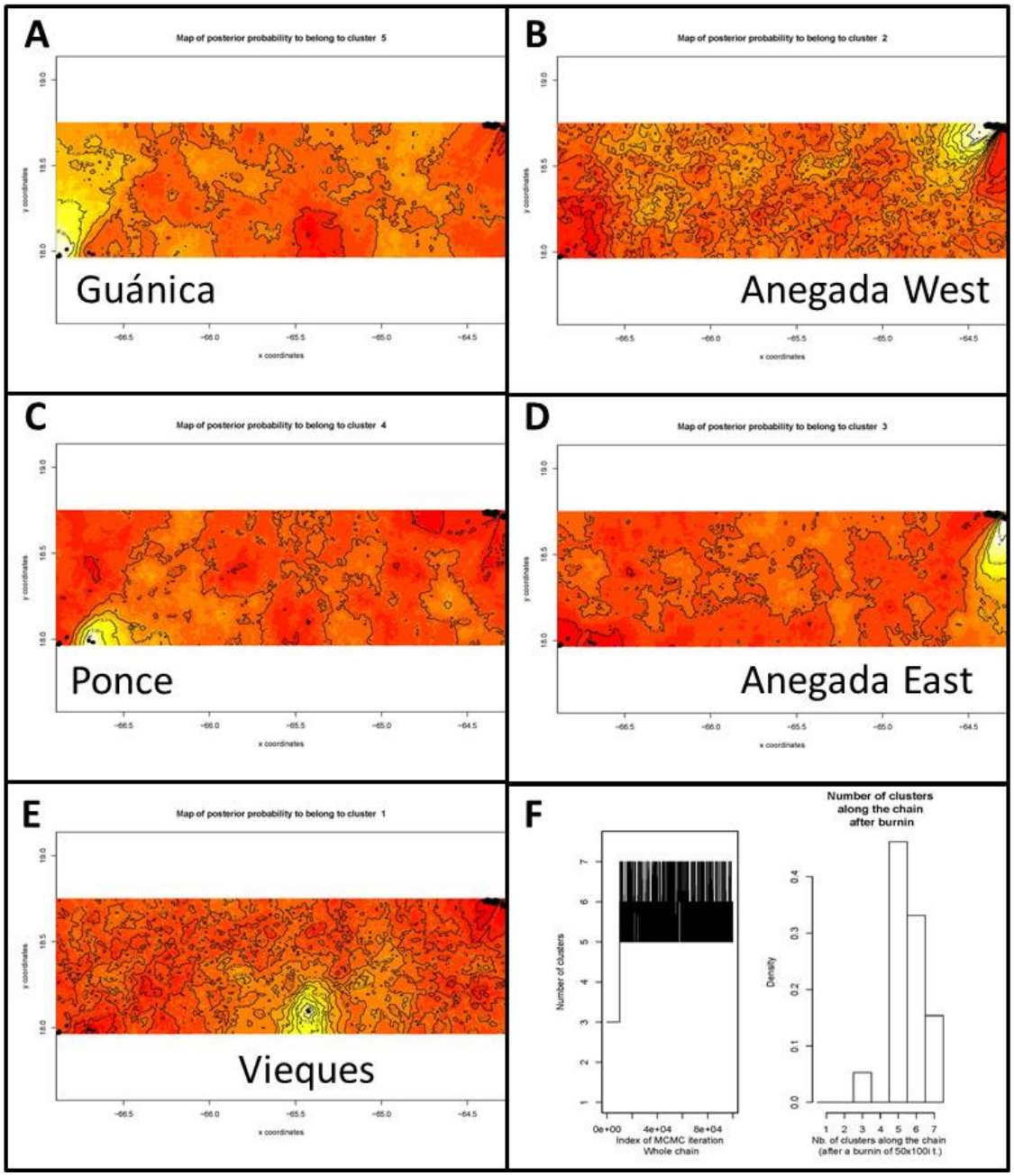

Figure 67: Populations and their spatial distribution estimated from wild samples using Bayesian methods in Geneland software without admixture, with the spatial model and uncorrelated allele frequencies. Maps of posterior probability to belong to each of the five populations (clusters) are shown in plates A – E with the highest probabilities indicated by lighter colours. Sampled individuals included in the analysis are represented by black dots. Populations were inferred through MCMC and the number of populations along the chain is depicted in plate F.

Based on Bayesian analyses of extant wild samples, five populations of *V. rupicola* occur across the Puerto Rican Bank: Anegada west, Anegada east, Guánica, Ponce and Vieques (Figure 68). The geographical extent and observations of individual *V. rupicola* plants will be further discussed and illustrated with more clarity in 4.4.4. Main conclusions.

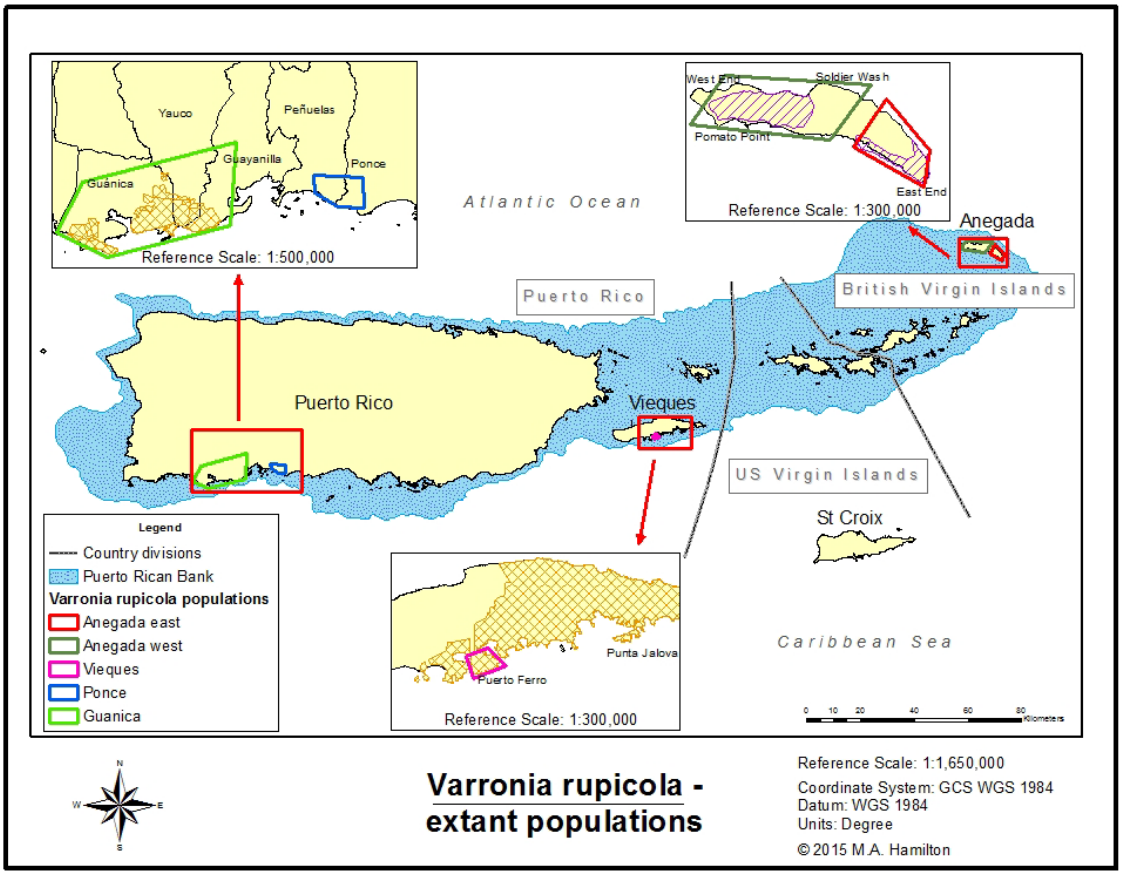

Figure 68: Populations of *Varronia rupicola* across the Puerto Rican Bank with coloured polygons to demarcate populations identified: Anegada west = Dark green polygon; Anegada east = Red polygon; Guánica (Guánica and Yauco) = Light green polygon; Ponce (Peñuelas and Ponce) = Blue polygon; Vieques = Pink polygon.

Inter-population genetic structure

Population pairwise genetic distances were calculated in GenAlEx and Arlequin to enable comparisons to be made of the different methods available in each. Results of pairwise population analyses giving F_{ST} calculated via Nei's (Nei, 1973, 1977; Nei and Chesser, 1983) distance method (Table 24) and those calculated via Weir and Cockerham's (1984) frequency method (Table 25) varied for analysis group A (extant, wild) samples; however, the variation was minor and all values showed low levels of between population differentiation and subdivision.

Table 24: Pairwise population heterozygosity analysis (pairwise F_{ST}) results and migrants per generation (*Nm*) for analysis group A samples calculated in GenAlEx software with F_{ST} values based on Nei's genotypic number calculations (Nei, 1973, 1977; Nei and Chesser, 1983) shown below the diagonal and estimations of migrants per generation (*Nm* = [(1/F_{ST})-1]/4) above the diagonal.

Population	Anegada East	Anegada West	Vieques	Ponce	Guánica
Anegada East	~	6.129	1.190	1.971	2.594
Anegada West	0.039	~	0.918	2.117	2.946
Vieques	0.174	0.214	~	0.679	0.781
Ponce	0.113	0.106	0.269	~	2.338
Guánica	0.088	0.078	0.242	0.097	~

Values for Nei's method ranged from 0.039 between the Anegada populations to 0.269 between Vieques and Ponce populations. The same populations showed minimum (0.064) and maximum (0.300) values using Weir and Cockerham's method. Due to software limitations, *P*-values were only calculated via AMOVA analysis in Arlequin. These were significant across the pairwise population calculations thus rejecting the null hypothesis of no genetic differentiation between populations.

Table 25: F_{ST} values for pairwise population analysis with F_{ST} values based on Weir and Cockerham (1984) **method below the diagonal followed by an F_{ST} *P*-value significance indicator where "***" = 0.1% nominal level. Above the diagonal are *P*-values obtained using Arlequin software.**

Population	Anegada East	Anegada West	Vieques	Ponce	Guánica
Anegada East	~	0.000	0.000	0.000	0.000
Anegada West	0.064***	~	0.000	0.000	0.000
Vieques	0.177***	0.262***	~	0.000	0.000
Ponce	0.137***	0.118***	0.300***	~	0.000
Guánica	0.119***	0.120***	0.277***	0.165***	~

Migrants per generation (*Nm*) values for analysis group A samples were also calculated between the populations (see Table 24). Vieques showed the lowest amount of migration when compared to all other populations, whereas the two Anegada populations showed the highest amount of between population migrations. While this measure has become less favoured according to Whitlock and McCauley (1999), it is shown here due to the support the calculations give to other analyses that will be discussed later.

Arlequin software was used to calculate Slatkin's linearised distances (Slatkin, 1995). Wild samples (analysis group A) collected across the Puerto Rican Bank were divided into five populations and geographical distances between the median values of geographical coordinates across all samples for each population were calculated. The resulting matrices, combined below into a single matrix (Table 26), were used for isolation by distance (IBD) hypothesis testing in GenAlEx.

Table 26: Pairwise population matrix developed for isolation by distance hypothesis testing showing results of Slatkin (1995) **linearised F_{ST} values below the diagonal and pairwise population matrix of geographical distances (km) above the diagonal for analysis group A samples.**

Population	Anegada East	Anegada West	Vieques	Ponce	Guánica
Anegada East	~	8.34	138.30	265.40	284.70
Anegada West	0.069	~	132.60	258.50	277.70
Vieques	0.215	0.355	~	133.50	153.50
Ponce	0.159	0.134	0.429	~	19.98
Guánica	0.135	0.136	0.382	0.198	~

To check the significance of correlation between Slatkin's linearised F_{ST} and geographical distances, Mantel tests were performed on the two matrices to calculate the squared correlation coefficient between observed and predicted values parameter (R^2). The null hypothesis of no significant relationship was not rejected as the isolation by distance hypothesis testing shows no significance (P = 0.179) across the five populations. The smallest population, Vieques, with only six extant individuals shows the highest Slatkin linearized F_{ST} values (Table 26, Figure 69) and lowest allelic richness (see Table 21).

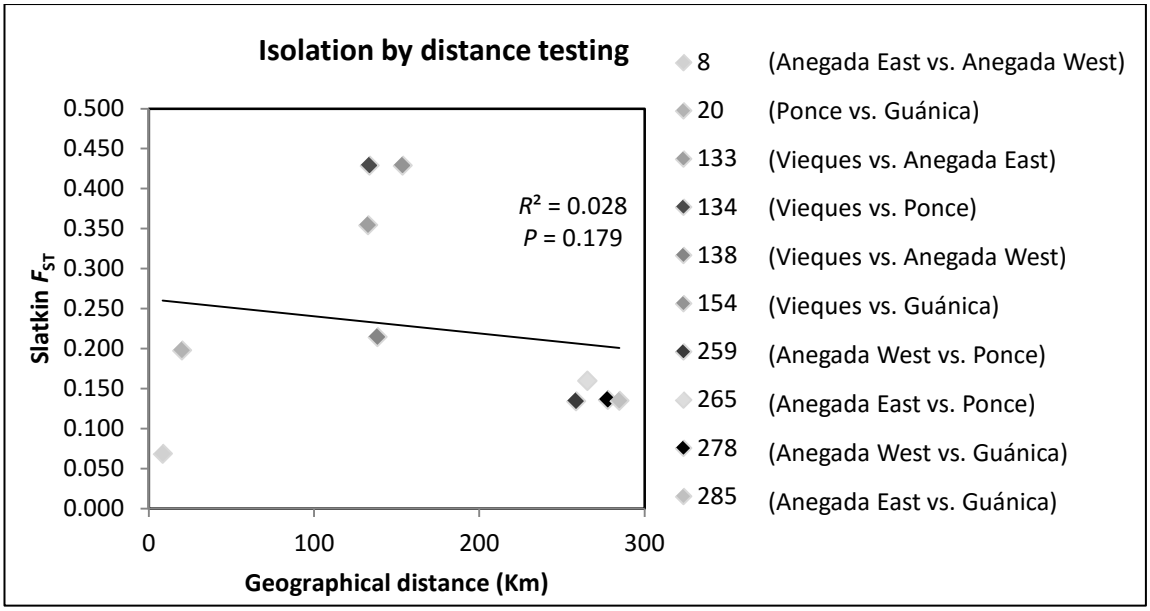

Figure 69: Isolation by distance test results derived from Mantel test calculations using pairwise population matrices of Slatkin linearized F_{ST} and geographical distances (km) performed in GenAlEx software. R^2 indicates variance explained by the model; in this case, 2.8% of the genetic distance between pairs of populations can be attributed to the geographical distance between these populations for nuclear microsatellites.

Results of GenAlEx population assignment tests (see Appendix 10: Population assignment tests) are summarised in Table 27. Three samples from the largest population, Anegada West, were assigned to other populations following testing. These reassignments were for sample 447 to the Anegada East population and samples 378 and 444 to the Ponce population. Overall, 99% of the samples were maintained in their originally assigned population.

Table 27: Summary of population assignment tests (Paetkau *et al.*, 1995, 2004) **for extant wild samples showing outcome per sample reported as remaining in the 'Assigned Population' or reassigned to 'Other Population'.**

Population	Assigned Population	Other Population
Anegada East	25	~
Anegada West	116	3
Guánica	72	~
Ponce	38	~
Vieques	6	~
Total assignments	***257***	***3***
Percentage assigned	**99%**	**1%**

To explore demography and detect recombination, paired loci associations were calculated to test the null hypothesis of no association between loci. All populations showed LD (Table 28) with the largest number of linked loci coming from the largest population, Anegada West. The smallest population, Vieques, with only six samples and two monomorphic loci that could not be calculated, showed the smallest number of linked loci.

Table 28: Number of linked loci per population across eight diploid-acting microsatellites with summary and overall linkage disequilibrium (LD) data divided by source population (Population) denoted by sequence identifier (Locus) and "^" denoting that values for two loci were not calculated for Vieques due to these loci, VRBREYV and VRBL3FZ, being monomorphic.

Locus	Anegada East	Anegada West	Guánica	Ponce	Vieques
VRgr3_2	1	3	4	0	0
VRBS43W	0	4	3	2	1
VRB5M1O	1	4	4	3	2
VR_gr271_2	0	4	2	2	0
VRE18LG	0	0	0	1	0
VRBREYV	1	5	5	0	~
VRBL3FZ	2	3	2	2	~
VRA5NLR	1	3	2	0	1
Overall	**6**	**26**	**22**	**10**	**4^**

4.4. Discussion and conclusions

Species conservation strategies can be greatly improved through a better understanding of genetic diversity and spatial structure, especially when coupled with detailed ecological and biological information. This research has sought to identify the genetic structure of *V. rupicola* and to inform the species management through detection of populations with unique genetic diversity as this will be key to the species survival in the future through adaption to changing environmental conditions (Fay *et al.*, 2009; Kramer and Havens, 2009). To that end, ten polymorphic microsatellite loci were selected and analysed. Of these, two loci, VRD2DN4 and VR_gr27_2, were polyploid-acting with five and ten alleles, respectively. These results provide support for *V. rupicola* being a complex polyploid and the discovery of eight alleles per sample across multiple samples for VR_gr27_2 further supports the idea that the species is an octoploid as eight alleles in a single individual should only be possible in an octoploid (or higher polyploid). The remaining eight loci were diploid-acting as they showed a maximum of two alleles. The eight loci that amplified from a unique site (one set of homeologues) suggest that the species is an allopolyploid that arose from crosses between species with disparate genomes (Collevatti *et al.*, 1999).

All diploid-acting loci were polymorphic for analysis group A (extant wild samples) at the country and regional levels; however, a 95% mean value for polymorphic loci across the populations was observed as two loci, VRBREYV and VRBL3FZ, were monomorphic for the smallest population, Vieques. For analysis group A, 3.5% and 6.9% data was missing from the diploid-acting loci, VRgr3_2 and VRBREYV, respectively. All samples missing data for these loci originated from Anegada and led to magnification of the missing data percentages when undertaking population level analyses as discussed below in 4.4.2. Allelic Diversity. Data missing for extant wild samples from all other loci was less than 4%.

Allelic dropout, when one or more copies fail to amplify by PCR at a locus, is not thought to be the cause of these missing data as high quality samples were used, other loci for these samples successfully amplified and the samples were repeated to minimise the potential of genotyping error (Wang *et al.*, 2012). These missing data, especially for the Anegada material, could suggest a mutation in the primer region as this can result in the inability of the loci to be successfully amplified (Paetkau and Strobeck, 1995).

Due to the limitations of commonly used software packages, analysis options for the two polyploid-acting loci were very limited. As the majority of microsatellite data was diploid-acting, most of the analyses focused on these eight loci. For these reasons, a range of techniques were employed to explore the data available and are discussed below.

4.4.1. Karyology

Gene duplication causes genetic variation and genome enlargement through the addition of gene fragments, genes, chromosomes (aneuploidy) or entire genomes (polyploidy) (Wayne and Miyamoto, 2006; Vandamme, 2009). Genomes of polyploids are highly dynamic and according to Soltis *et al.* (2014) undergo rapid changes in gene content and expression following polyploidisation. Polyploidy can occur through the union of genomes of two different species, allopolyploidy, or through the duplication of a single species genome, autopolyploidy (Leitch and Bennett, 1997; Soltis and Soltis, 1999; Petrov and Wendel, 2006).

Through observations of root tip cells at mitotic metaphase made during this research, it is clear that *V. rupicola* is a complex polyploid. Following the assertion of Heubl *et al.* (1990) that *Varronia* species have a base number of $x = 9$, counts made by the author would suggest that *V. rupicola* is an octoploid. Limited observations of *V. bahamensis* suggest that it is also a complex polyploid; however, formal counts were not possible and a ploidy level is not known.

Flow cytometry showed identical genome size measurements for *V. rupicola* samples originating from the islands Puerto Rico and Anegada. This suggests that plants in the two

countries have a shared ploidy. Results of genotyping (*i.e.* all samples showed similar numbers of alleles per loci) support this assertion. Comparison of *V. rupicola* and *V. bahamensis* samples showed the latter to have a smaller genome and supports the findings of phylogenetic research (see 3.4.3. Main conclusions) that places the two species separately.

Further studies are required to formally establish the ploidy level, ploidy type (e.g. allopolyploidy) and genome size of either species; however, the research presented here has provided the initial results required to interpret population genetic data.

4.4.2. Allelic Diversity

Missing data per locus was less than 4% overall except for the loci VRgr3_2 and VRBREYV as previously discussed. As these samples all originated from Anegada, population level analyses significantly magnified the missing data to the point that for Anegada East VRgr3_2 and VRBREYV were missing 24% and 8% data, respectively, and Anegada West was missing 10% data for VRBREYV. At the population level, all other loci had less than 5% missing data for analysis group A. Those loci with >5% missing data can be problematic for analyses performed during this research particularly pairwise distance-based methods (*i.e.* AMOVA, Mantel) and results provided from these loci must be interpreted with caution (Dufresne *et al.*, 2014). GenAlEx allows missing data to be interpolated such that average genetic distances are interpolated for each population level pairwise value; however, this can lead to bias, especially if large numbers of missing data are not minimized (Peakall and Smouse, 2012b). Loci missing >5% data were not considered in analyses undertaken with Arlequin (Excoffier and Lischer, 2011). Comparison of results for pairwise distance-based methods do not suggest that the missing data have introduced excessive bias; however, further analyses and simulation would be required to test this assumption (Wang *et al.*, 2012).

Alleles were summarised per locus, by ploidy group and overall using analysis group C samples (see Table 20). One diploid-acting locus, VRD2DN4, and one polyploid-acting locus, VRE18LG, lacked private alleles. All other loci contained private alleles, ranging from 1-13. The loci with ranges >60bp showed the highest number of private alleles (*Pa*) with the overall highest (*Pa* = 10) observed in the largest population, Anegada West (see Table 22). Vieques, the smallest population showed the lowest number of private alleles (*Pa* = 3). Over half (56%) of the private alleles were restricted to the British Virgin Islands where the majority of extant plants survive (see Chapter 2: Biogeography of *Varronia rupicola*). These results suggest that decreasing population size has a proportional effect on the number of private alleles within and among the populations (see Figure 64) and also suggests limited gene flow between the countries as

seen in the low (1.863) overall value of *Nm* and highly significant (*P*-value<0.001) total inbreeding coefficient (0.290).

Eight shared alleles have been found between the two BVI populations. For Puerto Rico, there are four alleles shared between Guánica and Ponce, one allele shared between Guánica and Vieques and one shared between Ponce and Vieques that are not found in the British Virgin Islands. The Puerto Rican populations of Guánica, Ponce and Vieques, have four, seven and three private alleles, respectively. There are ten and five private alleles found in the BVI populations of Anegada West and Anegada East, respectively. The high number of population specific private alleles further supports that overall gene flow is low.

One allele found in extant wild material from Anegada and detected in historical herbarium specimens from Puerto Rico was not found in extant wild material from the latter. This indicates that the allele has been lost from Puerto Rico as the sampling included the vast majority of extant (known) individuals in the wild and from *ex-situ* collections. Two alleles found in *ex-situ* collections from Anegada were not detected in extant wild material from that country. One of these alleles originated from a historical herbarium specimen and *ex-situ* collections and one only from *ex-situ* collections. Due to minimal records for the living collections, it is not clear whether the sampled plants in the *ex-situ* collection originated from wild collected seed or are propagules from another *ex-situ* collection; therefore, the allele only detected from *ex-situ* material could represent an undetected (due to sampling) natural allele that is potentially lost from the wild or represents a new allele originating from the *ex-situ* collections where several species of *Varronia* coexist. The allele detected in both historical herbarium specimens and *ex-situ* collections appears to represent either an undetected natural allele due to the sampling strategy or is one that has potentially been lost from the wild. This allele, along with all other rare alleles, is of particular importance for conservation planning and will be discussed further in 5.2. Conservation strategies.

4.4.3. Genetic Diversity

Puerto Rican Bank regional structure

Determining spatial population genetic structure through inference of the number of populations (*K*) by statistical models can be very challenging. The complexity of this process is discussed in a review by Sisson (2005) and highlighted in several studies (Françoıs *et al.*, 2006; Chen *et al.*, 2007; Guillot, 2009; Durand *et al.*, 2009; François and Durand, 2010; Cheng *et al.*, 2013) trying to determine the most robust models and software packages available for the task. In this research, a range of different approaches and software packages were employed to infer *K* and explore the different results obtained for analysis group A (wild extant samples)

data using the diploid- and polyploid-acting loci as a combined data set and separately depending on the limitations of the different software packages used.

Principal coordinates analysis (PCoA) of all ten loci detected three separate groupings of individuals based on genetic distances, one for each island, with poor resolution within the islands of Puerto Rico and Anegada (see Figure 66). A broad grouping of Anegada and Vieques is shown and provides insight for the best *K* results seen from the estimations following Evanno *et al.* (2005) of $K = 2$.

Bayesian techniques implementing Markov chain Monte Carlo (MCMC) methods to detect population boundaries were employed using Geneland and Structure software packages. These packages assume cluster members being at HWE and the presence of cluster-specific minimum allele frequencies (Guillot *et al.*, 2009). These assumptions are often unrealistic in wild populations and estimations of *K* using Bayesian clustering models may have no biological grounding depending on the data derived from a given sampling strategy (François and Durand, 2010). All populations detected by this research deviated from HWE; however, the results of PCoA analyses, which do not rely on the HWE assumption, were not considerably different from the results of Bayesian methods (see Table 23, Figure 66). Therefore, it seems that the HWE deviation has not had a significant negative impact on the Bayesian clustering results. For the analyses performed (see Table 23), the selection of ancestry model (with or without admixture) showed no effect on the results. Including a spatial model in Geneland or prior information about collecting location in Structure had a significant impact on the results as did the choice of allelic frequency model. François and Durand (2010) also found the inclusion of spatial information to be beneficial for analyses when comparing different software programmes. Guillot *et al.* (2005a) reported the overestimation of *K* using the correlated model; therefore, results from Geneland and Structure using spatial information and uncorrelated data, resulting in $K = 5$, were selected for post-processing (see Figure 67). The possible effects of populations not at HWLE on Bayesian methods could be further explored using packages that assume loci are unlinked (Gao *et al.*, 2007) as part of future research.

Despite 99% of the samples maintaining their originally assigned population, there were three samples that were reassigned (see Appendix 10: Population assignment tests, Table 27). Two of these samples, 378 and 444, originally assigned to Anegada West were reassigned to Ponce due to log likelihood values (illustrated in Figure 70 for convenience). The grouping of these samples with the other Anegada West samples suggests that these are possibly immigrants

and were correctly placed in the originally assigned population (Paetkau *et al.*, 2004). As such, the original population assignments appear extremely robust.

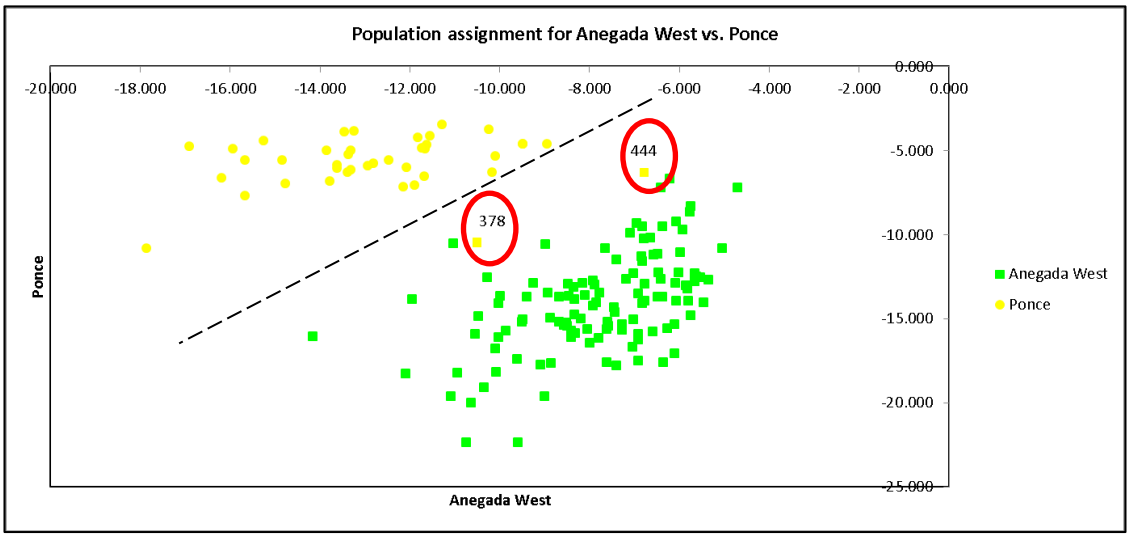

Figure 70: Pairwise population assignment graph for Anegada West vs. Ponce using the frequency method of Paetkau *et al.* (Paetkau *et al.*, 1995, 2004) **showing two samples, 378 and 444, originally assigned to Anegada West reassigned to Ponce; however, these are closely grouped with the other Anegada West samples as depicted by the dashed line.**

Overall, results showing clear separation of the three islands and further separation of extant individuals on the islands of Anegada and Puerto Rico into two populations each also appear to be the most biologically meaningful given the geographical separation (see Figure 57), historical land-use factors (see Figure 31) and on-going habitat fragmentation (see 2.4.2. Land cover). As such, the estimation of five clusters was chosen and population analyses were based on these groupings. These will also be the most practical groupings for conservation purposes at the country level; however, the broad grouping of Anegada and Vieques should be noted for the potential of *in-situ* conservation measures and population management. This will be discussed further in 5.2. Conservation strategies.

Regional variation between the two countries was the second most significant source of variation (25%) behind genetic variation within populations (65%). The variation among populations within countries (11%) was the smallest source (see Figure 65). Overall values for differentiation between populations were positive (F_{ST} = 0.214; G''_{ST} = 0.510) and the values were highly significant (P<0.001) confirming that population pairs have genetic differentiation. These results support the decision to select five populations instead of two as suggested by other methods (e.g. best K estimations following Evanno *et al.* (2005)). The results also show the need for a regional approach to conservation for the species to capture the large amount of variation at the individual level within populations.

An overall, low-level and significant (P<0.001) deviation from HWE (HWE_{DEV} = -0.169) was observed at the regional level across all loci as well as low levels of homozygosity and weak inbreeding (F_{IS} = 0.252 and F_{IT} = 0.290) overall (see Table 20) with the inbreeding coefficients highly significant at the regional level. As previously discussed, these results suggest that some caution should be taken in relation to the Bayesian techniques used to detect population boundaries as the packages assume cluster members being at HWLE. Although the HWE deviation results are significant, the values are low overall and the selected Geneland results all converged at the same value (K = 5) when using the uncorrelated allele frequencies and spatial models together. The presence of excess homozygosity and inbreeding in addition to significant genetic differentiation between populations indicate reduced gene flow at the species level.

Nonetheless, the isolation by distance (IBD) null hypothesis was not rejected as the overall value across the five populations (see Figure 69) was not significant (P=0.179). The smallest population, Vieques, with only six extant individuals remaining on the island showed the highest IBD values and suggests a relict population and possible bottlenecking. IBD could only explain 2.8% of the genetic distance between populations and therefore distance between islands is not the main underlying cause of the reduced gene flow observed in this research.

Country level analyses (see Table 22) were undertaken to compare with regional results and check for high levels of variation in heterozygosity between the countries. The findings of these analyses are discussed in the following sections.

Puerto Rican structure

Three populations could be detected based on results of the Bayesian analyses and *F*-statistics across the islands of Vieques and Puerto Rico with the latter having two populations defined as Ponce and Guánica. Observed heterozygosity was lower for Puerto Rico (*Ho* = 0.430) than the overall species value (*Ho* = 0.501). Observed heterozygosity was larger for Guánica (*Ho* = 0.470), the largest population in Puerto Rico, than the country value. Observed heterozygosity was proportional to population size across Puerto Rico suggesting that reduction in population size is having a negative impact on heterozygosity as all values were smaller than expected. Puerto Rico also had significant *P*-values for all HWE deviations across loci, but overall deviation from HWE was similar to the regional value. Overall inbreeding values for Puerto Rico are higher than the regional (taxon) value and all Puerto Rico populations had larger inbreeding values than those observed for the BVI. This suggests that the smaller and more spatially separate populations detected in Puerto Rico are exchanging less genetic material than those in BVI.

The Guánica population showed an overall low level of deviation from HWE with six loci having significant individual values (P<0.05) for HWE deviation. Guánica showed the second highest values (n = 22) for linkage disequilibrium (LD) with only one locus not showing signs of LD (see Table 28). Most loci showed low inbreeding values with an overall highly significant (P<0.001) value of 0.175 indicating that the population is inbreeding; however, it has the smallest value for the Puerto Rico populations and is a similar value to the smallest BVI population, Anegada East.

Overall levels of HWE deviation for Ponce were low and five individual loci exhibited HWE deviation with significant individual values (P<0.05). Five loci showed LD and Ponce had low to moderate overall levels with ten linked loci (Table 28). All but one locus showed inbreeding and with an overall value of 0.196 that was highly significant (P<0.001) indicating that the population is inbreeding.

The Vieques population level data must be analysed with caution as there were only six samples available for the analyses; two loci are monomorphic resulting in values not being calculated for many indices of those loci; and overall values are for only six loci compared to eight for other populations. With these issues in mind, only one locus showed significant deviation from HWE and overall HWE was the lowest for all populations. Vieques also had the lowest LD values overall (n = 4) with only three loci exhibiting LD. Vieques had the largest inbreeding value (F_{IS} = 0.239) over all populations (see Table 22), and the value was significant (P-value<0.05). This suggests that the small population size and close proximity of the six individuals is leading to higher levels of inbreeding than any other population.

Further research is needed to determine the flower morphology, pollination and dispersal (see 5.3.5. Reproduction biology and dispersal), particularly on Vieques, as these factors may be playing a significant role in the results observed here (e.g. lack of flowers with reciprocal floral forms for outcrossing).

British Virgin Islands structure

Two populations, Anegada East and Anegada West, were defined for the island of Anegada in the BVI based on results of the Bayesian analyses and F-statistics. Observed heterozygosity (Ho = 0.572) was higher for BVI and both of the populations in the country than the overall species value (Ho = 0.501) or Puerto Rico. The overall deviation from HWE was lower for the BVI (see Table 22) and significant P-values were observed for all HWE deviations across loci. As for Puerto Rico, observed heterozygosity was proportional to population size in BVI suggesting that reduction in population size is having a negative impact on heterozygosity as all values

were smaller than expected. Overall inbreeding values for BVI are considerably lower than the regional (taxon) value. This suggests that the larger and less spatially separated populations detected on Anegada are exchanging more genetic material than those in BVI. This was also supported by the results of pairwise population heterozygosity analyses and migrants per generation (see Table 24, Table 25).

Anegada West is the largest extant population of the species (519 observed individuals) and had the largest number of samples (n = 116) available for analysis. All loci for the Anegada West population showed HWE deviation with significant *P*-values ($P<0.01$) observed across loci. A highly significant (P-value<0.001) F_{IS} value of 0.120 (see Table 22) was observed for Anegada West indicating that the population is inbreeding; however, this was the lowest value observed across the five populations. The largest number of linked loci (see Table 28) was observed from Anegada West (n = 26) and all but one loci showed LD.

Anegada East (240 observed individuals, 25 sampled) showed significant ($P<0.05$) HWE deviation for five loci and the highest overall HWE deviation value (-0.098) of the five populations. Due to a significant (P-value<0.05) F_{IS} value of 0.176, Anegada East is inbreeding, but at levels lower than all of the Puerto Rico populations except Guánica. Five loci showed LD and overall Anegada East had the second lowest levels with six linked loci.

4.4.4. Main conclusions

Wild samples (n = 260) of *V. rupicola* collected from three islands with extant individuals in the PRB were combined with samples from five *ex-situ* collections (n = 114) and historical herbarium specimens (n = 6) to undertake population analyses and explore the extant genetic diversity of the species. Ten polymorphic nuclear microsatellites were genotyped for samples of *V. rupicola* and resulted in observation of 107 alleles. Private alleles were observed for each country and population. Eight microsatellites were diploid-acting with a maximum of two alleles. The remaining two microsatellites were polyploid-acting with five or eight alleles per sample supporting the theory that *V. rupicola* is a complex polyploid. Due to the observation of ca. 72 chromosomes and up to eight alleles per sample for one of the nuclear microsatellite loci coupled with the stated $x = 9$ base number of Heubl *et al.* (1990), the species is thought to be an octoploid.

Testing of the ten microsatellites selected for *V. rupicola* population analyses for cross-species transferability was successful for 50% of the loci (see Table 19) for one or more species across ten samples of *V. bahamensis*, three samples of *V. bullata*, two samples of *V. lima* and four samples of *V. polycephala*. Further testing is required to determine if these microsatellites are polymorphic for these species and interpretable for population level studies. Of particular

importance is the failure of *V. bahamensis*, the closest relative (see 3.4.3. Main conclusions) and species most often confused with *V. rupicola*, to successfully amplify for 60% of the microsatellites and did not return matching alleles for those amplified. This further supports the separation of these two species.

Significant (P<0.05) deviation from Hardy-Weinberg equilibrium (HWE) was observed at the regional level overall and within all populations; however, the overall value for the species was low (HWE_{DEV} = -0.169). Low levels of homozygosity and weak inbreeding (F_{IS} = 0.252 and F_{IT} = 0.290) were observed overall, and these were significant (P<0.05). Isolation by distance was not shown to be significant (P=0.179), but Vieques, the smallest population, did show higher IBD values than all other populations. Deviations from HWE and evidence of LD should be further explored to see if other factors are causing the departure from random mating [e.g. apomictic *Varronia* species have been suggested (Spoon and Kesseli, 2008)].

Cluster analyses based on Bayesian techniques (see Table 23) converged at the same value of K (n = 5) when using uncorrelated allele frequencies and spatial information together. Although fine scale resolution for the islands of Anegada and Puerto Rico (see Figure 66) were not detected with PCoA, clear separation for the three islands was detected. Population assignment testing following Paetkau *et al.* (Paetkau *et al.*, 1995, 2004) showed 99% assignment values providing very strong evidence for assignments. The three reassigned samples (see Figure 70) are possibly immigrants and were correctly placed in the originally assigned population (Paetkau *et al.*, 2004). The estimation of five clusters (see Figure 67) appears to be the most biologically meaningful given on-going habitat fragmentation, geographical separation and knowledge of the historical land-use factors (see 2.4.4. Main conclusions).

Population assignments are also supported by the elevation findings from Chapter 2 (see Figure 32). The two populations on Anegada (illustrated in Figure 71 for convenience) overlap in altitudinal range such that Anegada East is found from 0.50 to 3.71 m asl and Anegada West occurs between 0.90 to 7.45 m asl; however, these populations do not correspond to the range of the Vieques population (illustrated in Figure 72 for convenience) found 12 m asl or either of the Puerto Rican populations (illustrated in Figure 73 for convenience) found between 60 and 214 m asl. The altitudinal ranges of populations on the last two islands do not overlap either. Ponce samples ranged from 60 to 128 m asl whereas Guánica samples used for population analyses ranged from 133 to 214 m asl. The links to geological findings and the conservation implications of this research will be further explored in 5.1. Conservation implications.

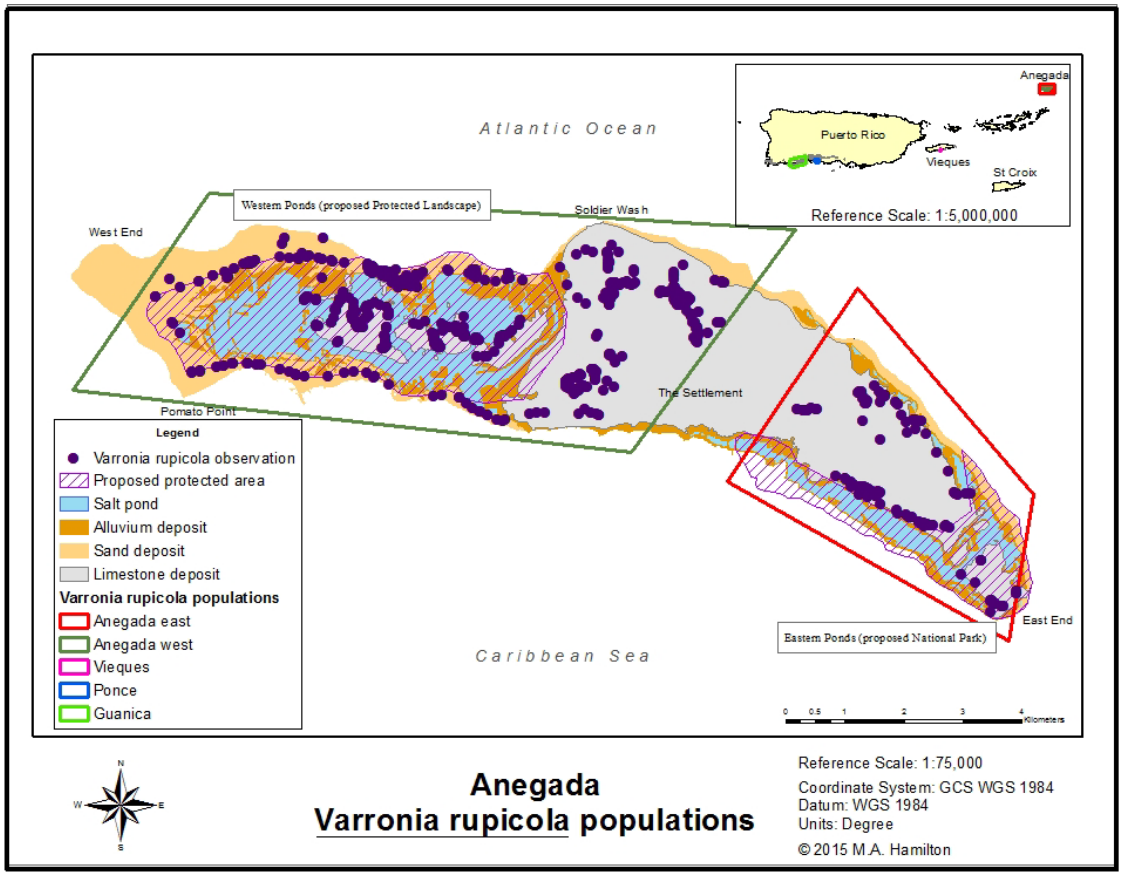

Figure 71: Map of *Varronia rupicola* populations on Anegada with observation records (purple circles) and coloured polygons to demarcate populations: Anegada west = Dark green polygon; Anegada east = Red polygon.

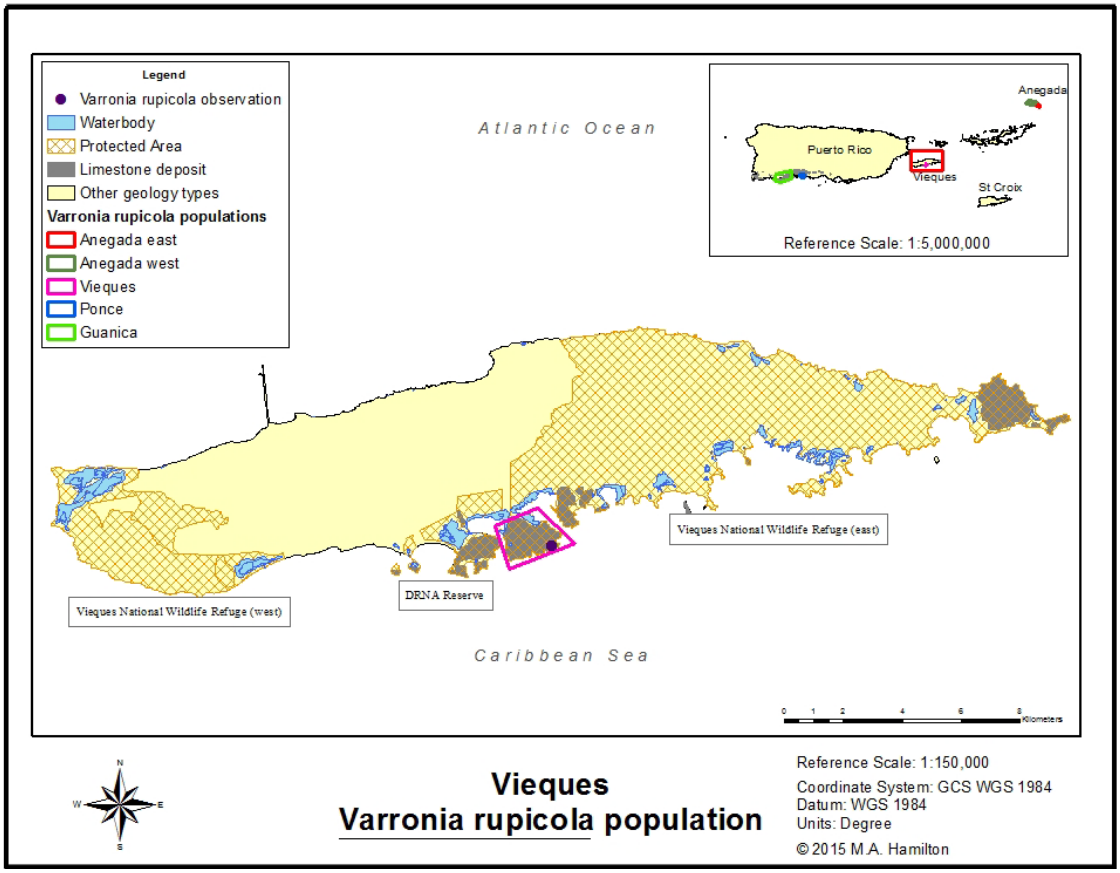

Figure 72: Map of *Varronia rupicola* population on Vieques with observation records (purple circles) and coloured polygons to demarcate populations: Vieques = Pink polygon.

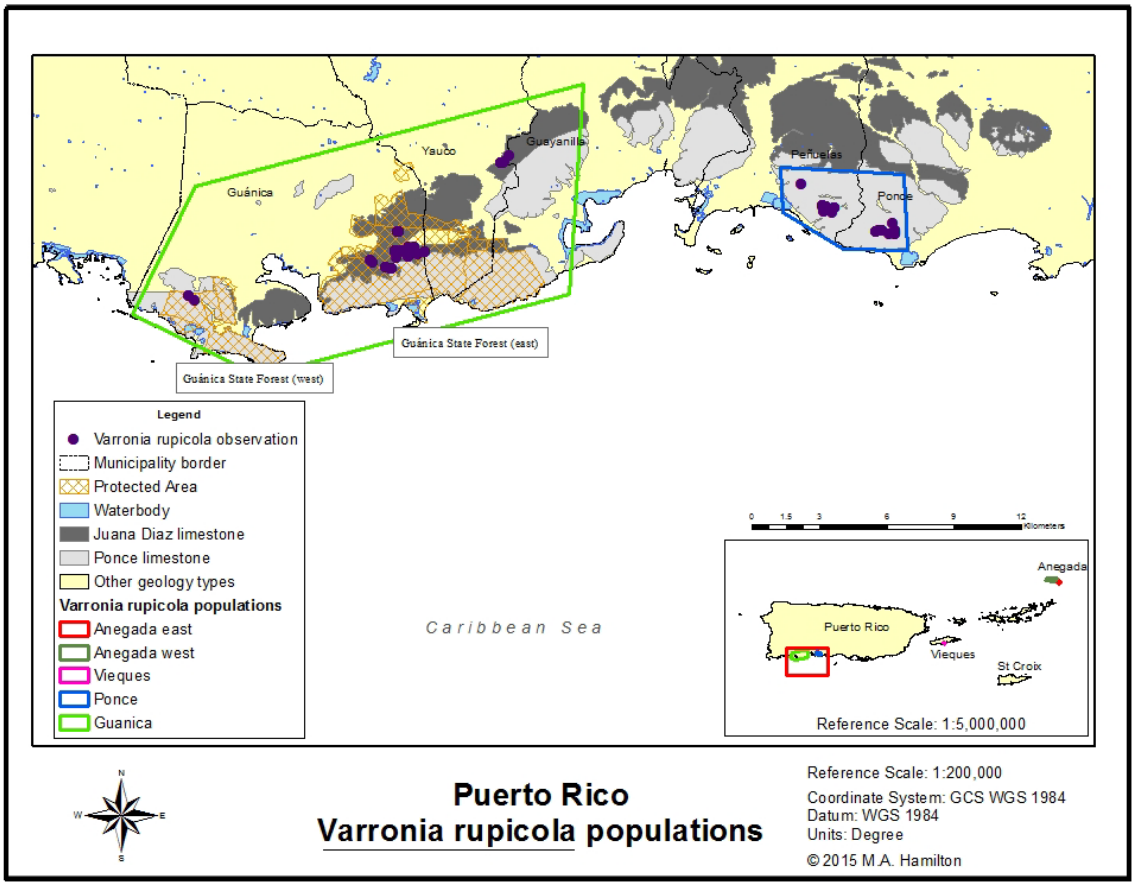

Figure 73: Map of *Varronia rupicola* populations on Puerto Rico with observation records (purple circles) and coloured polygons to demarcate populations: Guánica (Guánica and Yauco) = Light green polygon; Ponce (Peñuelas and Ponce) = Blue polygon.

This research set out to provide answers for a series of important questions for the conservation and management of *V. rupicola* genetic diversity.

- **First, are populations on the islands of Anegada, Vieques and Puerto Rico genetically distinct from one another?** Yes. Clear separation is observed through analysis methods employed [e.g. PCoA (see Figure 66), cluster analyses (see Figure 67)] and private alleles are observed for each island (see Table 22). Also, all populations show highly significant genetic differentiation (see Table 25).
- **Second, are there genetically distinct populations found within any of the islands?** Yes. Separation is shown in cluster analyses using Bayesian techniques for five populations (see Figure 68): one on Vieques (see Figure 72) and two on each of the islands of Anegada (see Figure 71) and Puerto Rico (see Figure 73). Significant values for genetic differences between populations (pairwise F_{ST}) were observed as well as private alleles for each population (see Table 22).
- **Third, has genetic diversity been impacted by a reduction in population size?** Apparently so. Vieques shows the highest inbreeding coefficient value and the lowest allelic diversity. These values are proportional to the size of the population across the

five populations (see Figure 64) which all show lower observed (*Ho*) than expected *(He*) heterozygosity.

- **Fourth, do existing *ex-situ* collections adequately represent the extant genetic diversity of wild populations?** No. Existing *ex-situ* collections only capture 44% of the 43 private alleles found in the wild. Existing *ex-situ* collections currently capture 14 of the 25 British Virgin Islands private alleles and five of 20 Puerto Rican private alleles, all from the Guánica population resulting in none of the private alleles in the Vieques or Ponce populations being captured.
- **Fifth, do existing *ex-situ* collections or samples from historical specimens reveal a loss of wild genetic diversity?** Yes. Two alleles detected in existing *ex-situ* collections or historical specimens from Anegada were not detected in the wild. One allele detected in historical specimens from Puerto Rico was not detected in the extant wild or *ex-situ* samples. This allele was detected in Anegada from extant wild samples suggesting it is lost from Puerto Rico.

The implications of these findings will be explored further (see Chapter 5: Discussion, conservation implications and research opportunities) with a specific focus on how the remaining genetic diversity observed in extant populations is linked to substrates and land cover across the native range of the species. This will be explored in the context of existing conservation measures, including protected areas and *ex-situ* collections, and recommendations for the species conservation and future research will be made based on the research presented here.

Chapter 5: Discussion, conservation implications and research opportunities

Based on the findings of this research, it is clear that *V. rupicola* and *V. bahamensis* are different but closely related species which occur in separate geographical areas and that other *Varronia* species sampled are more distantly related. This was specifically observed through:

- phylogenetic analyses using the *trnL*^{UAA} *intron* resolved the type material and modern samples of *V. rupicola* and *V. bahamensis* together, but in separate clades;
- combined ITS and *trnL-trnF* data resolved samples of the two species from across their native ranges as sister species in a well-supported (MP (96%), ML (95%) and BI (100%)) clade;
- flow cytometry showed that samples of *V. rupicola* from both BVI and Puerto Rico had the same genome size which was larger than that of a *V. bahamensis* sample from the northern Bahamas;
- of the ten microsatellites selected for *V. rupicola* that amplified for all samples collected across the PRB, six did not amplify in *V. bahamensis* samples collected across the Bahaman Archipelago;
- *V. rupicola* occurs on a very limited number of substrates on three islands in the PRB;
- micromorphological differences observed by the author during exploratory research between these species as discussed below in 5.3. Research opportunities.

This research brought to light several historical collections not previously known to workers in the PRB and provided new observation and voucher records through field survey as well as the confirmation of site and historical records from other workers across the native range of the species. The extant native range of *V. rupicola* (see Figure 21) is now known to be the islands of Puerto Rico (south-western coastal municipalities of Guánica, Yauco, Peñuelas and Ponce); Vieques (Puerto Ferro); and Anegada (across the island in 27 localities). This has provided a snap-shot of the extant metapopulation and enabled detailed assessment of the species biogeography.

Varronia rupicola plants were only found on specific substrates across its native range. On the island of Anegada (see Figure 22) the species was recorded on Pleistocene limestone and Quaternary deposits of sand. Pliocene limestone was found to support the species on the island of Vieques (see Figure 23), whereas substrates on the island of Puerto Rico, Juana Diaz and Ponce limestone formations (see Figure 24), were found to be the oldest deposits (Miocene origin) supporting the species. The limestone substrates found on each island are not known to occur on other islands in the PRB.

Overlying the substrates supporting the species, *V. rupicola* plants were found to be associated with specific land cover types across its native range. The regional habitat classifications of Kennaway *et al.* (2008) for the Virgin Islands and Kennaway & Helmer (2007) for Puerto Rico showed the species to prefer 'Deciduous, Evergreen Coastal and Mixed Forest or Shrubland with Succulents' on Anegada and 'Semi-Deciduous and Drought Deciduous Forest on Karst/limestone (includes semi-evergreen forest)' on Vieques and Puerto Rico. The latter classification is not found on Anegada, whereas the former is found in Puerto Rico although the species was not recorded in that land cover type in the country.

Varronia rupicola was found, in both countries and all three islands (see Figure 28), to occur within protected areas (established and proposed) containing 32% of the remaining intact preferred habitat of the species. The area of preferred land cover types for *V. rupicola* contained within these protected areas varies widely with the smallest proportion protected (15%) per island found on Anegada where the largest number of extant individuals remain. The potential consequences of this variation and the on-going loss of suitable habitat across the three islands on the substrates known to support the species are discussed further in the following sections. Maps below include no new data and are provided to assist the reader with visualisation of the discussion without referring back to other chapters.

5.1. Conservation implications

The implications of the current distribution and composition of protected areas across the PRB for the conservation of *V. rupicola* vary significantly between the islands. This is due to many factors including the numbers of extant individuals, the amount of remaining preferred habitat and the overall area of substrates that support the species. On each island, these factors may be significantly impacted by future climate change, especially in relation to elevation.

Chapter Three determined the placement of *V. rupicola* and its relationship with morphologically similar and often confused species. This was paramount for conservation planning as the confusion, particularly with *V. bahamensis*, has led to on-going issues with voucher curation and literature references to the two species distributions which negatively impact conservation recommendations and action. The findings of Chapter Four indicate that there are five populations found in the PRB with varying levels of allelic and genetic diversity as well as low levels of inbreeding. These findings are of particular significance for the species conservation and must be taken into account when developing management plans. In Chapter Two, the species biogeography was explored and the findings show that the species ecological preferences and native range put it at risk of extinction as discussed below in relation to the species population genetics.

5.1.1. Geology and land use

The limited area of occupancy (AOO), reliance on intact habitat and isolation to relatively small areas of specific substrates make *V. rupicola* particularly susceptible to impacts from human activities. Historical land use is poorly understood across the native range of *V. rupicola* due to a lack of detailed records and maps along with poor oral history; however, long-term habitat modification has been documented across the PRB (Schomburgk, 1832; Eggers, 1879; D'Arcy, 1971, 1975; Murphy and Lugo, 1990; Lugo *et al.*, 1996; Molina Colón and Lugo, 2006; Ramjohn *et al.*, 2012) since before the species was described by Urban in 1899. On the island of Anegada, historical agricultural features were digitised by the author (see 2.3.2. Land cover). The resulting features with all observations recorded of the species and the populations defined by this research are shown in Figure 74. Impelling evidence of the impact of historical land use on Anegada are shown through the nearly continuous corridor of disturbance in the middle of the island overlaid with the observations that show a distinct (1.75 km minimum) gap between plants occurring in the two identified populations.

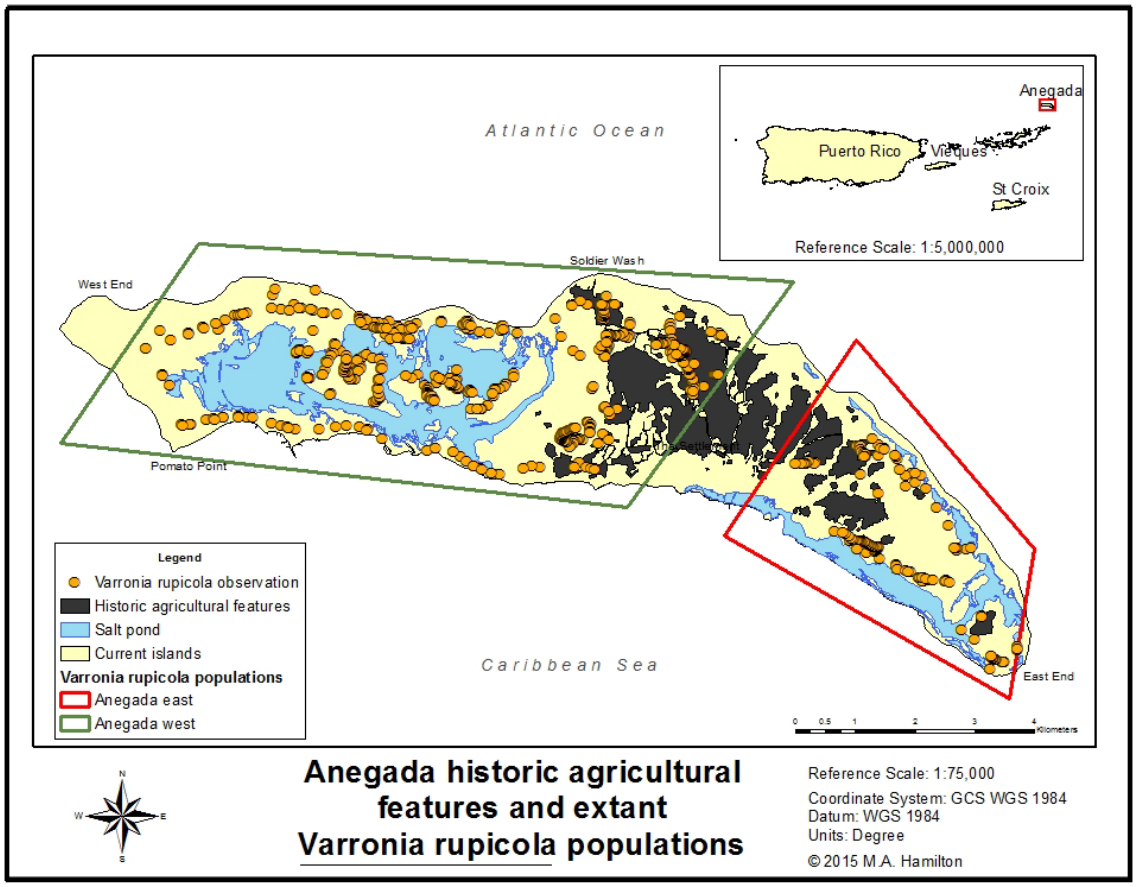

Figure 74: Map showing historical agriculture features as well as populations detected and observations of extant *Varronia rupicola* recorded between 2012 and 2015 on Anegada.

The isolation of extant individuals at a single location on the island of Vieques suggests that the historical habitat disturbance as well the potential impact on pollinators and dispersers during the 60 year period of use by the U.S. Navy has had a hugely negative impact on the species.

This assertion is supported by results of genetic analyses that showed the lowest number of private alleles and the lowest allelic richness for Vieques as well as the highest values for IBD and inbreeding.

Historical disturbance on Puerto Rico is well documented with Figueroa-Colón (1996) stating that 90% of the Puerto Rican forests have been modified and only ca. 1% of the mature vegetation was untouched. The separation of plants on Puerto Rico into two populations suggests that historical disturbance has impacted gene flow. The area between these populations where no modern records exist is centred on the municipality of Guayanilla. Of particular importance in this area are the developments of an oil refinery and a major highway that cut between the populations detected.

The historical disturbance that led to habitat loss is also the greatest current threat to the species. Quarrying and landfills are common developments across the species native range (Huggins *et al.*, 2007), particularly in areas not already developed for anthropogenic infrastructure. This research found that the limestone geology that supports the species is very limited with only 19.80 km^2 (10%) of Pleistocene limestone on Anegada, 6.28 km^2 (3%) of Pliocene limestone on Vieques and 165.82 km^2 (86%) of Miocene limestone formations (Juana Diaz limestone (67.83 km^2) and Ponce limestone (97.99 km^2)) on Puerto Rico. With such a small area of potential habitat to begin, all development and disturbance on these limestone formations can have a significant negative impact on the species, particularly as *V. rupicola* is only found in a limited number of habitats covering these substrates.

Urban developments on the limestone formations that support *V. rupicola* lead to fragmentation of the species already limited, preferred habitat. In 2014, 20.04 km^2 of the species preferred habitat remained on Anegada (see Figure 25) while only 2.86 km^2 remained on Vieques (see Figure 26). Significantly more habitat remained on Puerto Rico with 65.06 km^2 (see Figure 27) found to be overlying the specific substrates that support the species. The limited numbers of individuals and locations mean that *V. rupicola* is also threatened by habitat degradation caused by feral animals, recreation and human-induced fire, even within protected areas.

The currently limited human population on Anegada have a much less significant impact on the landscape compared to the Puerto Rican islands; however, Anegada has the potential for island-wide development (BVI Department of Town and Country Planning, 1993) with local community support and road improvement works underway. The species currently has no legal protection in BVI and there are no existing protected areas on Anegada. The Government of the Virgin Islands has produced plans for two new protected areas on Anegada that would

encompass part of the substrates and preferred habitat of the species on the island. Although a welcome addition to the BVI protected areas network, these areas will provide very little protection of *V. rupicola* preferred habitat as only 3.17 km^2 falls within these proposed areas (see Figure 40). Nonetheless, the proposed protected areas on Anegada will secure a significant proportion of the extant individuals with 39% of those observed between 2012 and 2015 (Figure 75). The proposed Western Ponds Protected Landscape will capture over half (51%) of the Anegada West individuals (total = 519) while the proposed Eastern Ponds National Park will only capture 13% of the Anegada East individuals (total = 240).

During field surveys, the author observed high levels of habitat disturbance including the operation of heavy machinery to clear vegetation and anthropogenic development within the boundary of the proposed Western Ponds Protected Landscape. *Varronia rupicola* plants were killed, in significant numbers, without regard to the species globally threatened status. Feral livestock roam free grazing and degrading the landscape within the boundaries of both proposed protected areas and across the entire island. These animals, often malnourished and unable to find adequate amounts of fresh water, have altered the landscape immeasurably. Their continued, unmanaged presence in the landscape is environmentally unsustainable along with being an animal welfare issue.

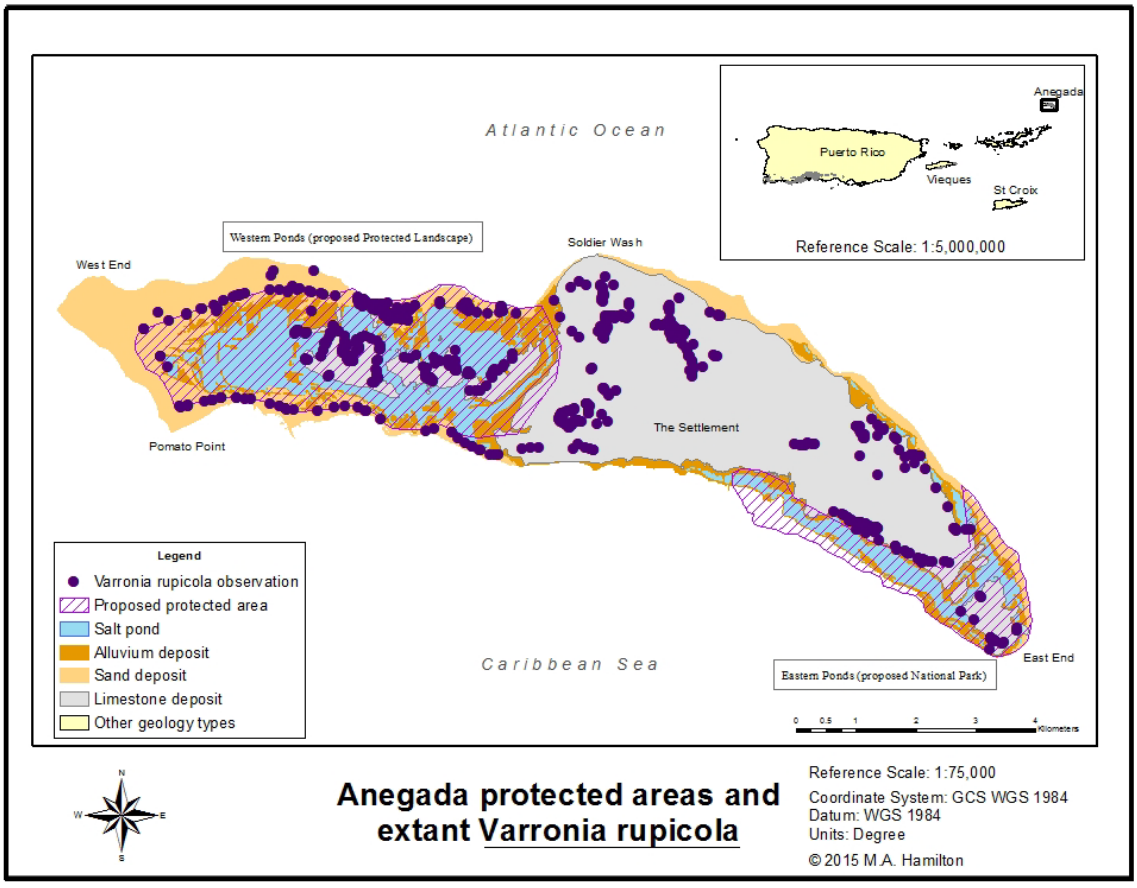

Figure 75: Map showing proposed protected areas, Quaternary deposits (sand and alluvium) and Pleistocene limestone deposits on Anegada with extant *Varronia rupicola* recorded between 2012 and 2015.

The Vieques NWR is home to the six *V. rupicola* recorded on the island during this research at a single location on the Puerto Ferro peninsula. Although all of the extant individuals and all the substrates known to support the species and its preferred habitat (2.86 km^2 remaining in 2014 (see Figure 41)) are found within the Vieques NWR (eastern tract) or the adjacent DRNA reserve (Figure 76), the species future survival and the conservation of the remaining genetic diversity on the island is not certain as both of these areas experience significant levels of on-going disturbance and degradation. The single location and tiny area (ca. 50 m^2) occupied by the species adjacent to an access road make it highly susceptible to extirpation from the island. Road works undertaken in the years immediately prior to this research without proper management were thought to have resulted in the extirpation of the species from Vieques. Fortunately, *V. rupicola* re-established through propagules (seed and/or root shoots) and was located during this research. Activities allowed/undertaken in the area in future need to be fully assessed and overseen to ensure that the species and its habitat are not impacted again as a single event could result in the loss of all genetic diversity found on the island.

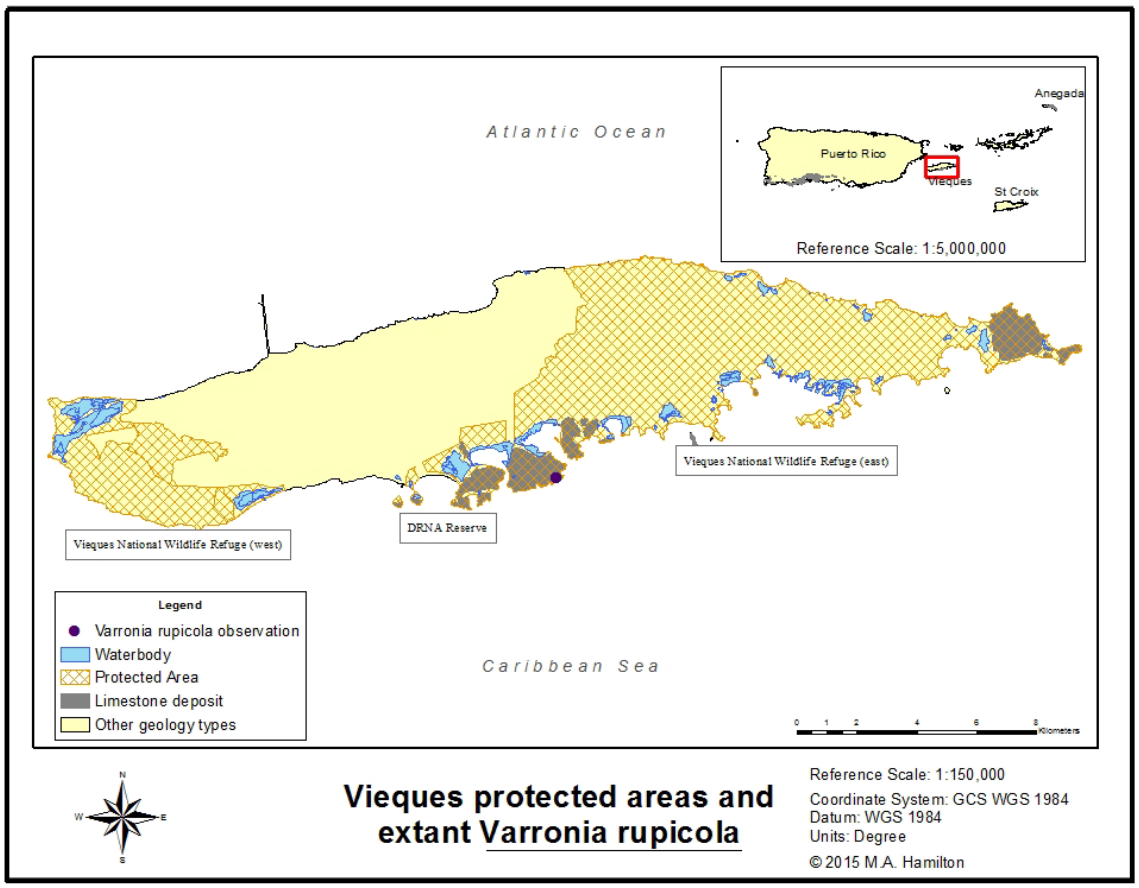

Figure 76: Map showing protected areas and Pliocene limestone deposits on Vieques with the location of the six extant *Varronia rupicola* recorded on the Puerto Ferro peninsula between 2012 and 2015.

The largest island, Puerto Rico, also has the most human inhabitants. Significantly more anthropogenic issues were observed on Puerto Rico during this research which include increased disturbance through recreational activities, development/maintenance of

infrastructure and vandalism (e.g. fire). These issues are documented (U.S. Fish and Wildlife Service, 2010) and were observed during this research being as prevalent, if not more so, within protected areas as they are outside of them and are directly impacting *V. rupicola* and its habitat. Guánica State Forest in south-western Puerto Rico is divided into two main tracts of land that are separated by significant anthropogenic disturbance and Guánica Bay. Both tracts of the forest were found to support extant *V. rupicola* with the eastern tract containing the greater number of plants (n = 94) when compared to the three found in the western tract. Both tracts of forest suffer from arson attacks/escaped fires and significant levels of unauthorised trail clearance for recreational use as well as disturbance caused by clearance for accessing electrical distribution infrastructure. However, the plants and their habitat have a much better chance of survival compared to those found in unprotected areas where conversion from intact, mature forest to urban development is ongoing and documented during this research. There is currently only 22.08 km^2 of the species preferred habitat within existing protected areas (see Figure 42), mainly within Guánica State Forest. This results in 59% of the extant plants under protection and 86% of the Guánica population (total = 113) under protection (Figure 77). This means that none of the Ponce population (total = 52) or its genetic diversity is protected *in-situ*.

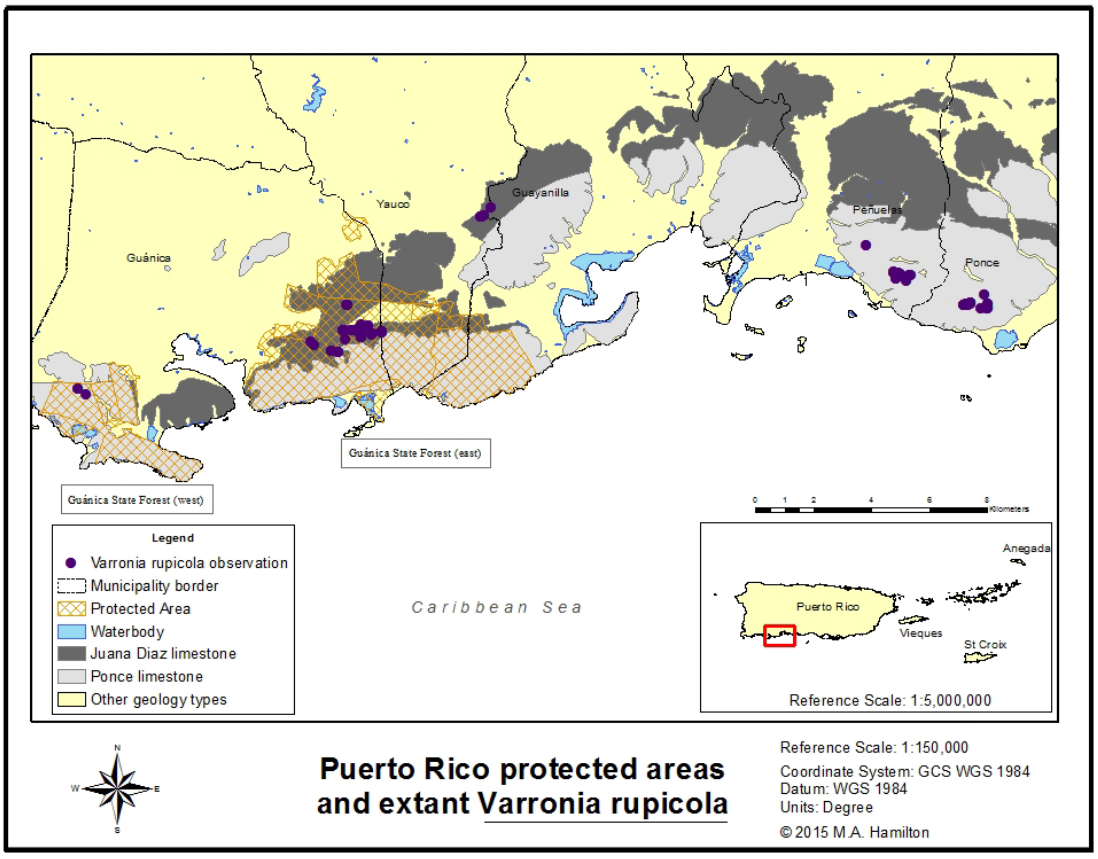

Figure 77: Map showing extant *Varronia rupicola* recorded between 2012 and 2015 as well as protected areas and Miocene limestone deposits on Puerto Rico within the species native range.

5.1.2. Sea level rise

Past sea level rise

Past sea level rise has undoubtedly had a significant impact (positive and negative) on the species genetic diversity through connectivity between as well as isolation of the islands and substrates that are home to extant *V. rupicola*. Substantially larger areas of limestone substrates and sandy deposits overlying limestone were available for colonisation during periods of lower seas. This was particularly true during the LIG when limestone known to support extant plants across the three islands was fully formed. This may have resulted in considerably larger populations and increased gene flow as the exposed land would have opened opportunities for the movement of genetic material (*i.e.* pollen and seed) from non-avian organisms (e.g. reptiles).

Within the past 8,500 years, the Virgin Islands were separated from Puerto Rican islands and within a short period (1,500 years) the individual islands of the PRB were isolated with slightly larger land masses. During the period up to 3,000 ybp the sea level rose to near current levels and has since been fairly constant. Therefore, the sea separating the islands with *V. rupicola* would have probably limited gene flow to avian seed dispersal over the past 7,000 years. The limited gene flow between islands coupled with the limited area of exposed limestone substrates and subsequent anthropogenic disturbance of the habitats covering those substrates led to further limitation of gene flow within islands and the development of the five populations detected by this research.

Future sea level rise

The implications of future sea level rise for the long-term survival of *V. rupicola* depend mainly on the human response and the amount of rise experienced. If there are active, global efforts to curb GHG emissions and keep the global temperature from rising above the 2 °C threashold (IPCC, 2012), lower levels of sea rise may be experienced. If these efforts are undertaken in conjunction with coastal defences and mitigation strategies, the impacts could be minimal over the coming centuries (Traill *et al.*, 2011). Alternatively, continued or accelerated GHG emissions and abandonment of coastal areas could have hugely negative impacts on *V. rupicola*, its habitat and our planet, generally.

The lowest lying island within the species native range is Anegada which has a maximum elevation of just over 8 m. The impacts of the four IPCC RCP scenarios for 2100 (IPCC, 2013a) were explored for Anegada (see Figure 35) and showed a minimal direct impact on extant individuals of *V. rupicola* (see Figure 36). If sea levels rise significantly more over the coming centuries, the survival of *V. rupicola* on the island is in doubt through direct inundation of the

land and increased salinity of the fresh water lens. The scenarios of 1 m, 2 m, 3 m and 6 m for 2100 to 2300 showed significant impacts across Anegada with the species current locations being all but lost with a 6 m increase (Figure 78). The entire Anegada East population lies below 3.8 m asl and only ten individuals in the Anegada West population are found above 6 m asl. Active measures and international collaborations will be required to secure the genetic diversity currently found on Anegada in light of these data using a suite of techniques.

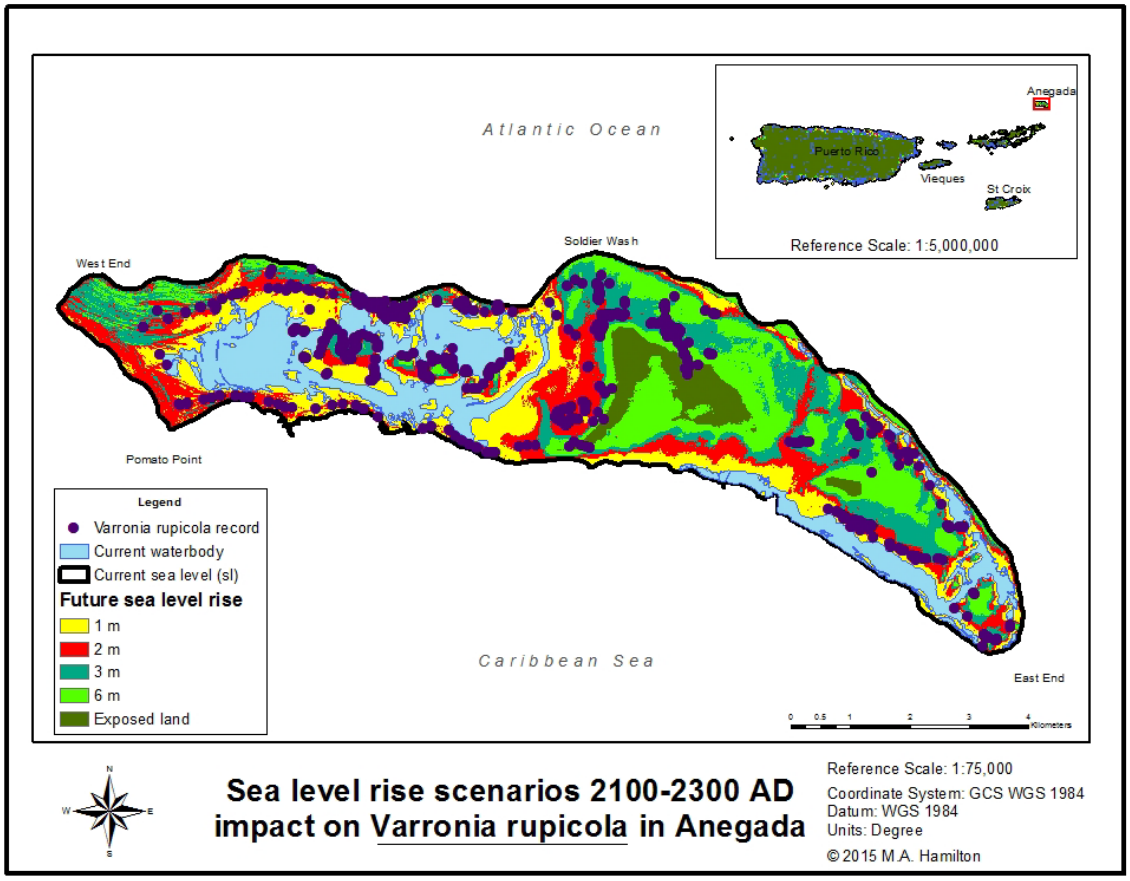

Figure 78: Map showing the impact of future sea level rise scenarios for 2100 to 2300 AD (Bellard *et al.*, 2014) **on extant *Varronia rupicola* on the island of Anegada**

The situation on Vieques and Puerto Rico is not as dire, at least for the direct impact of rising seas. The 1 m, 2 m, 3 m and 6 m scenarios for 2100 to 2300 (see Figure 34) showed that *V. rupicola* plant locations on Vieques would not be affected due to the population being found 12 m asl (Figure 79). Similarly, the Ponce and Guánica populations on Puerto Rico Island will not be affected as they occur above 60 m and 133 m asl (Figure 80), respectively.

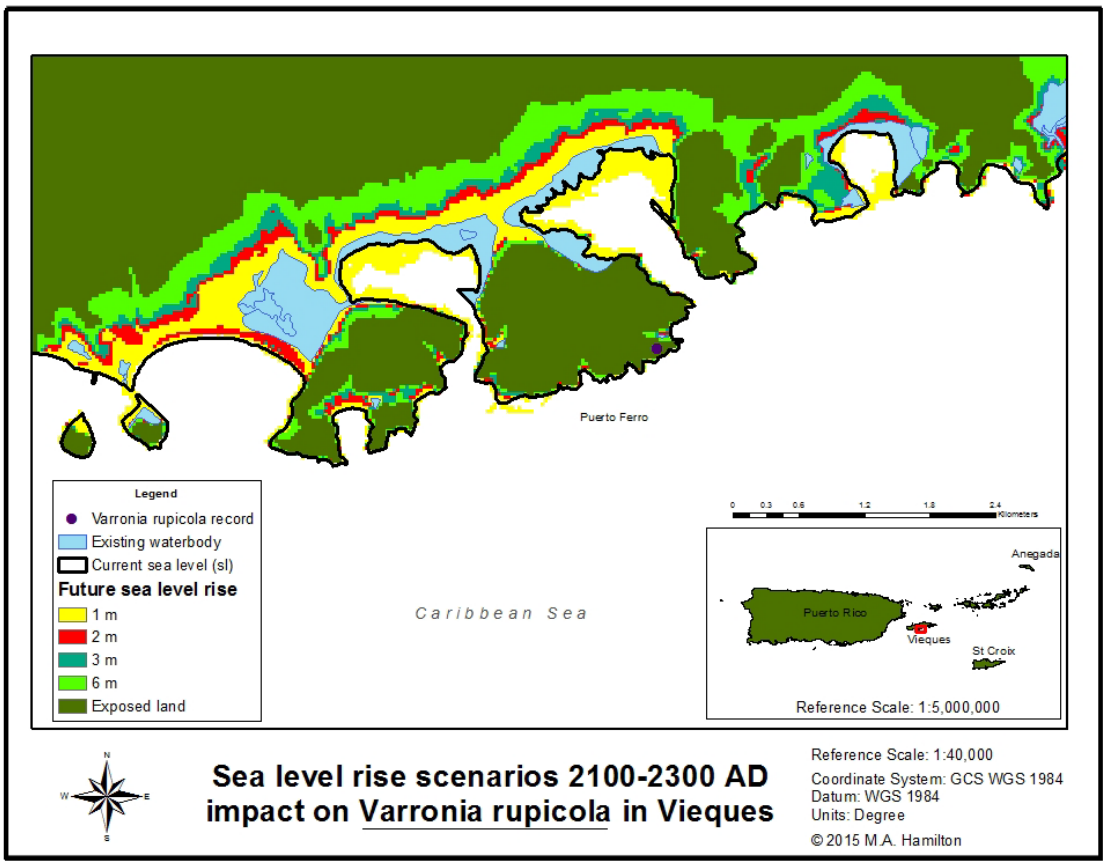

Figure 79: Map showing the impact of future sea level rise scenarios for 2100 to 2300 AD (Bellard *et al.*, 2014) **on extant *Varronia rupicola* along the southern coast of Vieques Island.**

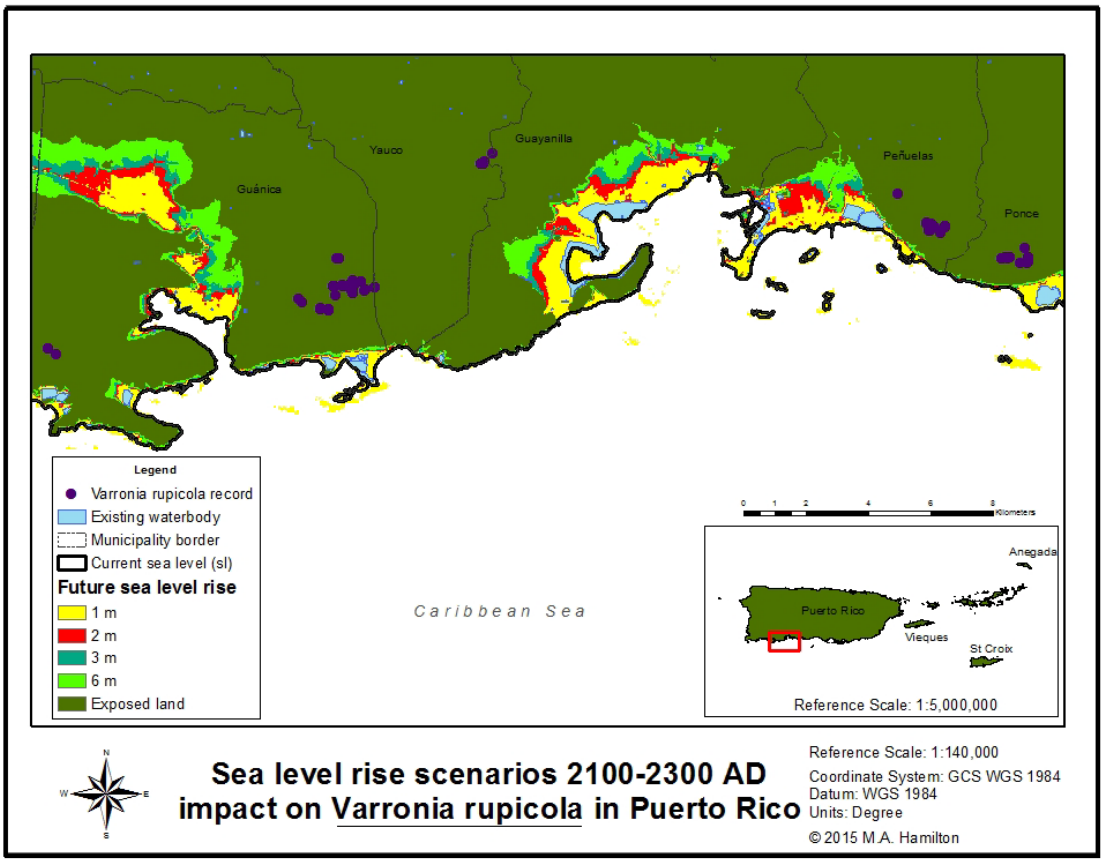

Figure 80: Map showing the impact of future sea level rise scenarios for 2100 to 2300 AD (Bellard *et al.*, 2014) **on extant *Varronia rupicola* along the southern coast of Puerto Rico Island.**

Rising sea levels could have significant indirect impacts on *V. rupicola* populations across the three islands. Anegada could potentially become an untenable option for human habitation in the coming century or be developed such that all available land is completely altered for human use. Depending on the overall sea level rise experienced and the human response, the island could remain as a refuge for the species or become uninhabitable. The latter due to lost habitat through combined sea level rise and human development on the remaining exposed land. Further loss of genetic diversity and higher levels of inbreeding should be expected unless active conservation measures are implemented to counteract these impacts.

Rising seas around Vieques could isolate the Puerto Ferro peninsula such that it becomes an isolated cay separate from the main island. This phenomenon could also be repeated around the adjacent Pliocene limestone formations. This could potentially limit human disturbance in these areas and result in a positive outcome for the species long-term conservation. The isolation of the Vieques and Anegada populations on smaller land masses with less elevation would mean that they would be more susceptible to the issues discussed in 5.1.3. Natural disasters.

Outside of existing protected areas on Puerto Rico Island the indirect impacts could be the most detrimental as the human population seeks refuge from rising seas in areas with higher elevation close to existing infrastructure. Given the significantly higher elevations of the Puerto Rican populations, isolation and reduction of the extant habitat directly through sea level rise will not be an issue; therefore, the protection of these populations is paramount for the species long-term survival as discussed in 5.2. Conservation strategies.

5.1.3. Natural disasters

The native range of *V. rupicola* is prone to natural disasters, particularly hurricanes and tsunamis (Parsons and Geist, 2009; Elsner and Jagger, 2010; Atwater *et al.*, 2012). The International Panel on Climate Change (IPCC, 2012) state that a 2 °C to 5 °C rise in the daily maximum of global temperature by the end of 2100 will likely result in higher wind speeds in tropical cyclones. This is of particular concern for *V. rupicola* as the Caribbean Sea has a very high ocean heat capacity which drives hurricane intensity (Elsner and Jagger, 2010). Although *V. rupicola* and the other native species found in the PRB dry forests are adapted to cyclonic activity, more intense storms brought on by climate change in a relatively short period may prove to be too much for these species to cope with, particularly in already altered landscapes where water retention is lowered by exposure to direct sunlight and drying winds.

The impact of storm surge associated with higher sea levels and more intense hurricanes could result in greater levels of inundation, especially on low-lying Anegada. These surges have the

capacity to change the landscape profoundly through alteration of habitats due to salt intrusion of the freshwater lens and vegetation mortality (Sah *et al.*, 2010). Intensive storms can also cause major changes to the coastlines with up to 30 meters of gain or loss recorded in a single storm event (UNESCO, 1989). Quaternary deposits on Anegada are highly susceptible to storm surge and could lead to the opening/closing of sea connections to the interior salt ponds as has been documented by past events (Atwater *et al.*, 2012). If significant alterations were made to the Quaternary deposits, *V. rupicola* populations could be directly impacted by further habitat loss and fragmentation as well as indirectly through salinisation of the freshwater lens.

The native range of *V. rupicola* has been struck by several documented tsunamis originating from earthquakes in the region (Parsons and Geist, 2009) or from as far away as Europe (Atwater *et al.*, 2012). The low-lying nature of Anegada makes *V. rupicola* populations particularly susceptible to extirpation through tsunami induced impacts. These can be either direct mortality from overwash or indirect mortality caused by salt water intrusion of the freshwater lens. Increased sea levels will only exacerbate the potential impacts of tsunamis in the future.

Depending on the amount of sea level rise experienced, the *V. rupicola* population on Vieques could be impacted by future tsunamis. Parsons and Geist (2009) state that Vieques experienced 6.1 m tsunami induced waves in the past. A similar magnitude tsunami following 6 m sea level rise or greater magnitude following less severe sea rise would have the potential to impact the Vieques population.

Tsunamis are not expected to have any direct impacts on Puerto Rican *V. rupicola* populations due to the species altitudinal range (>60 m asl); however, the indirect impacts could be significant if the human population was forced to develop new infrastructure (*i.e.* roads, power transmission) and urban developments at higher elevations. The latter should not impact populations in existing protected areas, but the former could impact protected populations due to easements allowed for such infrastructure on public land. Developing a robust management plan for the species that takes the above issues into account will require significant resources and support from a wide range of stakeholders as discussed in the next section.

5.2. Conservation strategies

Effective conservation management should aim to preserve the maximum genetic diversity of a species through capture of allelic richness as well as genetically different populations in order to enable adaption to environmental change (Frankham *et al.*, 2002). Defining distinct

conservation units for *V. rupicola* should focus on the detected genetic, observed ecological and existing political boundaries across the species native range. Genetic data presented here suggest that a single evolutionary significant unit (ESU) is present in the PRB; however, the five distinct populations detected should be conserved such that the species potential for adaptation and evolutionary success are maximised (Crandall *et al.*, 2000). As the loss of any population would result in reduced adaptation potential and lower diversity, each of the five populations should be defined as separate management units (MUs) in the short-term (Moritz, 1994). Movement of individuals between MUs may be warranted if significant inbreeding is detected (Moritz, 1999), populations are small or at risk of extirpation due to external factors (e.g. anthropogenic disturbance, natural disasters). As these data presented here have met these criteria, management plans should consider movement of genetic material between MUs; however, careful consideration must also be given to the potential for outbreeding depression (Frankham *et al.*, 2011) and experimental trails are suggested (see 5.2.3. Conservation introduction).

Given the limited total area of substrates and habitats that are known to support *V. rupicola*, a range of different conservation strategies will need to be employed to ensure the survival of the species in the face of on-going habitat loss and the potential impacts of climate change discussed previously. Every effort should be made to designate further protected areas that will capture more of the species preferred habitat and genetic diversity as *V. rupicola* is more likely to survive stochastic events in the long term in a network of areas that facilitate gene flow (Soulé, 1985). This is particularly important on Puerto Rico where none of the Ponce population is protected *in-situ*.

Changes to existing and introduction of new management practices are required to ensure the species is not adversely affected in protected areas across the PRB. The predominance of the species to grow along roads, trails and powerline clearings in Puerto Rico has led to several documented (U.S. Fish and Wildlife Service, 2010) and observed (this research) instances where individual *V. rupicola* plants have been damaged or even killed. New measures to control feral animals on Anegada, fire on Puerto Rico and unsanctioned vegetation clearance across the species native range are urgently needed. This will require close collaboration between conservationists, land managers/owners, maintenance practitioners and the local communities. While not impossible, this will not be easily accomplished; therefore, complementary and engaging approaches are needed to garner local support.

An awareness raising campaign should be started with the main goals of enabling the identification of the species by non-botanists and highlighting the global importance of the

species to local communities. This campaign should include the production of posters for display in protected areas and public facilities, species fact sheets for distribution to local communities and schools, presentations about the species to local community groups and schools as well as local and national government officials. Communities must be made aware that *V. rupicola* is part of the local, national and cultural heritage and the species is in decline.

To assist fundraising and awareness raising efforts, the species should be reassessed for the IUCN Red List based on the findings of this research. Initial assessment using this new information indicate that the species should be downlisted from 'Critically Endangered', but will remain in a threatened category. Following re-assessment, targeted communications with BVI Government officials should be undertaken to promote the species inclusion in national legislation for protected status such that across its range *V. rupicola* has legal protection. The global assessment and threat status should also be highlighted to Puerto Rican as well as United States Government officials and agencies to facilitate and promote cross border conservation initiatives.

The IUCN Species Survival Commission (SSC) guidelines (2013) for conservation translocation, or moving organisms from one place to another deliberately for a positive conservation outcome, provide a detailed framework for developing conservation plans for *V. rupicola*. Restoration of degraded habitat and reinforcement plantings (IUCN/SSC, 2013) to bolster numbers of individuals should be undertaken within existing protected areas and beyond the species recorded native range as conservation introductions (*i.e.* assisted colonisation (IUCN/SSC, 2013)). In the dry forests that support suitable habitat for *V. rupicola* in south-western Puerto Rico, the soil is predominantly high in organic matter and found in pockets between exposed limestone (Murphy and Lugo, 1990; Lugo *et al.*, 1996; Monsegur, 2009). Anegada soils on exposed limestone are similar to those described for Puerto Rico; however, *V. rupicola* also occurs in the Anegada Ridge Plain Formation where the soils are composed of sand with organic matter (Gore, 2013). Habitat restoration and reinforcement plantings using nursery grown plants in thin soils on rocky substrates as well as freely drained sandy soils can be very challenging and will require supplemental irrigation to ensure establishment and survival (Gilman *et al.*, 2009).

Compounding the challenges of restoring habitats and establishing new *V. rupicola* plantings are the potential impacts of insect pests (Serra *et al.*, 2003; Malumphy *et al.*, 2012) as well as climate change induced drying and prolonged droughts (IPCC, 2012). With dry forests already suffering a water deficit for up to ten months of the year (Miller and Lugo, 2009), climate change causing more intense droughts over the coming century could prove to be too much

for *V. rupicola* to cope with, especially in degraded habitats. Building resilience through habitat restoration and bolstering populations with supplemental plantings could be key measures for securing the species survival.

Climate change induced stress could result in plants being more susceptible to existing and new pests throughout the PRB. Two pest insects were found attacking *V. rupicola* across Anegada (Malumphy *et al.*, 2015) during the course of this research. The fact that one of these pests was a new record for the BVI (Malumphy *et al.*, 2015) suggests that increased biosecurity is needed to reduce the spread of established pests across the PRB and introduction of new pests to the region.

The populations detected by this research and the remaining genetic diversity are thought to be the result of adaptation to specific ecological niches, habitat fragmentation (*i.e.* sea level rise and subsequent anthropogenic disturbance) and potentially many more factors not explored in this research. This has significant implications for management planning, particularly if separate MUs are to be maintained (Moritz, 1994). In the short-term, the precautionary approach of maintaining the five populations separately is suggested; however, the threats to the species survival may become so great that maintaining separate populations is impossible. To evaluate the variations observed in habitat, substrate and elevation as well as the possible deleterious effects of mixing populations, further research should be undertaken and will be discussed further in 5.3. Research opportunities.

In light of the currently limited *in-situ* protection and the challenges discussed previously, several different conservation measures should also be employed including *in-situ* reinforcement coupled with habitat restoration, *ex-situ* collections and conservation introductions (IUCN/SSC, 2013). Suggested approaches are discussed in the following sections.

5.2.1. *In-situ* conservation

Once established, the two proposed protected areas on Anegada would offer opportunities for habitat restoration and conservation translocations (*i.e.* population reinforcement) as both areas have experienced significant degradation through feral animal grazing/trampling and anthropogenic disturbance. Excluding the feral animals, or preferably permanently removing them, would have an immediate positive impact on the habitat and enable planting activities to be undertaken without grazing pressure on the establishing plant material. Small exclosures (20 x 20 m) could be trialled to demonstrate proof of concept for population reinforcement and restoration plantings on sand and limestone within the protected areas if feral animal exclusion or management across the landscape is not achievable in the short-term. Overall, reinforcement and restoration plantings should be planned such that areas with the highest

elevation are included to build resilience against sea level rise and natural disasters. Within The Settlement, the grounds of the rock iguana head start facility would make an ideal location for reinforcement plantings of the Anegada West population. Using this location would minimise monitoring/maintenance costs and the plantings could serve as an educational and outreach resource once adequate interpretation and NPTVI staff training is provided. As the location is already fenced to exclude feral animals, planting activities could be undertaken immediately using rescued seedlings from road verges/areas slated for development. Subsequently, further plants grown from seed could be planted at the site to bolster the collection.

In the country of Puerto Rico and within the known historical range of *V. rupicola*, there are two established protected areas, Guánica State Forest and Vieques NWR, with extant individuals. Both protected areas offer opportunities for habitat restoration of degraded dry forest overlying the limestone substrates known to support the species. The eastern tract of the Vieques NWR includes the former U.S. Navy Eastern Maneuver Area where extensive modification of the landscape resulted from the military's use that included exercises that left UXO in the landscape. The removal of identified UXO is underway and often requires the vegetation to be clear-cut. There are plans for subsequent restoration (CH2M HILL, 2012b) and this could include specific habitat improvement for *V. rupicola* as well as population enhancement through reinforcement plantings using material from the Vieques population. Both of the Puerto Rican protected areas have public access (though currently limited in the Vieques NWR) that leads to habitat degradation through recreational uses (e.g. unauthorised trail cutting, fires and pollution). Habitat restoration, especially in areas of the western tract of Guánica State Forest that were burnt or cleared for powerline maintenance could potentially provide a significant amount of new habitat for reinforcement plantings of *V. rupicola* sourced from the Guánica population. Areas for potential habitat restoration in the Guánica State Forest eastern tract are numerous; however, large areas of intact habitat exist that are less susceptible to anthropogenic disturbance and would be ideal for reinforcement plantings of the Guánica population with minimal resources required. The species should also be planted in close proximity to the forest headquarters in the eastern tract for ease of monitoring/maintenance and to reduce the risk of vandalism. These plantings could also serve as an educational and outreach resource if adequate interpretation and forest staff training is provided. Existing plants held in the forest nursery could be used immediately to undertake these plantings, particularly near the forest headquarters.

5.2.2. *Ex-situ* conservation

The on-going loss and fragmentation of habitat as well as the potential for future threats from sea level rise and natural disasters puts *V. rupicola* populations at risk of extinction. Limited resources and habitat protection compounded by small population sizes mean that *in-situ* conservation measures alone leave populations at risk. To overcome these limitations, *in-situ* activities should be complemented by *ex-situ* conservation measures. This would also provide future opportunities for population reinforcement, assisted colonisation and conservation translocation to be undertaken using these *ex-situ* collections.

The success of an *ex-situ* collection to preserve the genetic diversity of *V. rupicola* will depend on the representativeness of the material. Capturing all the extant genetic diversity in living collections of plants is possible, but most likely impractical due to nursery and planting space constraints. Alternatively, seed banking is an economical and safe way to preserve the genetic diversity of an orthodox (desiccation tolerant) species like *V. rupicola* in a small space with few long-term resource needs (Li and Pritchard, 2009). The representativeness of the seed collection and its subsequent ability to supply adequate material for population reinforcement, assisted colonisation and conservation translocation will be determined by the sampling strategy. The strategy employed must ensure that genetically distinct populations are collected throughout the species range and a high proportion of extant individuals are sampled (Hoban and Strand, 2015).

During this research, conservation collections (plants and seeds) were documented at several locations (see Appendix 1: *Varronia rupicola* records - Cultivated material held in *ex-situ* collections). Material originating from Anegada was found at three institutions: The J.R. O'Neal Botanic Garden, Tortola, BVI; Royal Botanic Gardens, Kew, UK; and Fairchild Tropical Botanic Garden, Dade County, Florida, USA. A single seed collection is held in the Millennium Seed Bank. These Anegada collections are all thought to have originated from the Anegada West population; therefore, securing collections (seed/cuttings) from across the Anegada East population is paramount. Further collections from the Anegada West population should be secured as well, ensuring that new material is collected from across the population.

Conservation collections from Puerto Rico have also been established at three locations: Royal Botanic Gardens, Kew, UK; Department of Natural and Environmental Resources (DRNA), Guánica State Forest, Guánica, Puerto Rico; and USFWS, Cabo Rojo NWR, Cabo Rojo, Puerto Rico. All of these Puerto Rican collections are thought to have originated from the Guánica population; therefore, securing material (seed/cuttings) from across the Ponce population and the six individuals on Vieques is crucial for the species conservation. As no seed collections are

known to exist from Puerto Rico, collections should be secured, ensuring that seed are collected from across the three populations.

Maintaining living plants in botanic gardens is beneficial, especially for research and educational purposes; however, these collections must be maintained to ensure records are accurate and also monitored to ensure that the species does not escape (*i.e*. in tropical locations like Florida outside the species native range). If the material will be used for population reinforcement, assisted colonisation and conservation translocation, progeny should be destroyed unless they are from known crosses or asexual propagation to ensure that the potential negative effects of hybridisation, inbreeding and outbreeding are minimised.

5.2.3. Conservation introductions

Conservation introductions should be undertaken following the IUCN/SSC guidelines (2013) and planned such that areas with low elevation (<5 m asl) are generally avoided to ensure resilience against sea level rise and natural disasters. There may be instances were plantings below 5 m asl are warranted to test the species ecological adaptation, develop nursery stock on soils with higher moisture content/retention or make use of intact habitat within established protected areas. If plantings are undertaken in such areas, the potential loss of the material must be taken into account and mirrored collections containing the same genetic diversity should be maintained elsewhere for security.

There are no known areas of suitable limestone substrate within BVI other than Anegada to consider for conserving *V. rupicola*. There are deposits of Quaternary sand within protected areas on several other islands in BVI; however, these are outside the species known range and will not afford long-term resilience against sea level rise and natural disasters as these are all less than 5 m asl. As discussed previously, conservation introductions in these areas should only be considered as experimental or potentially short-term measures.

Given the lack of suitable areas within BVI to conserve the genetic diversity detected in the two Anegada populations of *V. rupicola*, conservation introductions to other islands should be considered, particularly within protected areas with limestone deposits known to support the species. Multivariate analyses (*i.e*. PCoA) showed an overall grouping of Anegada and Vieques population samples. This could justify a conservation introduction of Anegada material to Vieques. Following the UXO removal at Punte Este and subsequent habitat restoration, Anegada material could be planted within the Vieques NWR to provide added resilience against sea level rise.

Vieques also has a second protected area adjacent to the Vieques NWR containing Pliocene limestone and suitable habitat for *V. rupicola* which is managed by the DRNA. This area would be ideal for conservation introductions using material from the six extant plants in the Vieques population. The DRNA protected area also has extensive Quaternary sand deposits that have been heavily degraded. These areas could be ideal locations for educational and outreach plantings of the species. Interpretation could be used to raise awareness in the local public about the negative impact of arson/escaped fires and unauthorised trail development.

Along the southern coast of Puerto Rico Island, Ponce limestone occurs within three protected areas outside the recorded native range of *V. rupicola*: Los Morrillos de Cabo Rojo, Cabo Rojo NWR and the small off-shore island of Isla Caja de Muertos (Monroe, 1980). The three areas offer the opportunity for conservation introductions of material from all five populations (mixed or separated) for research and conservation.

The Cabo Rojo NWR includes areas with both Miocene limestone and Quaternary sand deposits. As part of the general restoration efforts on the refuge and as a specific conservation action for *V. rupicola*, a small planting (n = 30) of the species was undertaken in November 2012 by the USFWS using nursery grown plants originating from wild source seed collected in Yauco and Guánica (Morales and Martinez, 2013). Many potential sites for further conservation introductions within the refuge exist and should be explored urgently, particularly for material from the Ponce population. Of particular note is an area on the extreme south-western edge of the refuge where habitat similar to that on Anegada overlies Quaternary sand deposits. This area should be considered for experimental conservation introduction plantings, particularly of Anegada sourced material.

Los Morrillos de Cabo Rojo is adjacent to the Cabo Rojo NWR and has many similarities (e.g. aspect, elevation) to the Puerto Ferro peninsula within the Vieques NWR where the Vieques population of *V. rupicola* exists. Unfortunately, the area has been heavily degraded through anthropogenic disturbance, mainly recreational activities and arson. This could be an ideal location to establish a fully representative planting of the Vieques population. The accessibility and proximity to the USFWS regional office would make monitoring straightforward while isolating the Vieques material from other areas that could be used for plantings of other populations. Further plantings of the species in this area should only be undertaken following a broader habitat restoration programme and an awareness raising campaign to reduce the potential for escaped fires/arson in the area.

Isla Caja de Muertos should be considered for conservation introductions following a thorough habitat assessment and survey for *V. rupicola*. If no *V. rupicola* plants are found, the island

could be used for the preservation of the species using material from one or many source populations. This could be an ideal location for the protection of Anegada sourced genetic material as well as the Critically Endangered iguana, *Cyclura pinguis*, which is also extant on Anegada. More straightforward and potentially less controversial would be the conservation introduction of genetic material from the nearest population, Ponce, on mainland Puerto Rico which has no *in-situ* protection.

Depending on the resources available for subsequent monitoring and research, these protected areas could be used to conserve a single population each (low-medium resource input) or to bring multiple populations together to test the genetic consequences (medium-high resource input). The opportunities for research into the mixing of individuals from different populations, common garden experiments and sowing/planting techniques will be discussed along with other topics in the following section.

5.3. Research opportunities

Development of a comprehensive species management and recovery plan that is applicable across political boundaries and legal frameworks throughout the PRB requires robust and scientifically sound evidence. On-going collaborations with a network of individuals and institutions interested in conserving *V. rupicola* have enabled an active research and conservation programme to be established for the species. Further investigations will be undertaken in the field and the lab to study the species phenology, reproductive biology and seed dispersal to inform the long-term management of the species. The research undertaken and presented here has also set the stage for further cyto-, phylo- and population genetic studies to be undertaken of endemic Caribbean *Varronia* and *Cordia* species. This could lead to significant contributions to the scientific literature for an understudied and often threatened group of species.

The established network of protected areas offer opportunities to undertake quantitative genetic studies (e.g. reciprocal transplant, common garden experiments) that Kramer and Havens (2009) state are largely neglected but of major importance due to the risks associated with the introduction of genetically different material. Frankham *et al.* (2011) showed that previous estimates of outbreeding depression are conflated and likely to occur when individuals from different populations with different karyotypes and from different environments are brought together after separation for more than 500 years. Undertaking quantitative genetic studies would provide much needed evidence for the suitability of *V. rupicola* population mixing as well as contributing much needed data for this field of study.

Another significant opportunity for research in the established protected areas is the development of sowing/planting techniques for *V. rupicola* and associated dry forest species with the aim of restoring degraded lands. These studies could establish protocols that ensure high survivorship which will maximise resources (*i.e.* plant material and funding). Ultimately this research could provide more conservation impact through delivering efficiencies that provide practitioners with more time to focus on other species/issues of concern.

This research allowed the author to work with a wide range of specialists across many different fields. This has established several necessary protocols/methodologies to enable further research as well as opening many areas of investigation. The areas with on-going research, that require further investigation to generate publishable results or offer new lines of inquiry are presented in the following sections.

5.3.1. Karyology

This research found traditional methods to observe root tip cells at mitotic metaphase and pollen grains at meiotic metaphase to be very difficult due to sticky Boraginaceae chromosomes (Bhattacharya, 1968; Gaviria, 1987). Establishing a new protocol for breaking the sticky chromosomes apart to enable counting would be a major advance for research of the *Varronia* group specifically and the family as a whole. This in turn would open several areas of investigation, particularly flow cytometry studies for *Varronia* species.

If a protocol is not available, material of a known diploid and tetraploid species could potentially be used to develop a flow cytometry library to confirm the ploidy level for *V. rupicola*. Securing living material of new *Varronia* species is the first step required to progress this research. Regardless of chromosome counts being successfully undertaken, further flow cytometry studies can be undertaken using the protocol established during this research for *V. rupicola* to generate publishable results for the species.

5.3.2. Phylogenetics

The ITS and *trnL-trnF* matrices generated by this research should be expanded to include new species of *Varronia* and *Cordia* native to the Caribbean and specifically the PRB. Further pDNA analyses using new regions (*i.e*. *matK*, *trnS-trnG* spacer and the group II *rpl16* intron) should also be undertaken using new and existing samples. These new and expanded matrices should be combined to produce informative phylogenetic trees of Caribbean *Varronia* and *Cordia* species with the aim of presenting better resolution of the interspecific relationships. This may, in turn, shed light on possible parentage for species of hybrid origin and open new lines of inquiry using next-generation sequencing (Soltis *et al.*, 2013). These are exciting possibilities

that could lead to the development of new model systems with far reaching research possibilities.

5.3.3. Population genetics

New *V. rupicola* plants have been discovered within the two Puerto Rican populations and the extremes of the Anegada East population since this research was completed. Samples of these new plants and any further discoveries, particularly on Puerto Rico and Vieques, should be analysed and the data combined with the results of this research to inform the species management discussed in 5.2. Conservation strategies.

Morphology data collected from individual plants sampled for genetic material during this research should be analysed jointly using multivariate and cluster analysis methods (*i.e.* PCoA and Bayesian techniques) with population genetics data. These combined data may provide added support for the populations detected and could possibly provide insight into phenotypic variation, especially flower morphology.

A subset of the microsatellite loci used in this research was successfully amplified for four other *Varronia* species. This offers many new research opportunities that will require limited time and resources to be invested before results can be acquired. This is particularly significant for other Caribbean endemic species like *V. lima* and *V. bahamensis* and potentially useful for threatened species such as *V. bellonis* that have yet to be tested but were found to be closely related to *V. rupicola*.

5.3.4. Palynology

Pollen of *Varronia* has been shown to differ from that of *Cordia s.s.* (Nowicke and Ridgway, 1973; Moncada and Herrera-Oliver, 1988; Miller and Nowicke, 1989; Heubl *et al.*, 1990; Nowicke and Miller, 1990). Initial micromorphological studies undertaken by the author using available pollen samples from *V. rupicola, V. lima* (see Figure 7) and *V. bahamensis* suggests that further research is warranted and could provide strong support for the results of phylogenetic analyses. Fresh pollen samples should be acquired from *Varronia* species native to the Caribbean, especially endemics included in phylogenetic analyses presented here, to inform development of phylogenetic trees. This area of research could occupy several years and offer a significant amount of new data.

5.3.5. Reproduction biology and dispersal

Understanding the reproductive biology of *V. rupicola* is fundamental to the species conservation. On-going research is exploring this topic in the wild and in controlled environments. The *ex-situ* collections held at Kew are being used to undertake a reproductive

biology study (Figure 81 (a) and (b)) to determine lengths of time for flowering, fruit development and dispersal as well as floral morphology variations. The results of this research will assist conservationists in planning monitoring and collecting programs and may help to identify reproductive impediments within wild populations due to flower morphology. The study may also help to determine if the departure from random mating detected in this research is the result of apomixes which has been suggested for other *Varronia* species (Spoon and Kesseli, 2008).

Working with herpetologist, Kelly Bradley, on Anegada, a controlled feeding study (Figure 81 (c)) using *V. rupicola* fruits presented to captive iguanas, *Cyclura pinguis,* strongly suggests that these two threatened species have a relationship in the wild. This led to a camera trapping programme on Anegada (Figure 81 (d)) to record *V. rupicola* pollinators and seed dispersers. This research is on-going and has also been extended to Puerto Rico with the assistance of USFWS biologist, Omar Monsegur. This research has the potential to unlock the secrets of gene flow across the PRB for *V. rupicola* and suggest new approaches to the conservation of the species. This is an exciting and potentially ground-breaking area of research that will bring together a range of taxonomic specialists and conservation practitioners.

To understand the potential of dispersed as well as stored seed, a longevity study is needed. This will provide insight into the species ability to remain in the seed bank for natural regeneration (Molina Colón and Lugo, 2006; Bossuyt and Honnay, 2008) and also establish the species storage behaviour enabling the design of a seed testing and recollection protocol (Li and Pritchard, 2009). These are very important considerations for the species long-term conservation as the seed longevity will have many implications for the strategies discussed previously in 5.2. Conservation strategies for *V. rupicola*.

Figure 81: Examples of reproduction biology and dispersal research: (a-b) *V. rupicola* controlled pollination of *ex-situ* collections; (c) captive rock iguana, *Cyclura pinguis,* controlled feeding study using *V. rupicola* fruits; (d) camera trapping to record *V. rupicola* pollinators and seed dispersers. ©M.A. Hamilton.

5.3.6. Impacts of climate change

This research developed a robust GIS comprised of species observations, elevation, land cover and geologic data that can be used to develop models and project them into the future. Data loggers have been deployed across the native range of *V. rupicola* with the assistance of USFWS biologists in Puerto Rico and Vieques to record micro-climatic conditions for use in modelling habitat niche and future climate scenarios. Climatic data currently being collected will enable the inclusion of high resolution temperature and humidity readings in models that are currently unavailable.

The data loggers will also enable seed germination studies to be designed to explore the species response to dryer and hotter conditions than it currently experiences. The climatic data can also be used to determine periods when optimal conditions for seed germination are experienced which can be used for propagating the species by direct seeding in appropriate nursery or field situations. The results of this research can have a profound effect on the management decisions for *V. rupicola* discussed in 5.2. Conservation strategies.

5.4. Main conclusions

This research set out to investigate the distribution, biology and variability of *V. rupicola* in order to make informed management decisions for its conservation. The results of karyological and phylogenetic research presented here clearly show that *V. rupicola* is a distinct species endemic to the PRB. Biogeographical and population genetic research support this finding and provide insight into the structure as well as diversity of extant populations, their ecological preferences and the impacts of past sea level rise as well as anthropogenic disturbance.

Less than 1,000 individuals were observed during this research across the PRB in five detected populations. The largest population, Anegada West, was found to have 519 individuals and the greatest number of private alleles (*Pa* = 10). Anegada was also found to support the second largest population, Anegada East, with 240 individuals. Puerto Rico supports the third, Guánica, and fourth, Ponce, largest populations with 113 and 52 individuals, respectively. Vieques was found to have the smallest population with only six extant individuals.

Although Anegada holds 79% of extant individuals, it was only found to have just over half (56%) of the private alleles; therefore, the considerably smaller populations on Puerto Rico (20% of extant individuals) and Vieques (1% of extant individuals) still have a significant amount of the overall allelic diversity for the species with the smallest population, Vieques, found to have three private alleles. The historical range of the species includes locations on Puerto Rico and Vieques (see Figure 21) that were not found to support extant individuals. This

reduction in the species native range and the small populations observed on these islands has undoubtedly driven the reduction in allelic and genetic diversity detected by this research.

Throughout the PRB, *V. rupicola* plants were found within an extremely limited area of intact habitat (<90 km^2, see Figure 25, Figure 26, Figure 27) overlying a very limited number of substrates (see Figure 22, Figure 23, Figure 24) that cover <200 km^2 across the three islands where the species is extant. These islands do have protected areas (established or proposed, see Figure 28) that include intact habitat and *V. rupicola* individuals (see Figure 75, Figure 76, Figure 77); however, these areas contain less than a third (<30 km^2) of the remaining intact habitat (see Figure 40, Figure 41, Figure 42) across the PRB that supports *V. rupicola*. Unfortunately, considerable genetic diversity also remains unprotected as these protected areas do not include any of the Ponce population, meaning only 59% of the two Puerto Rican populations is protected, and only 39% of the two Anegada populations. Fortunately, all remaining intact habitat, all substrate known to support the species and the six extant *V. rupicola* on Vieques are found within protected areas.

Given the species limited AOO and protected habitat compounded by the fact that the two largest populations occur on Anegada below 8 m asl, the species is threatened with extinction. On-going habitat fragmentation and degradation will continue to be significant threats to the species. Climate change induced drying, increased cyclonic intensity and sea level rise (IPCC, 2012) will surely impact the species directly if global efforts to curb GHG emissions and keep the global temperature from rising above the 2 °C threashold (IPCC, 2012) are unsuccessful. If a 6 m sea level rise were to be experienced, 99% of the locations of the extant individuals on Anegada will be lost under the rising water. The indirect impacts of climate change could have similarly catastrophic impacts on *V. rupicola*, especially outside of protected areas as anthropogenic development is forced inland and up slope into areas with currently intact habitat. Populations within protected areas may also be impacted through storm surge or development of new infrastructure given easement in these areas, an already established practise. Natural disasters are also a potential threat to *V. rupicola* populations, particularly on Anegada where hurricane storm surge or tsunami run-up could kill plants directly by overwash or result in salinisation of the freshwater lens. These natural disasters could also result in similar indirect impacts as climate change induced sea level rise, particularly if the frequency of these events were to increase forcing coastal communities to seek refuge at higher elevation. Combined, the effects of sea level rise and natural disasters could push the species to extinction over the coming century.

This research suggests that genetic diversity has already been impacted by a reduction in population size as Vieques (six remaining plants) shows the highest inbreeding coefficient value and the lowest allelic diversity. Across the five populations, values for allelic diversity are proportional to the size of the population (see Figure 64) suggesting that reduction drives the loss of genetic diversity. Also, historical specimens and *ex-situ* collections reveal a loss of wild genetic diversity from Puerto Rico and Anegada. Therefore, it seems that an integrated approach to the species conservation is needed to preserve the *V. rupicola* gene pool and the species habitat across its native range.

Established *ex-situ* collections capture less than half (44%) of the private alleles found in the wild with none of the Vieques or Ponce private alleles captured. A single seed collection is known from the Anegada West population which means the remainder of the *ex-situ* collections are living plants that could easily be lost if resources are cut or natural disasters strike. Bolstering these collections should be a high priority for the species conservation.

An integrated management plan that is ambitious, yet implementable, should be designed based on the genetic and ecological results presented here (Heywood and Iriondo, 2003). The conservation strategy should include *in-situ* and *ex-situ* measures to maximise genetic diversity. These will potentially allow *V. rupicola* to adaptat to environmental change and new threats (e.g. sea level rise, drought, anthropogenic disturbance and pest attack).

This research allowed the observation of on-going habitat loss, future threats to the species as well as genetic structure and variability of *V. rupicola* through the combination of genetic and biogeographical data. This work is a valuable reference for developing a conservation strategy for the species and has also contributed new scientific knowledge of *V. rupicola* as well as several other *Varronia* and *Cordia* species native to the Caribbean.

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Appendix 1: *Varronia rupicola* records

The table below provides a complete list of *Varronia rupicola* records by year, location and type. Note that only dated, georeferenced observations are listed; therefore, many literature references are excluded.

Year Collected	Island	Wild vouchers	Cultivated vouchers	Observations
1886	Puerto Rico	2	0	0
1902	Puerto Rico	1	0	0
1913	Anegada	1	0	0
1913	Puerto Rico	1	0	0
1915	Puerto Rico	1	0	0
1918	Anegada	1	0	0
1943	Puerto Rico	1	0	0
1959	Puerto Rico	1	0	0
1964	Puerto Rico	1	0	0
1970	Anegada	2	0	0
1971	Anegada	1	0	0
1972	Puerto Rico	1	0	0
1978	Vieques	1	0	0
1987	Anegada	1	0	0
1990	Anegada	1	0	0
1992	Anegada	1	0	0
1992	Puerto Rico	1	0	0
1993	Puerto Rico	2	0	0
1994	Puerto Rico	1	0	0
1995	Puerto Rico	2	0	0
1996	Puerto Rico	1	0	0
1999	Puerto Rico	1	0	0
2000	Anegada	2	0	0
2002	Anegada	1	0	0
2003	Anegada	1	0	0
2004	Anegada	1	0	174
2004	Puerto Rico	1	0	0
2005	Anegada	0	0	45
2005	Vieques	4	0	0
2005	Puerto Rico	2	0	0
2006	Puerto Rico	1	0	0
2008	Fairchild, USA	0	1	0
2010	Kew, UK	0	1	0
2011	Anegada	0	0	3
2011	Puerto Rico	0	0	1
2012	Kew, UK	0	58	0
2012	Puerto Rico	13	0	3
2012	Vieques	6	0	0
2012	Anegada	2	0	8
2013	Kew, UK	0	29	0
2013	Tortola, BVI	0	17	0
2013	Fairchild, USA	0	19	0
2013	Puerto Rico	97	55	43
2013	Vieques	0	0	6
2013	Anegada	148	0	327
2014	Puerto Rico	18	0	1
2014	Anegada	0	0	55
2015	Puerto Rico	0	0	4
2015	Anegada	3	0	114
	Total	**326**	**180**	**784**

Voucher specimens[4,5]

Puerto Rico and Vieques

Vouchers from wild plants

Acevedo-Rodriguez, P. 7684, 12/10/1995, Guánica, Guánica Forest Reserve, (MAPR [MAPR26677!], NY, UPRRP [031039!], US [US520897]).

Acevedo-Rodriguez, P. 7756, 16/01/1996, Ponce, Canas, Off Rd. 2 behind the Holiday Inn Hotel, (NY [NY00842563!], UPRRP [031046!], US [US590489]).

Acevedo-Rodriguez, P. 10803, 29/01/1999, Guánica, Guánica Forest Reserve, along main road to ranger station, (UPRRP [36630!], US [US614075]).

Ackerman, J.D. 3827, 10/09/2005, Vieques, Puerto Ferro, (UPR, UPRRP [044069!]).

Axelrod, F. 7060, 06/10/1993, Ponce Bo Canas, North off Rt 2 just before prison, (UPRRP [026524!], MO [MO-878731]).

Axelrod, F. 13094, 22/07/2005, Vieques, US National Wildlife Refuge, along road to lighthouse peninsula (Punta Coneja), 100 m before lighthouse, (UPRRP [UPRRP11321!]).

Breckon, G.J. 4507, 20/12/1994, El Peñon, E of Peñon de Ponce, (JBSD, MAPR [MAPR26676!], NY, US).

Breckon, G.J. 7276, 23/06/2005, Vieques, US National Wildlife Refuge, East tract, Lighthouse Peninsula; along Lighthouse Road, c. 100m north of old lighthouse, (MAPR [MAPR26675!], UPRRP [41562]).

Breckon, G.J. 7516, 21/07/2005, Vieques, US National Wildlife Refuge, East tract, Lighthouse Peninsula; along Lighthouse Road, ca. 100m north of old lighthouse, (MAPR [MAPR26674!]).

Cedeno, J.A. & Aponte, I.J. 368, 08/01/1995, El Peñon, Peñuelas, Bo. Encarnación, (MO [MO-2256605!], SJ [SJ00251!]).

Gregory, L.E. 658, 12/02/1943, Guánica, (UPR [32944]).

Hamilton, M.A. 905, 18/07/2012, Bosque Estatal de Guánica, Guánica eastern tract, (K [K000679885!]).

[4] Voucher list compiled from an exhaustive search of literature and major herbaria of Europe and North America. Many of these specimens have not been listed in the literature previously.

[5] An "!" immediately after a herbarium code/barcode/accession number (e.g. K! or K [K000679885!]), indicates that the specimen, physical or digital copy, has been seen by the author.

Hamilton, M.A. 945, 03/05/2013, Bosque Estatal de Guánica, Guánica eastern tract, (K [K000679893!]).

Hamilton, M.A. 1044, 08/05/2013, Yauco, Barina, Catala Farm (K [K000679891!], MAPR!, UPRRP!).

Hamilton, M.A. 1046, 08/05/2013, Yauco, Barina, Catala Farm (K [K000679892!]).

Heller, A.A. 6245 09/12/1902, Limestone hills along coast three miles west of Ponce, (NY [NY00952750!], US [US971030]).

Kraus, F. s.n. 09/08/1992, Peñuelas, Ba. Encarnación, hills SE. of Tallaboa, just NW. of Peñon de Ponce, (SJ [SJ00253!]).

Liogier, A.H. 10629, 03/01/1964, Guánica insular forest, (NY [NY00952748!], US [US970980]).

Monsegur, O. 202, 20/03/2004, Peñuelas, Ba. Encarnación, to the north of road # 2 on the back area of an old junker to a small canyon, to the west of Carcel Las Cucharas, (MAPR [MAPR26673!]).

Monsegur, O. 356, 12/07/2005, Guánica Forest Reserve, from road 334 taking Las Cobanas trail, (MAPR [MAPR24899!]).

Monsegur, O. 467, 18/08/2005, Guánica Forest Reserve, El Fuerte Trail from DRNA office always taking the trails to the northeast leading to road 334, El Maniel, Vereda El Fuerte, (MAPR [MAPR29818!]).

Monsegur, O. 763, 17/06/2006, Peñuelas, Bo. Encarnación, North of highway 2, taking a drainage to the north, close to the boundary with El Peñon de Ponce, behind a gas station, (MAPR [MAPR30776!], UPPRP [045552!]).

Proctor, G.R. 48775, 14/04/1993, Peñuelas, Ba. Encarnación, hills SE. of Tallaboa, just NW. of Peñon de Ponce, (SJ [SJ00252!]).

Shafer, J.A. 1989, 13/03/1913, Limestone hill, Peñon, Guayanilla to Tallaboa, (NY [NY00952747!], US [US970971]).

Sintenis, P.E.E. 3731 (syntype), 10/02/1886 *prope* Guánica *in declivibus umbrosis montis* El Maniel *ad* La Ballena *versus*, (B [destroyed], HBG [HBG507449!], JE [JE00000805!], K [K000212605!, K000213358!], LD [LD1407637!, LD1407697!], MO [MO-694636!], NY [NY01085777!], P [P03540816!, P03860090!, P03860089!], S [S12-18801!], US [US00110719!], WU [WU0040311, W1900-0000177!], Z [Z-000001932!]).

Sintenis, P.E.E. 4879 (syntype), 29/07/1886, *Inter* Guayanilla *et* Barinas *in declivibus ad* Los Indios, (B [destroyed], MPU [MPU019554!]).

Stevens, F.L. 9104, 29/07/1915, Guánica, (NY [NY00038089!]).

Woodbury, R.O. s.n., 18/09/1959, along road 116 east of Ensenada, (UPR [UPR02869]).

Woodbury, R.O. s.n., 1972, Guayanilla, (MO [MO-2158001!], NY [NY00952749!], UPR [28233]).

Woodbury, R.O. s.n., 25/06/1978, Vieques, Punta Jalova, (SJ [SJ00249!]).

DNA samples (georeferenced with field images – no voucher specimen)

Hamilton, M.A. 906, 907, 908, 909, 910, 912, 913, 914, 915, 916, 917, 918, 18/07/2012, Bosque Estatal de Guánica, Guánica eastern tract.

Hamilton, M.A. 929 to 934, 30/07/2012, Eastern Vieques National Wildlife Refuge, Puerto Ferro.

Hamilton, M.A. 943, 944, 946, 947, 03/05/2013, Bosque Estatal de Guánica, Guánica eastern tract.

Hamilton, M.A. 951 to 960, 05/05/2013, Bosque Estatal de Guánica, Guánica eastern tract.

Hamilton, M.A. 995 to 1021, 07/05/2013, Bosque Estatal de Guánica, Guánica eastern tract.

Hamilton, M.A. 1043, 1045, 1047 to 1051, 08/05/2013, Yauco, Barina, Catala Farm.

Hamilton, M.A. 1054 to 1077, 09/05/2013, Ponce, Canas, Off Rd. 2 behind the Holiday Inn Hotel.

Hamilton, M.A. 1078, 1079, 1081 to 1092, 10/05/2013, Peñuelas, Bo. Encarnación, N of Highway 2.

Hamilton, M.A. 1093 to 1100, 11/05/2013, Bosque Estatal de Guánica, Guánica eastern tract.

Hamilton, M.A. 1349 to 1354, 03/03/2014, Bosque Estatal de Guánica, Guánica eastern tract.

Hamilton, M.A. 1356 to 1357, 04/03/2014, Ponce, Canas, Off Rd. 2 behind the Holiday Inn Hotel.

Hamilton, M.A. 1359 to 1361, 04/03/2014, Bosque Estatal de Guánica, Guánica western tract.

Hamilton, M.A. 1362 to 1365, 06/03/2014, Peñuelas, Bo. Encarnación, N of Highway 2.

Hamilton, M.A. 1366 to 1368, 07/03/2014, Bosque Estatal de Guánica, Guánica eastern tract.

British Virgin Islands - Anegada

Vouchers from wild plants

Acevedo-Rodriguez, P. 10957, 10/4/2000, Anegada, N of Flamingo Pond, (MAPR [MAPR26743], US [US711911]).

Acevedo-Rodriguez, P., 11542, 21/11/2000, Anegada, Middle Key, (UPRRP [39267], US [US708421]).

Britton, N. L. & Fishlock, W. C. 955, 19/02/1913, Anegada, West End, (NY [NY01360743!], US [US757425]).

D'Arcy, W.G. 4838, 31/07/1970, Anegada, Scattered plants around the sandy plain near West End, (MO [MO-2158003!]).

D'Arcy, W.G. 4971, 04/08/1970, Near East End, (MO [MO-878732!]).

D'Arcy, W.G. 5077, 04/02/1971, Anegada, Ca. 1 mi. east of The Settlement on the limestone plain, (A!, FAU, MO [MO-2158000!], SIU [SIU-09877!]).

Fishlock, W.C. 41, 10/1918, Anegada, junction of rocky and sandy parts, (NY [NY01360760!]).

Hamilton, M.A. 880, 04/07/2012, Anegada, Cow Wreck Bay, (BVI [OT0001334!], K [K000816540!], MAPR!).

Hamilton, M.A. 881, 04/07/2012, Anegada, Cow Wreck Bay, (BVI [OT0001335!], K [K000816543!], MAPR!).

Hamilton, M.A. 1192, 27/07/2013, Anegada, Pomato Point, (BVI [OT0001350!], K [K000818120!], MAPR!).

Hamilton, M.A. 1194, 28/07/2013, Anegada, West End, (BVI [OT00001351!], K [K000818130!], MAPR!).

Hamilton, M.A. 1196, 28/07/2013, Anegada, Flamingo Pond, (BVI [OT0001352!], K [K000818129!], MAPR!).

Hamilton, M.A. 1198, 28/07/2013, Anegada, Bones Bight, (BVI [OT0001353!], K [K000818128!], MAPR!).

Hamilton, M.A. 1213, 29/07/2013, Anegada, Keel Point, (BVI [OT0001354!], K [K000818127!], MAPR!).

Hamilton, M.A. 1215, 29/07/2013, Anegada, Flamingo Pond, (BVI [OT0001355!], K [K000818126!], MAPR!).

Hamilton, M.A. 1220, 29/07/2013, Anegada, Bones Low Point, (BVI [OT0001356!], K [K000818132!], MAPR!).

Hamilton, M.A. 1225, 30/07/2013, Anegada, Warner, (K [K000818133!]).

Hamilton, M.A. 1253, 31/07/2013, Anegada, Pearl Point, (BVI [OT0001357!], K [K000818135!], MAPR!).

Hamilton, M.A. 1269, 31/07/2013, Anegada, Saltheap Point, (BVI [OT0001362!], K [K000818134!], MAPR!).

Hamilton, M.A. 1282, 01/08/2013, Anegada, Warner, (BVI [OT0001358!], K [K000818131!], MAPR!).

Hamilton, M.A. 1289, 01/08/2013, Anegada, Windlass Low Point, (BVI [OT0001360!], K [K000818136!], MAPR!).

Hamilton, M.A. 1309, 02/08/2013, Anegada, Middle Cay, (BVI [OT0001359!], K [K000817098!], MAPR!).

Hamilton, M.A. 1503, 14/02/2015, Anegada, Warner, (K [K000819878!]).

Hamilton, M.A. 1506, 14/02/2015, Anegada, Warner, (K [K000819879!]).

Pollard, B.J. 1159, 28/11/2002, Anegada, Southern Dunes, 1km E of Anegada Reef Hotel, (K [cited by Wenger *et al.* (2010), specimen not located]).

Pollard, B.J. 1266, 14/12/2002, Anegada, West End, along road from West End along northern shore towards Bones Bight, (BVI [OT0001201!], K [K000367630!]).

Proctor, G.R. 43601, 30/05/1987, Anegada, West End, (SJ [SJ00250!]).

Proctor, G.R. 48393, 07/10/1992, Anegada, c. 0.3 mile SE of Bones Bight, (FTG [00067727!], MO [MO-2158002], SJ [SJ00248!]).

Smith, D.N. s.n. 10/09/1990, Anegada, Growing at the Slob [Sambeal Slob], near the air-strip, (FTG [00064942!]).

Walker, R. & Clubbe, C. 04, 21/11/2003, Anegada, Sambeal Slob, (K [K000297612!], BVI [OT0001083!], UPRRP [UPPRP09266]).

Walker, R. 55, 22/12/2004, Anegada, Next to Botanic Garden along Settlement Road, (K [K000297637!], BVI [OT0001091!]).

DNA samples (georeferenced with field images – no voucher specimen)

Hamilton, M.A. 1176-1191, 1193, 1195, 1197, 1199-1212, 1214, 1216-1219, 1221-1224, 1226-1252, 1254-1268, 1270-1281, 1283-1288, 1290-1308, 1310-1322, collected between 26/07/2013 and 05/08/2013, across the island of Anegada.

Hamilton, M.A. 1503, 14/02/2015, Anegada, Warner.

Cultivated material held in *ex-situ* collections

Royal Botanic Gardens, Kew held 87 individuals in glasshouse facilities during the period of this research.

Fairchild Tropical Botanic Garden, Dade County, Florida, USA held 19 individuals in outdoor garden beds during the period of this research.

J.R. O'Neal Botanic Garden, Tortola, British Virgin Islands held 17 individuals in a shade house nursery and outdoor garden beds during the period of this research.

Cabo Rojo National Wildlife Refuge, Cabo Rojo, Puerto Rico held 34 individuals in a shade house nursery and field plots during the period of this research.

Department of Natural and Environmental Resources, Guánica State Forest, Guánica eastern tract held 21 individuals in the forest shade house nursery during the period of this research.

Vouchers from cultivated plants

Abbott, J. 23959, 03/01/2008, Fairchild Tropical Botanic Garden, Dade County, Florida, USA (FTG [00142361!]).

Bancroft, N. 006, 08/06/2010, Royal Botanic Gardens Kew, Tropical Nursery, (K [K000703992!]).

Corcoran, M.R. UKOTsLC 0045, 05/07/2012, Royal Botanic Gardens Kew, Kew Quarantine House, (K [K000816585!]).

Hamilton, M.A. 936, 24/03/2013, Royal Botanic Gardens Kew, Kew Quarantine House, (K [K000679879!]).

Hamilton, M.A. 937, 24/03/2013, Royal Botanic Gardens Kew, Kew Quarantine House, (K [K000679880!]).

Hamilton, M.A. 938, 24/03/2013, Royal Botanic Gardens Kew, Kew Quarantine House, (K [K000679881!]).

Hamilton, M.A. 939, 24/03/2013, Royal Botanic Gardens Kew, Kew Quarantine House, (K [K000679882!]).

Hamilton, M.A. 940, 24/03/2013, Royal Botanic Gardens Kew, Kew Quarantine House, (K – wood sample [K000679883!]).

Hamilton, M.A. FTG 91544B, 14/08/2013, USA, Dade County, Fairchild Tropical Botanic Garden (FTG!, K [K000818122!]).

Hamilton, M.A. FTG U-0067A, 14/08/2013, USA, Dade County, Fairchild Tropical Botanic Garden (FTG!, K [K000818121!]).

Hamilton, M.A. 1118, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000818062!]).

Hamilton, M.A. 1119, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000818063!]).

Hamilton, M.A. 1120, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000817096!]).

Hamilton, M.A. 1121, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000817094!]).

Hamilton, M.A. 1122, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000818069!]).

Hamilton, M.A. 1123, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000818068!]).

Hamilton, M.A. 1124, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000817091!]).

Hamilton, M.A. 1125, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000817086!]).

Hamilton, M.A. 1126, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000817088!]).

Hamilton, M.A. 1127, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000817087!]).

Hamilton, M.A. 1128, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000818070!]).

Hamilton, M.A. 1129, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000817095!]).

Hamilton, M.A. 1130, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000818064!]).

Hamilton, M.A. 1131, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000817090!]).

Hamilton, M.A. 1132, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000818065!]).

Hamilton, M.A. 1133, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000818067!]).

Hamilton, M.A. 1134, 11/07/2013, Tortola, J.R. O'Neal Botanical Gardens National Park, (K [K000817097!]).

Hamilton, M.A. 1323, 05/10/2013, Royal Botanic Gardens Kew, Kew Quarantine House, (K [K000679920!]).

Hamilton, M.A. 1324, 05/10/2013, Royal Botanic Gardens Kew, Kew Quarantine House, (K [K000679921!]).

Hamilton, M.A. 1329, 05/10/2013, Royal Botanic Gardens Kew, Kew Quarantine House, (K [K000679922!]).

Hamilton, M.A. 1330, 05/10/2013, Royal Botanic Gardens Kew, Kew Quarantine House, (K [K000679923!]).

Hamilton, M.A. 1332, 05/10/2013, Royal Botanic Gardens Kew, Kew Quarantine House, (K [K000679924!]).

Hamilton, M.A. 1334, 05/10/2013, Royal Botanic Gardens Kew, Kew Quarantine House, (K [K000679925!]).

Hamilton, M.A. 1336, 05/10/2013, Royal Botanic Gardens Kew, Kew Quarantine House, (K [K000679926!]).

DNA samples (georeferenced with images – no voucher specimen)

Corcoran, M.R. s.n., 2012, one sample of plant grown from wild collected seed on Anegada held under accession 2005-1557, Royal Botanic Gardens, Kew.

Corcoran, M.R. s.n., 2012, 48 samples of individual plants grown from seed collected from cultivated plants held under accession 2009-2709, Royal Botanic Gardens, Kew.

Corcoran, M.R. s.n., 2012, two samples from individual plants held under accession 2008-3140; seed collected from cultivated mother plant held under accession 2005-1557, Royal Botanic Gardens, Kew.

Corcoran, M.R. s.n., 2012, four samples from individual plants held under accession 2011-1273; seed collected from plants grown from wild seed collected on Anegada held under various accessions, Royal Botanic Gardens, Kew.

Hamilton, M.A. 880A, 880B and 880-1 to 880-9, 24/03/2013, eleven samples from seedlings grown from wild seed collected on Anegada.

Hamilton, M.A. 961 to 994, 06/05/2013, restoration plot [CULTIVATED], Cabo Rojo National Wildlife Refuge.

Hamilton, M.A. 1022 to 1042, 07/05/2013, forest nursery [CULTIVATED], Bosque Estatal de Guánica, Guánica eastern tract.

Hamilton, M.A. FTG91544A, FTG91544C, FTG91544D, FTG91544E, FTG91544F, FTG941068B, FTG941068C, FTG941068E, FTG941068F, FTG941068G, FTG941068H, FTGVOL1, FTGVOL2, FTGVOL3, FTGVOL4, FTGVOL5, FTGVOL6, 14/08/2013, Original seed source from Anegada with subsequent collection from cultivated plants and volunteers in outdoor displays, USA, Dade County, Fairchild Tropical Botanic Garden

Hamilton, M.A. 1325-1328, 1331, 1333, 1335, 1337 05/10/2013, leaf samples of plants grown in the Kew Quarantine House, Royal Botanic Gardens Kew, from wild seed collected on Puerto Rico.

Appendix 2: Survey points by substrate and regional habitat class

The following table provides the total number of sampling points across the Puerto Rican Bank by island with presence/absence records per substrate type and percentage as well as per regional habitat class based on Kennaway & Helmer (2007) **for the country of Puerto Rico and Kennaway *et al.*** (2008) **for the U.S and British Virgin Islands and percentage. Pleistocene limestone and Quaternary sand deposits occur on the island of Anegada. Pliocene limestone deposits occur on the island of Vieques. Miocene deposits of Juana Diaz and Ponce limestone occur on the island of Puerto Rico. Note: 'Semi-Deciduous and Drought Deciduous Forest on Karst (includes semi-evergreen forest)' = 'Deciduous forest on karst'; 'Deciduous, Evergreen Coastal and Mixed Forest or Shrubland with Succulents' = 'Mixed Forest'; 'Pasture, Hay or Inactive Agriculture (e.g. abandoned sugar cane)' = 'Pasture'**

Island	Survey points	Absence records	Limestone	% on limestone	Other	% on other	High-Medium Density Urban	Low-Medium Density Urban	Pasture	Mixed Forest	Deciduous forest on karst	Other habitats
Anegada	666	514	292	57%	222	43%	1	52	17	355	0	89
		Presence records	Limestone	% on limestone	Other	% on other	High-Medium Density Urban	Low-Medium Density Urban	Pasture	Mixed Forest	Deciduous forest on karst	Other habitats
		152	78	51%	74	49%	3	36	3	110	0	0
Vieques	514	Absence records	Limestone	% on limestone	Other	% on other	High-Medium Density Urban	Low-Medium Density Urban	Pasture	Mixed Forest	Deciduous forest on karst	Other habitats
		513	47	9%	466	91%	0	65	136	1	103	208
		Presence records	Limestone	% on limestone	Other	% on other	High-Medium Density Urban	Low-Medium Density Urban	Pasture	Mixed Forest	Deciduous forest on karst	Other habitats
		1	1	100%	0	0%	0	0	0	0	1	0
Puerto Rico	760	Absence records	Limestone	% on limestone	Other	% on other	High-Medium Density Urban	Low-Medium Density Urban	Pasture	Mixed Forest	Deciduous forest on karst	Other habitats
		734	424	58%	310	42%	56	64	107	49	268	190
		Presence records	Limestone	% on limestone	Other	% on other	High-Medium Density Urban	Low-Medium Density Urban	Pasture	Mixed Forest	Deciduous forest on karst	Other habitats
		26	26	100%	0	0%	0	0	0	0	26	0

Appendix 3: *Varronia rupicola* observations made between 2012 and 2015

The tables below show *Varronia rupicola* observations (presence and opportunistic records) made across the species' native range between 2012 and 2015 divided by habitat and substrate per island. Table A. Shows values for refined habitat types of Kennaway *et al.* (2008) for Anegada and Gould *et al.* (2008) for Puerto Rico and Vieques. Table B. Shows values for regional habitat types of Kennaway & Helmer (2007) **for the country of Puerto Rico and Kennaway *et al.* (2008) for the U.S and British Virgin Islands. Pleistocene limestone and Quaternary sand deposits occur on the island of Anegada. Pliocene limestone deposits occur on the island of Vieques. Miocene deposits of Juana Diaz and Ponce limestone occur on the island of Puerto Rico.**

A. Refined habitat types of Kennaway *et al.* (2008) for Anegada and Gould *et al.* (2008) for Puerto Rico and Vieques						
Island	**Number of observations**	**Refined habitats**	**Limestone**	**%**	**Sand**	**%**
Anegada	479	Deciduous, Evergreen and Mixed Forest and Shrubland with or without Succulents	449	94%	30	6%
	100	Evergreen Coastal Shrubland	26	26%	74	74%
	59	Low Density Urban	12	20%	47	80%
	9	Pasture, Hay, Abandoned Agriculture or Other Grassy Areas	7	78%	2	22%
	4	High-Medium Density Urban	0	0%	4	100 %
Vieques	6	Mature secondary lowland dry limestone semi-deciduous forest	6	100%	0	0%
Puerto Rico	159	Mature secondary lowland dry limestone semi-deciduous forest	159	100%	0	0%
	6	Young secondary lowland dry limestone semi-deciduous forest	6	100%	0	0%
Total observations	**822**	**Total observations by substrate and percentage**	**665**	**81%**	**157**	**19%**

B. Regional habitat types of Kennaway & Helmer (2007) **for the country of Puerto Rico and Kennaway *et al.* (2008) for the U.S and British Virgin Islands**						
Island	**Number of observations**	**Habitat**	**Limestone**	**%**	**Sand**	**%**
Anegada	579	Deciduous, Evergreen Coastal and Mixed Forest or Shrubland with Succulents	475	82%	104	18%
	59	Low Density Urban	12	20%	47	80%
	9	Pasture, Hay or Inactive Agriculture	7	78%	2	22%
	4	High-Medium Density Urban	0	0%	4	100%
Vieques	6	Semi-Deciduous and Drought Deciduous Forest on Karst (includes semi-evergreen forest)	6	100%	0	0%
Puerto Rico	165	Semi-Deciduous and Drought Deciduous Forest on Karst (includes semi-evergreen forest)	165	100%	0	0%
Total observations	**822**	**Total observations by substrate and percentage**	**665**	**81%**	**157**	**19%**

Appendix 4: New samples used for phylogenetic analysis

The following table provides the species binomial (Genus, Species), Identification number (ID), voucher number (Voucher) and source material (Source) for samples included in the five analyses undertaken, A-E, indicating if the sample was included using plus (+) or minus (-). Source material abbreviations: Silica = Silica dried leaf material; HS = leaf material from herbarium specimen. An asterisk (*) beside the specific epithet indicates type material.

A	B	C	D	E	Genus	Specific epithet	ID	Voucher	Source
+	+	+	+	+	*Cordia*	*borinquensis*	83	MAH924	Silica
+	+	+	+	+	*Cordia*	*collococca*	137	MAH948	Silica
+	+	+	+	+	*Cordia*	*obliqua*	138	MAH949	Silica
+	+	+	+	+	*Cordia*	*rickseckeri*	72	MAH926	Silica
+	+	+	+	+	*Cordia*	*rickseckeri*	241	MAH1052	Silica
+	+	+	-	-	*Cordia*	*rickseckeri*	308	MAH875	Silica
+	+	+	+	+	*Cordia*	*rickseckeri*	522	SB111	Silica
+	+	+	+	+	*Varronia*	*bahamensis*	67	MAH863	Silica
+	-	+	-	-	*Varronia*	*bahamensis**	74	K000583257	HS
+	+	+	+	+	*Varronia*	*bahamensis*	307	K000297743	HS
+	+	+	+	+	*Varronia*	*bahamensis*	519	FTG2009-0504A	Silica
+	+	+	+	+	*Varronia*	*bahamensis*	525	MAH861	Silica
+	+	+	+	+	*Varronia*	*bahamensis*	526	FTG00151995	HS
+	-	+	+	-	*Varronia*	*bellonis*	119	K000817006	HS
+	+	+	+	+	*Varronia*	*bellonis*	120	K000817007	HS
+	+	+	+	+	*Varronia*	*bullata*	69	MAH780	Silica
+	+	+	+	+	*Varronia*	*bullata*	84	MAH925	Silica
+	+	+	+	+	*Varronia*	*bullata* spp. *humilis*	139	MAH950	Silica
+	-	+	+	-	*Varronia*	*bullata* spp. *humilis*	242	MAH1053	Silica
+	+	+	-	-	*Varronia*	*lima*	85	MAH922	Silica
+	+	+	+	+	*Varronia*	*lima*	86	MAH923	Silica
+	+	+	+	+	*Varronia*	*lucayana*	309	MARC55	Silica
+	+	+	-	-	*Varronia*	*macrostachya*	292	K1936-31901	Silica
+	+	+	+	+	*Varronia*	*nesophila*	549	MAH773	Silica
+	+	+	+	+	*Varronia*	*polycephala*	81	MAH927	Silica
+	+	+	+	+	*Varronia*	*polycephala*	88	MAH920	Silica
+	+	+	+	+	*Varronia*	*polycephala*	131	MAH941	Silica
+	+	+	+	+	*Varronia*	*polycephala*	302	K000679889	HS
+	-	+	-	-	*Varronia*	*rupicola**	3	K000212605	HS
+	-	+	-	-	*Varronia*	*rupicola**	4	K000213358	HS
+	+	+	+	+	*Varronia*	*rupicola*	6	MAH880	Silica
+	+	+	+	+	*Varronia*	*rupicola*	7	MAH934	Silica
+	+	+	+	+	*Varronia*	*rupicola*	96	MAH913	Silica
33	**28**	**33**	**27**	**25**	**Total per analysis**				

Appendix 5: GenBank sequences used in phylogenetic analysis

The following table provides the species binomial and GenBank number (GenBank #) for samples included in the four analyses undertaken, A-E, by indicating if the sample was included using plus (+) or minus (-).

A	B	C	D	E	Genus	Specific epithet	GenBank #
+	+	-	-	-	*Cordia*	*aberrans*	JF332090
+	+	-	-	-	*Cordia*	*acutifolia*	JF332066
+	+	-	-	-	*Cordia*	*alliodora*	JF332103
-	-	+	-	-	*Cordia*	*americana*	KF158212
+	+	-	-	-	*Cordia*	*anabaptista*	JF332088
+	+	-	-	-	*Cordia*	*bicolor*	JF332068
+	-	-	-	-	*Cordia*	*boissieri*	EU862046
+	+	-	-	-	*Cordia*	*bordasii*	JF332105
+	+	-	-	-	*Cordia*	*collococca*	JF332092
+	+	-	-	-	*Cordia*	*correae*	JF332061
+	-	-	-	-	*Cordia*	*croatii*	EU862048
+	+	-	-	-	*Cordia*	*cymosa*	JF332083
-	-	+	-	-	*Cordia*	*decandra*	EF688851
+	+	-	-	-	*Cordia*	*decandra*	EF688903
+	+	-	-	-	*Cordia*	*decipiens*	JF332070
+	+	-	-	-	*Cordia*	*dentata*	JF332104
+	+	-	-	-	*Cordia*	*dichotoma*	JF332093
+	+	-	-	-	*Cordia*	*diversifolia*	JF332112
+	+	-	-	-	*Cordia*	*dwyeri*	JF332079
+	+	-	-	-	*Cordia*	*ecalyculata*	JF332057
+	+	-	-	-	*Cordia*	*elaeagnoides*	JF332106
+	+	-	-	-	*Cordia*	*exaltata*	JF332074
+	+	-	-	-	*Cordia*	*gerascanthus*	JF332100
+	+	-	-	-	*Cordia*	*glazioviana*	JF332109
+	+	-	-	-	*Cordia*	*goeldiana*	JF332102
+	+	-	-	-	*Cordia*	*guineensis*	JF332096
-	-	+	-	-	*Cordia*	*guineensis*	KF158203
+	+	-	-	-	*Cordia*	*incognita*	JF332110
+	+	-	-	-	*Cordia*	*insignis*	JF332098
+	+	-	-	-	*Cordia*	*lasiocalyx*	JF332081
+	+	-	-	-	*Cordia*	*leslieae*	JF332062
+	+	-	-	-	*Cordia*	*liesneri*	JF332063
+	+	-	-	-	*Cordia*	*lucidula*	JF332082
-	-	+	-	-	*Cordia*	*lutea*	KF158215
+	+	-	-	-	*Cordia*	*megalantha*	JF332101
+	+	-	-	-	*Cordia*	*monoica*	JF332095
-	-	+	-	-	*Cordia*	*monoica*	KF158202
+	+	-	-	-	*Cordia*	*myxa*	KC155288
+	+	-	-	-	*Cordia*	*naidophila*	JF332071
-	-	+	-	-	*Cordia*	*nevillii*	HQ412979
-	-	+	-	-	*Cordia*	*nodosa*	HQ286269

A	B	C	D	E	Genus	Specific epithet	GenBank #
+	+	-	-	-	*Cordia*	*nodosa*	JF332072
+	+	-	-	-	*Cordia*	*oncocalyx*	JF332108
+	+	-	-	-	*Cordia*	*panamensis*	JF332060
+	+	-	-	-	*Cordia*	*panicularis*	JF332084
+	+	-	-	-	*Cordia*	*pilosa*	JF332089
+	+	-	-	-	*Cordia*	*porcata*	JF332080
+	+	-	-	-	*Cordia*	*rufescens*	JF332086
+	+	-	-	-	*Cordia*	*saccellia*	JF332111
-	-	+	-	-	*Cordia*	*saccellia*	KF158208
+	+	-	-	-	*Cordia*	*scabrifolia*	JF332067
+	+	-	-	-	*Cordia*	*sebestena*	JF332107
-	-	+	-	-	*Cordia*	*sebestena*	KC542482
+	+	-	-	-	*Cordia*	*sellowiana*	JF332069
-	-	+	-	-	*Cordia*	*sonorae*	KF158214
+	+	-	-	-	*Cordia*	*sprucei*	JF332075
+	+	-	-	-	*Cordia*	*superba*	JF332085
+	+	-	-	-	*Cordia*	*taguahyensis*	JF332087
+	+	-	-	-	*Cordia*	*tetrandra*	JF332076
+	+	-	-	-	*Cordia*	*toqueve*	JF332059
+	+	-	-	-	*Cordia*	*trachyphylla*	JF332078
+	+	-	-	-	*Cordia*	*trichoclada*	JF332077
+	+	-	-	-	*Cordia*	*trichotoma*	JF332099
+	-	-	-	-	*Cordia*	*ucayaliensis*	JF332073
-	-	+	-	-	*Cordia*	*weddellii*	KF158189
-	-	+	+	+	*Heliotropium*	*angiospermum*	HQ286151
+	+	-	-	+	*Heliotropium*	*angiospermum*	HQ286121
+	-	-	-	-	*Varronia*	*ambigua*	EU862043
+	-	-	-	-	*Varronia*	*anderssonii*	HM443749
+	-	-	-	-	*Varronia*	*angustifolia*	AY321619
+	-	-	-	-	*Varronia*	*bifurcata*	AF402575
+	-	-	-	-	*Varronia*	*bonplandii*	AY321620
-	-	+	-	-	*Varronia*	*bonplandii*	KC542473
+	-	-	-	-	*Varronia*	*bullata*	AY176084
-	-	+	-	-	*Varronia*	*bullata*	KF158211
+	+	-	-	-	*Varronia*	*curassavica*	JF332114
+	+	-	-	-	*Varronia*	*discolor*	JF332123
+	+	-	-	-	*Varronia*	*glandulosa*	JF332115
+	+	-	-	-	*Varronia*	*globosa*	JF332121
+	-	-	-	-	*Varronia*	*guanacastensis*	EU862054
+	+	-	-	-	*Varronia*	*harleyi*	JF332118
+	-	-	-	-	*Varronia*	*inermis*	EU862055
+	-	-	-	-	*Varronia*	*lauta*	AY321622
+	-	-	-	-	*Varronia*	*lenis*	AF402577
+	+	-	-	-	*Varronia*	*leucocephala*	JF332124
+	+	-	-	-	*Varronia*	*leucomalloides*	JF332120

A	B	C	D	E	Genus	Specific epithet	GenBank #
+	-	-	-	-	*Varronia*	*leucophlyctis*	HM443754
+	-	-	-	-	*Varronia*	*lima*	AY321624
+	-	-	-	-	*Varronia*	*longipedunculata*	AY321625
+	-	-	-	-	*Varronia*	*macrocephala*	HM443757
+	-	-	-	-	*Varronia*	*martinicensis*	EU862056
+	+	-	-	-	*Varronia*	*mayoi*	JF332119
+	+	-	-	-	*Varronia*	*multispicata*	JF332113
+	-	-	-	-	*Varronia*	*oaxacana*	EU862058
+	-	-	-	-	*Varronia*	*podocephala*	EU862060
+	-	-	-	-	*Varronia*	*polycephala*	HM443762
+	-	-	-	-	*Varronia*	*pringlei*	EU862062
+	-	-	-	-	*Varronia*	*revoluta*	HM443764
+	+	-	-	-	*Varronia*	*sessilifolia*	JF332116
+	-	-	-	-	*Varronia*	*spinescens*	AY701587
+	+	-	-	-	*Varronia*	*striata*	JF332122
+	+	-	-	-	*Varronia*	*tarodaea*	JF332117
88	**64**	**14**	**1**	**2**	**Total per analysis**		

Appendix 6: Population genetics samples

The following table provides the Voucher number (Voucher), Identification number (Sample ID), Collection location (Location), Population identifier (Population), Origin of source material (Origin) and Origin of material for *ex-situ* material (*Ex-situ* origin) for 380 samples. If the sample was included or excluded in the three analysis groups, A-C, it is indicated using plus (+) or minus (-), respectively.

Source material abbreviations: Wild = Silica dried leaf material; Cultivated = *ex-situ* material; HS = leaf material from herbarium specimen; N/A = not applicable. Samples originate from extant *ex-situ* plants, extant wild plants or herbarium specimens of dead plants. All wild and *ex-situ* plant material originated from silica dried leaf samples. Herbarium specimen material originated from air-dried herbarium vouchers. Source material abbreviations for *ex-situ* plants: BVI = Anegada source material; PR = Puerto Rico source material.

Population abbreviations for *ex-situ* plants: Cabo Rojo = Cabo Rojo National Wildlife Refuge, Puerto Rico; England = Royal Botanic Gardens, Kew, UK; Florida = Fairchild Tropical Botanical Garden, Miami, Florida, USA; Guánica nursery = Guánica State Forest nursery, Puerto Rico; Tortola = J.R. O'Neal Botanic Garden nursery, Road Town, Tortola, BVI. Population abbreviations for wild plants: Anegada East = eastern Anegada, British Virgin Islands including the localities of East End and Warner; Anegada West = western Anegada, British Virgin Islands including the localities of Bones Bight, Bones Low Point, Bumber Well Cay, Capt. Auguste George Airport, Citron Bush, Cow Wreck Bay, Flamingo Pond, Jack Bay, Keel Point, Low Cay, Middle Cay, North Raibin Slob, Nutmeg Point, Pearl Point, Pomato Point, Sambeal Slob, Setting Point, Soldier East Point, Soldier Point, Saltheap Point, The Settlement, Vagabond Pond, West End, Windlass Bight and Windlass Low Point; Guánica = the municipalities of Guánica and Yauco on the island of Puerto Rico; Ponce = the municipalities of Peñuelas and Ponce on the island of Puerto Rico; Vieques = the peninsula of Puerto Ferro on Vieques island.

A	B	C	Voucher	Sample ID	Location	Population	Origin	*Ex-situ* origin
+	+	+	MAH881	5	Anegada	Anegada West	Wild	N/A
+	+	+	MAH880	6	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1176	311	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1177	312	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1178	313	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1179	314	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1180	315	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1181	316	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1182	317	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1183	318	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1184	319	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1185	320	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1186	321	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1187	322	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1188	323	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1189	324	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1190	325	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1191	326	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1192	327	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1193	328	Anegada	Anegada West	Wild	N/A

A	B	C	Voucher	Sample ID	Location	Population	Origin	*Ex-situ* origin
+	+	+	MAH1194	329	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1195	330	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1196	331	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1197	332	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1198	333	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1199	334	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1200	335	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1201	336	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1202	337	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1203	338	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1204	339	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1205	340	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1206	341	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1207	342	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1208	343	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1209	344	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1210	345	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1211	346	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1212	347	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1213	348	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1214	349	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1215	350	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1216	351	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1217	352	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1219	354	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1221	356	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1222	357	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1223	358	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1224	359	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1225	360	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1226	361	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1227	362	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1228	363	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1229	364	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1231	366	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1232	367	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1233	368	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1234	369	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1235	370	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1236	371	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1237	372	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1238	373	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1239	374	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1240	375	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1242	377	Anegada	Anegada West	Wild	N/A

A	B	C	Voucher	Sample ID	Location	Population	Origin	*Ex-situ* origin
+	+	+	MAH1243	378	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1244	379	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1245	380	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1246	381	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1247	382	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1248	383	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1249	384	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1250	385	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1251	386	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1252	387	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1253	388	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1254	389	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1255	390	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1257	392	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1258	393	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1260	395	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1261	396	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1262	397	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1263	398	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1264	399	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1265	400	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1266	401	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1267	402	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1268	403	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1269	404	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1270	405	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1271	406	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1272	407	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1273	408	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1274	409	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1275	410	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1276	411	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1277	412	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1278	413	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1279	414	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1280	415	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1281	416	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1282	417	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1283	418	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1284	419	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1285	420	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1286	421	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1287	422	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1288	423	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1289	424	Anegada	Anegada West	Wild	N/A

A	B	C	Voucher	Sample ID	Location	Population	Origin	*Ex-situ* origin
+	+	+	MAH1290	425	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1291	426	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1292	427	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1293	428	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1294	429	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1295	430	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1296	431	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1297	432	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1298	433	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1299	434	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1300	435	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1301	436	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1302	437	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1303	438	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1304	439	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1305	440	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1306	441	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1307	442	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1308	443	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1309	444	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1310	445	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1311	446	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1312	447	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1313	448	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1314	449	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1315	450	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1316	451	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1317	452	Anegada	Anegada West	Wild	N/A
+	+	+	MAH1318	453	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1319	454	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1320	455	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1321	456	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1322	457	Anegada	Anegada East	Wild	N/A
+	+	+	MAH1241	530	Anegada	Anegada West	Wild	N/A
-	+	+	MAH961	150	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH962	151	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH963	152	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH964	153	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH965	154	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH966	155	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH967	156	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH968	157	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH969	158	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH970	159	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH971	160	Puerto Rico	Cabo Rojo	Cultivated	PR

A	B	C	Voucher	Sample ID	Location	Population	Origin	*Ex-situ* origin
-	+	+	MAH972	161	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH973	162	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH974	163	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH975	164	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH976	165	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH977	166	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH978	167	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH979	168	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH980	169	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH981	170	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH982	171	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH983	172	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH984	173	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH985	174	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH986	175	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH987	176	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH988	177	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH989	178	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH990	179	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH991	180	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH992	181	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH993	182	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	MAH994	183	Puerto Rico	Cabo Rojo	Cultivated	PR
-	+	+	2005-1557_2	1	England	England (BVI)	Cultivated	BVI
-	+	+	MAH880A	300	England	England (BVI)	Cultivated	BVI
-	+	+	MAH880B	301	England	England (BVI)	Cultivated	BVI
-	+	+	MAH880-1	509	England	England (BVI)	Cultivated	BVI
-	+	+	MAH880-2	510	England	England (BVI)	Cultivated	BVI
-	+	+	MAH880-3	511	England	England (BVI)	Cultivated	BVI
-	+	+	MAH880-4	512	England	England (BVI)	Cultivated	BVI
-	+	+	MAH880-5	513	England	England (BVI)	Cultivated	BVI
-	+	+	MAH880-6	514	England	England (BVI)	Cultivated	BVI
-	+	+	MAH880-7	515	England	England (BVI)	Cultivated	BVI
-	+	+	MAH880-8	516	England	England (BVI)	Cultivated	BVI
-	+	+	MAH880-9	517	England	England (BVI)	Cultivated	BVI
-	+	+	MAH1323	494	England	England (PR)	Cultivated	PR
-	+	+	MAH1324	495	England	England (PR)	Cultivated	PR
-	+	+	MAH1325	496	England	England (PR)	Cultivated	PR
-	+	+	MAH1327	498	England	England (PR)	Cultivated	PR
-	+	+	MAH1328	499	England	England (PR)	Cultivated	PR
-	+	+	MAH1329	500	England	England (PR)	Cultivated	PR
-	+	+	MAH1330	501	England	England (PR)	Cultivated	PR
-	+	+	MAH1331	502	England	England (PR)	Cultivated	PR
-	+	+	MAH1332	503	England	England (PR)	Cultivated	PR
-	+	+	MAH1333	504	England	England (PR)	Cultivated	PR

A	B	C	Voucher	Sample ID	Location	Population	Origin	*Ex-situ* origin
-	+	+	MAH1334	505	England	England (PR)	Cultivated	PR
-	+	+	MAH1335	506	England	England (PR)	Cultivated	PR
-	+	+	MAH1336	507	England	England (PR)	Cultivated	PR
-	+	+	MAH1337	508	England	England (PR)	Cultivated	PR
-	+	+	FTG_91544-A	475	Florida	Florida	Cultivated	BVI
-	+	+	FTG_91544-B	476	Florida	Florida	Cultivated	BVI
-	+	+	FTG_91544-C	477	Florida	Florida	Cultivated	BVI
-	+	+	FTG_91544-D	478	Florida	Florida	Cultivated	BVI
-	+	+	FTG_91544-F	479	Florida	Florida	Cultivated	BVI
-	+	+	FTG_91544-E	480	Florida	Florida	Cultivated	BVI
-	+	+	FTG_941068-B	481	Florida	Florida	Cultivated	BVI
-	+	+	FTG_941068-C	482	Florida	Florida	Cultivated	BVI
-	+	+	FTG_941068-E	483	Florida	Florida	Cultivated	BVI
-	+	+	FTG_941068-F	484	Florida	Florida	Cultivated	BVI
-	+	+	FTG_941068-G	485	Florida	Florida	Cultivated	BVI
-	+	+	FTG_941068-H	486	Florida	Florida	Cultivated	BVI
-	+	+	FTG_U-0067-A	487	Florida	Florida	Cultivated	BVI
-	+	+	FTG_VOL1	488	Florida	Florida	Cultivated	BVI
-	+	+	FTG_VOL2	489	Florida	Florida	Cultivated	BVI
-	+	+	FTG_VOL3	490	Florida	Florida	Cultivated	BVI
-	+	+	FTG_VOL4	491	Florida	Florida	Cultivated	BVI
-	+	+	FTG_VOL5	492	Florida	Florida	Cultivated	BVI
-	+	+	FTG_VOL6	493	Florida	Florida	Cultivated	BVI
-	+	+	MAH1022	211	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1023	212	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1026	215	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1027	216	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1028	217	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1029	218	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1030	219	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1031	220	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1032	221	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1033	222	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1034	223	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1035	224	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1036	225	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1037	226	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1038	227	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1039	228	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1040	229	Puerto Rico	Guánica nursery	Cultivated	PR
-	+	+	MAH1041	230	Puerto Rico	Guánica nursery	Cultivated	PR
+	+	+	MAH918	80	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH905	89	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH906	90	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH907	91	Puerto Rico	Guánica	Wild	N/A

A	B	C	Voucher	Sample ID	Location	Population	Origin	*Ex-situ* origin
+	+	+	MAH908	92	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH909	93	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH910	94	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH912	95	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH913	96	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH914	97	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH915	98	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH916	99	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH917	100	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH943	132	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH944	133	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH945	134	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH946	135	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH947	136	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH951	140	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH952	141	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH953	142	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH954	143	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH955	144	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH956	145	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH957	146	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH958	147	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH959	148	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH960	149	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH995	184	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH996	185	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH997	186	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH998	187	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH999	188	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1000	189	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1001	190	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1002	191	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1003	192	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1004	193	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1005	194	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1006	195	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1007	196	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1008	197	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1009	198	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1010	199	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1011	200	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1012	201	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1013	202	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1014	203	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1015	204	Puerto Rico	Guánica	Wild	N/A

A	B	C	Voucher	Sample ID	Location	Population	Origin	*Ex-situ* origin
+	+	+	MAH1016	205	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1017	206	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1018	207	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1019	208	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1020	209	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1021	210	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1043	232	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1044	233	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1045	234	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1046	235	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1047	236	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1048	237	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1049	238	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1050	239	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1054	243	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1055	244	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1056	245	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1057	246	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1058	247	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1059	248	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1060	249	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1061	250	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1062	251	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1063	252	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1064	253	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1065	254	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1066	255	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1067	256	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1068	257	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1069	258	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1070	259	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1071	260	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1072	261	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1073	262	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1074	263	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1075	264	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1076	265	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1077	266	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1078	267	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1079	268	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1081	270	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1082	271	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1083	272	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1084	273	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1085	274	Puerto Rico	Ponce	Wild	N/A

A	B	C	Voucher	Sample ID	Location	Population	Origin	*Ex-situ* origin
+	+	+	MAH1086	275	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1087	276	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1088	277	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1089	278	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1090	279	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1091	280	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1092	281	Puerto Rico	Ponce	Wild	N/A
+	+	+	MAH1093	282	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1094	283	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1095	284	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1096	285	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1097	286	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1098	287	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1099	288	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1100	289	Puerto Rico	Guánica	Wild	N/A
+	+	+	MAH1051	299	Puerto Rico	Guánica	Wild	N/A
-	+	+	MAH1118	458	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1119	459	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1120	460	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1121	461	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1122	462	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1123	463	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1124	464	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1125	465	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1126	466	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1127	467	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1128	468	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1129	469	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1130	470	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1131	471	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1132	472	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1133	473	Tortola	Tortola	Cultivated	BVI
-	+	+	MAH1134	474	Tortola	Tortola	Cultivated	BVI
+	+	+	MAH934	7	Vieques	Vieques	Wild	N/A
+	+	+	MAH933	8	Vieques	Vieques	Wild	N/A
+	+	+	MAH932	9	Vieques	Vieques	Wild	N/A
+	+	+	MAH931	10	Vieques	Vieques	Wild	N/A
+	+	+	MAH929	12	Vieques	Vieques	Wild	N/A
+	+	+	MAH930	305	Vieques	Vieques	Wild	N/A
-	-	+	SJ_00248	293	Anegada	Anegada West	HS	N/A
-	-	+	SJ_10111	295	Anegada	Anegada West	HS	N/A
-	-	+	SJ_15176	296	Puerto Rico	Puerto Rico	HS	N/A
-	-	+	SJ_10108	297	Puerto Rico	Puerto Rico	HS	N/A
-	-	+	SJ_10107	298	Puerto Rico	Puerto Rico	HS	N/A
-	-	+	SJ_10109	294	Vieques	Vieques	HS	N/A

Appendix 7: Oligonucleotide testing

The following table provides the microsatellite (Locus), identifier used in this study (Primer code), size of the PCR product (PCR Product Size), description of the ability to determine the loci as monomorphic or polymorphic (Mono/Polymorphic) and description of the interpreted behavior of alleles (Alleles) for primer pairs tested. The selection of a locus for further investigation is indicated in the column 'Loci selected' using plus (+) for selected or minus (-) for rejected.

Loci Selected?	Locus	Primer code	PCR Product Size	Mono/Polymorphic	Alleles
+	VRgr3_2	P1.2	287	Polymorphic	Diploid acting
-	VRgr280_2	P3.4	223	Further optimization required to assess	N/A
+	VRBS43W	P5.6	268	Polymorphic	Diploid acting
-	VRgr48_2	P7.8	147	Monomorphic	N/A
+	VRBREYV	P9.10	287	Polymorphic	Diploid acting
-	VRA7EYM	P11.12	166	Further optimization required to assess	N/A
+	VRA7EYM	P13.14	221	Polymorphic	Polyploid acting
+	VREDKKW	P15.16	188	Polymorphic	Polyploid acting
-	VRCB795	P17.18	141	Monomorphic	N/A
-	VRC39CG	P19.20	233	Monomorphic	N/A
-	VRBSY0Y	P21.22	234	Monomorphic	N/A
-	VRE111H	P23.24	132	Further optimization required to assess	N/A
-	VRgr81_4	P25.26	295	Monomorphic	N/A
-	VRgr109_2	P27.28	281	Monomorphic	N/A
+	VRBL3FZ	P29.30	203	Polymorphic	Diploid acting
+	VRD2DN4	P31.32	179	Polymorphic	Polyploid acting
-	VRBC387	P33.34	204	Further optimization required to assess	N/A
-	VREZDWJ	P35.36	206	Monomorphic	N/A
-	VRBBWS9	P37.38	101	Monomorphic	N/A
-	VRDLD44	P39.40	143	Further optimization required to assess	N/A
-	VRAZXR2	P41.42	128	Monomorphic	N/A
-	VREQIGY	P43.44	251	Monomorphic	N/A
-	VRCG2MT	P45.46	258	Monomorphic	N/A
-	VRDXSVZ	P47.48	108	Further optimization required to assess	N/A
+	VRB5M1O	P49.50	223	Polymorphic	Diploid acting
-	VRCQS1L	P51.52	263	Further optimization required to assess	N/A
-	VRBFM20	P53.54	113	Further optimization required to assess	N/A
+	VR_gr271_2	P55.56	230	Polymorphic	Diploid acting
-	VRB6Q8H	P57.58	275	Further optimization required to assess	N/A
-	VRC4F2A	P59.60	247	Monomorphic	N/A
-	VR_gr103_3	P61.62	204	Further optimization required to assess	N/A

Loci Selected?	Locus	Primer code	PCR Product Size	Mono/Polymorphic	Alleles
-	VRCM6UB	P63.64	258	Further optimization required to assess	N/A
-	VRA7LYT	P65.66	227	Further optimization required to assess	N/A
+	VRC00AE	P67.68	124	Polymorphic	Polyploid acting
-	VRDLD44	P69.70	299	Further optimization required to assess	N/A
-	VRCR6TK	P71.72	279	Further optimization required to assess	N/A
-	VR_gr186_2	P73.74	246	Further optimization required to assess	N/A
-	VRCG2N5	P75.76	138	Further optimization required to assess	N/A
-	VRCXMA7	P77.78	217	Further optimization required to assess	N/A
-	VR_gr230_3	P79.80	266	Monomorphic	N/A
+	VRA5NLR	P81.82	174	Polymorphic	Diploid acting
+	VR_gr27_2	P83.84	256	Polymorphic	Polyploid acting
+	VRE18LG	P85.86	158	Polymorphic	Diploid acting
-	VRDJFTL	P87.88	224	Monomorphic	N/A

Appendix 8: Microsatellite loci selections

The following table provides results for microsatellite loci selection. The table shows the microsatellite (Locus), identifier used in this study (Primer code), size of the PCR product (PCR Product Size), description of the interpreted behavior of alleles (Alleles) per locus and repeat motif (Motif) of each locus. The selection of a locus for further investigation or rejection due to an inability to interpret the alleles is indicated in the column 'Selected' using plus (+) for selected or minus (-) for rejected.

Locus	Primer code	PCR Product Size	Alleles	Motif	Selected
VRgr3_2	P1.2	287	Diploid acting	AAC	+
VRBS43W	P5.6	268	Diploid acting	AAT	+
VRBREYV	P9.10	287	Diploid acting	ACT	+
VRA7EYM	P13.14	221	Not interpretable	AAT	-
VREDKKW	P15.16	188	Not interpretable	AAT	-
VRBL3FZ	P29.30	203	Diploid acting	AG	+
VRD2DN4	P31.32	179	Polyploid acting	AC	+
VRB5M1O	P49.50	223	Diploid acting	AAT	+
VR_gr271_2	P55.56	230	Diploid acting	AAC	+
VRC00AE	P67.68	124	Not interpretable	AG	-
VRA5NLR	P81.82	174	Diploid acting	AAG	+
VR_gr27_2	P83.84	256	Polyploid acting	AC	+
VRE18LG	P85.86	158	Diploid acting	AG	+

The following table provides the alleles identified per microsatellite loci selected for genotyping using the manufacturer's identification code (Sequence ID). A list of alleles by size is recorded for each locus in the fields Allele 1, Allele 2, Allele 3, etc...

Locus	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8	Allele 9
VRgr3_2	271	274	277	285	288	291	294	297	300
VRBS43W	266	269	272	275	278	281	284	287	291
VRBREYV	274	283	286	289	292	295	298	301	304
VRA7EYM	~	~	~	~	~	~	~	~	~
VREDKKW	~	~	~	~	~	~	~	~	~
VRBL3FZ	214	216	218	220	222	224	~	~	~
VRD2DN4	188	198	200	202	208	~	~	~	~
VRB5M1O	212	215	218	221	224	227	230	232	235
VR_gr271_2	256	259	265	268	271	274	277	~	~
VRC00AE	~	~	~	~	~	~	~	~	~
VRA5NLR	208	211	214	217	220	223	226	~	~
VR_gr27_2	288	290	296	300	308	312	322	324	328
VRE18LG	172	174	~	~	~	~	~	~	~

Alleles identified per microsatellite loci selected (continued).

Locus	Allele 10	Allele 11	Allele 12	Allele 13	Allele 14	Allele 15	Allele 16	Allele 17	Allele 18
VRgr3_2	303	309	312	315	318	321	324	330	~
VRBS43W	294	297	300	319	341	344	~	~	~
VRBREYV	307	310	313	316	~	~	~	~	~
VRA7EYM	~	~	~	~	~	~	~	~	~
VREDKKW	~	~	~	~	~	~	~	~	~
VRBL3FZ	~	~	~	~	~	~	~	~	~
VRD2DN4	~	~	~	~	~	~	~	~	~
VRB5M1O	238	241	244	247	250	253	256	259	262
VR_gr271_2	~	~	~	~	~	~	~	~	~
VRC00AE	~	~	~	~	~	~	~	~	~
VRA5NLR	~	~	~	~	~	~	~	~	~
VR_gr27_2	330	~	~	~	~	~	~	~	~
VRE18LG	~	~	~	~	~	~	~	~	~

Alleles identified per microsatellite loci selected (continued).

Locus	Allele 19	Allele 20	Allele 21	Allele 22	Allele 23	Allele 24	Allele 25
VRgr3_2	~	~	~	~	~	~	~
VRBS43W	~	~	~	~	~	~	~
VRBREYV	~	~	~	~	~	~	~
VRA7EYM	~	~	~	~	~	~	~
VREDKKW	~	~	~	~	~	~	~
VRBL3FZ	~	~	~	~	~	~	~
VRD2DN4	~	~	~	~	~	~	~
VRB5M1O	268	277	280	286	292	295	298
VR_gr271_2	~	~	~	~	~	~	~
VRC00AE	~	~	~	~	~	~	~
VRA5NLR	~	~	~	~	~	~	~
VR_gr27_2	~	~	~	~	~	~	~
VRE18LG	~	~	~	~	~	~	~

Appendix 9: Private alleles detected for microsatellite loci

Private alleles were detected for ten microsatellite loci using *Varronia rupicola* analysis group C samples. Summary data is divided by 'Country', 'Island' and 'Population' for microsatellites (Locus), private alleles (Allele), and source of the samples (Origin) containing those alleles. Abbreviations: BVI = British Virgin Islands; WILD = Found in extant wild samples; CULT = Found in extant *ex-situ* samples; HS = Found in herbarium specimen samples; "*" = Lost from Puerto Rico, extant in Anegada; "^" = Only found in *ex-situ* and/or historical material; "[1]" = Source population is unknown (likely from Anegada West).

Country	Island	Population	Locus	Allele	Origin
BVI	Anegada	Anegada West	VRgr3_2	297	WILD
BVI	Anegada	Anegada West	VRgr3_2	300	WILD & CULT
BVI	Anegada	Anegada East & West	VRgr3_2	303	WILD & CULT
BVI	Anegada	Anegada West	VRgr3_2	309	WILD & CULT
BVI	Anegada	Anegada West	VRgr3_2	312^	CULT & HS
BVI	Anegada	Anegada East & West	VRgr3_2	315	WILD & CULT
BVI	Anegada	Anegada East & West	VRgr3_2	318	WILD & CULT
BVI	Anegada	Anegada East	VRgr3_2	321	WILD
BVI	Anegada	Anegada West	VRgr3_2	324	WILD
BVI	Anegada	Anegada West	VRBS43W	269	WILD
BVI	Anegada	Anegada East	VRBS43W	341	WILD
BVI	Anegada	Anegada East	VRBS43W	344	WILD
BVI	Anegada	Anegada East & West	VRBREYV	301	WILD
BVI	Anegada	Anegada East & West	VRBREYV	310	WILD & CULT
BVI	Anegada	Anegada East & West	VRBREYV	313	WILD
BVI	Anegada	Anegada West	VRBREYV	316	WILD & CULT
BVI	Anegada	*Provenance unknown*[1]	VRBL3FZ	214^	CULT
BVI	Anegada	Anegada East & West	VRB5M1O	244	WILD & CULT
BVI	Anegada	Anegada East	VRB5M1O	262*	WILD
BVI	Anegada	Anegada West	VR_gr271_2	265	WILD & CULT
BVI	Anegada	Anegada East	VR_gr271_2	277	WILD
BVI	Anegada	Anegada East & West	VRA5NLR	217	WILD & CULT
BVI	Anegada	Anegada West	VRA5NLR	223	WILD & CULT
BVI	Anegada	Anegada West	VRA5NLR	226	WILD & CULT
BVI	Anegada	Anegada West	VR_gr27_2	328	WILD & CULT
Puerto Rico	Puerto Rico	Guánica & Ponce	VRgr3_2	271	WILD & CULT
Puerto Rico	Puerto Rico	Guánica & Ponce	VRgr3_2	274	WILD & CULT
Puerto Rico	Puerto Rico	Guánica	VRgr3_2	285	WILD
Puerto Rico	Puerto Rico	Guánica	VRgr3_2	330	WILD
Puerto Rico	Puerto Rico	Guánica	VRBREYV	274	WILD & CULT
Puerto Rico	Puerto Rico	Guánica & Ponce	VRBREYV	286	WILD & CULT
Puerto Rico	Puerto Rico	Guánica & Ponce	VRBREYV	295	WILD & CULT
Puerto Rico	Puerto Rico & Vieques	Guánica & Vieques	VRB5M1O	212	WILD
Puerto Rico	Puerto Rico	Guánica	VRB5M1O	268	WILD
Puerto Rico	Puerto Rico	Ponce	VRB5M1O	277	WILD
Puerto Rico	Puerto Rico	Ponce	VRB5M1O	280	WILD

Country	Island	Population	Locus	Allele	Origin
Puerto Rico	Puerto Rico	Ponce	VRB5M1O	286	WILD
Puerto Rico	Puerto Rico	Ponce	VRB5M1O	292	WILD
Puerto Rico	Puerto Rico	Ponce	VRB5M1O	295	WILD
Puerto Rico	Puerto Rico	Ponce	VRB5M1O	298	WILD
Puerto Rico	Puerto Rico	Ponce	VR_gr271_2	259	WILD
Puerto Rico	Vieques	Vieques	VRBS43W	266	WILD
Puerto Rico	Vieques	Vieques	VRBS43W	319	WILD
Puerto Rico	Vieques	Vieques	VRA5NLR	220	WILD
Puerto Rico	Puerto Rico & Vieques	Ponce & Vieques	VR_gr271_2	274	WILD
Puerto Rico	Puerto Rico	Ponce	VRB5M1O	262*	HS

Appendix 10: Population assignment tests

Results of population assignment tests undertaken in GenAlEx for analysis group A samples using the frequency method test (Paetkau *et al.*, 1995, 2004). Results reported as log-likelihood values per sample (Sample ID) against each of five populations for analysis group A samples. The highest value indicates the most likely population and is the least negative for negative values. Population assignments following cluster analyses using Structure and Geneland software programmes are recorded under "Initial Population Assignment" per sample. Population assignment following frequency method test is recorded under "Population Assignment Post-testing" per sample. Re-assigned samples (n = 3) are highlighted using '*bold italics*'.

Sample ID	Initial Population Assignment	Population log-likelihood values					Population Assignment Post-testing
		Anegada East	Anegada West	Guánica	Ponce	Vieques	
357	Anegada East	-5.945	-9.652	-11.497	-12.187	-14.079	Anegada East
358	Anegada East	-5.311	-7.820	-7.473	-8.820	-9.362	Anegada East
359	Anegada East	-7.555	-12.732	-17.284	-17.354	-22.158	Anegada East
360	Anegada East	-6.661	-11.048	-14.749	-15.689	-20.079	Anegada East
361	Anegada East	-7.183	-8.509	-16.695	-15.795	-19.903	Anegada East
362	Anegada East	-10.236	-14.813	-13.316	-16.112	-19.556	Anegada East
363	Anegada East	-6.294	-10.062	-15.150	-13.902	-15.857	Anegada East
364	Anegada East	-8.293	-15.657	-12.978	-13.286	-13.334	Anegada East
366	Anegada East	-5.122	-10.040	-11.826	-13.371	-10.028	Anegada East
367	Anegada East	-5.757	-9.451	-12.508	-12.403	-13.362	Anegada East
368	Anegada East	-11.442	-12.410	-12.479	-18.057	-24.459	Anegada East
411	Anegada East	-5.914	-9.456	-13.991	-15.170	-14.158	Anegada East
412	Anegada East	-6.448	-8.813	-15.447	-14.791	-16.158	Anegada East
413	Anegada East	-8.969	-12.199	-14.429	-18.023	-19.653	Anegada East
414	Anegada East	-7.307	-12.610	-13.691	-14.474	-12.760	Anegada East
415	Anegada East	-6.320	-7.837	-13.887	-14.251	-16.079	Anegada East
416	Anegada East	-4.930	-6.014	-10.517	-9.021	-14.459	Anegada East
417	Anegada East	-6.408	-7.418	-11.614	-11.205	-14.760	Anegada East
418	Anegada East	-8.353	-12.452	-17.897	-16.439	-20.000	Anegada East
419	Anegada East	-8.388	-15.060	-18.353	-19.310	-17.778	Anegada East
453	Anegada East	-7.176	-11.023	-13.157	-15.231	-13.806	Anegada East
454	Anegada East	-7.129	-9.531	-13.835	-14.405	-14.584	Anegada East
455	Anegada East	-8.936	-12.384	-14.590	-16.802	-19.556	Anegada East
456	Anegada East	-7.655	-12.823	-19.208	-19.491	-22.477	Anegada East
457	Anegada East	-7.460	-13.146	-16.267	-17.768	-21.699	Anegada East
5	Anegada West	-14.253	-9.582	-16.730	-22.386	-24.176	Anegada West
6	Anegada West	-11.646	-8.679	-11.477	-15.204	-24.000	Anegada West
311	Anegada West	-13.777	-10.021	-14.245	-14.075	-23.699	Anegada West
312	Anegada West	-9.918	-8.408	-16.030	-15.747	-20.760	Anegada West
313	Anegada West	-11.941	-8.845	-15.292	-17.633	-19.903	Anegada West
314	Anegada West	-11.958	-8.558	-14.841	-15.412	-19.778	Anegada West
315	Anegada West	-8.177	-6.015	-11.074	-12.248	-18.079	Anegada West
316	Anegada West	-10.162	-5.345	-12.206	-12.705	-15.857	Anegada West
317	Anegada West	-7.958	-5.035	-11.614	-10.847	-15.477	Anegada West
318	Anegada West	-11.659	-8.150	-12.335	-12.889	-17.556	Anegada West
319	Anegada West	-13.015	-7.984	-18.488	-16.448	-19.255	Anegada West
320	Anegada West	-9.894	-5.748	-14.723	-14.810	-21.477	Anegada West
321	Anegada West	-8.868	-5.611	-12.430	-12.496	-21.778	Anegada West
322	Anegada West	-7.650	-6.817	-13.594	-14.115	-19.778	Anegada West
323	Anegada West	-10.414	-5.855	-11.509	-13.048	-15.778	Anegada West
324	Anegada West	-6.707	-5.792	-15.295	-13.950	-19.778	Anegada West
325	Anegada West	-11.600	-8.350	-13.905	-13.120	-19.097	Anegada West

Sample ID	Initial Population Assignment	Population log-likelihood values					Population Assignment Post-testing
		Anegada East	Anegada West	Guánica	Ponce	Vieques	
326	Anegada West	-10.953	-6.258	-18.966	-15.565	-21.061	Anegada West
327	Anegada West	-9.769	-7.917	-13.814	-14.238	-20.574	Anegada West
328	Anegada West	-9.146	-7.263	-13.314	-15.335	-17.556	Anegada West
329	Anegada West	-10.343	-8.192	-14.848	-15.015	-22.079	Anegada West
330	Anegada West	-16.564	-10.266	-15.924	-12.569	-11.113	Anegada West
331	Anegada West	-8.924	-5.815	-13.619	-13.241	-19.857	Anegada West
332	Anegada West	-13.138	-6.903	-17.072	-17.501	-17.857	Anegada West
333	Anegada West	-10.415	-6.099	-13.425	-15.356	-19.556	Anegada West
334	Anegada West	-10.534	-6.832	-12.326	-11.305	-17.556	Anegada West
335	Anegada West	-8.260	-6.407	-12.373	-12.674	-20.760	Anegada West
336	Anegada West	-10.140	-6.486	-14.845	-11.162	-20.158	Anegada West
337	Anegada West	-10.461	-9.249	-14.775	-12.890	-21.778	Anegada West
338	Anegada West	-10.297	-6.761	-14.648	-12.960	-19.477	Anegada West
339	Anegada West	-8.936	-6.780	-14.939	-10.238	-18.158	Anegada West
340	Anegada West	-11.577	-9.001	-20.771	-19.639	-22.158	Anegada West
341	Anegada West	-8.434	-6.580	-15.420	-15.765	-21.778	Anegada West
342	Anegada West	-12.498	-9.506	-15.116	-15.217	-21.875	Anegada West
343	Anegada West	-10.687	-6.374	-14.145	-13.687	-14.681	Anegada West
344	Anegada West	-9.250	-6.823	-13.726	-11.574	-17.255	Anegada West
345	Anegada West	-9.708	-6.817	-12.383	-9.542	-14.158	Anegada West
346	Anegada West	-10.499	-5.454	-13.205	-14.029	-16.158	Anegada West
347	Anegada West	-11.673	-6.375	-13.954	-9.516	-20.158	Anegada West
348	Anegada West	-10.024	-6.095	-15.360	-17.063	-21.176	Anegada West
349	Anegada West	-10.866	-7.084	-10.908	-9.903	-16.158	Anegada West
350	Anegada West	-14.315	-8.406	-15.275	-16.119	-17.857	Anegada West
351	Anegada West	-10.611	-8.090	-15.623	-13.593	-19.556	Anegada West
352	Anegada West	-10.655	-6.348	-16.141	-17.598	-22.079	Anegada West
354	Anegada West	-11.382	-7.392	-14.955	-17.780	-22.380	Anegada West
356	Anegada West	-16.014	-10.745	-19.949	-22.367	-22.033	Anegada West
369	Anegada West	-11.636	-8.975	-16.025	-10.607	-24.158	Anegada West
370	Anegada West	-8.561	-7.590	-12.795	-15.215	-20.760	Anegada West
371	Anegada West	-12.893	-11.079	-17.362	-19.632	-22.875	Anegada West
372	Anegada West	-13.639	-9.386	-13.246	-13.696	-21.398	Anegada West
373	Anegada West	-10.894	-6.204	-8.293	-6.705	-20.301	Anegada West
374	Anegada West	-9.780	-6.407	-7.755	-7.239	-16.760	Anegada West
375	Anegada West	-12.620	-9.988	-12.137	-13.646	-17.778	Anegada West
377	Anegada West	-9.884	-7.654	-12.456	-10.817	-19.398	Anegada West
378	Anegada West	-13.743	-10.508	-15.857	-10.457	-20.602	***Ponce***
379	Anegada West	-8.234	-5.765	-10.910	-8.655	-19.778	Anegada West
380	Anegada West	-8.896	-8.052	-16.200	-15.629	-17.857	Anegada West
381	Anegada West	-9.971	-7.610	-16.653	-15.645	-19.857	Anegada West
382	Anegada West	-18.008	-9.602	-15.971	-17.432	-25.699	Anegada West
383	Anegada West	-12.853	-7.432	-15.052	-14.602	-19.255	Anegada West
384	Anegada West	-10.744	-7.393	-12.071	-11.477	-17.255	Anegada West
385	Anegada West	-11.155	-10.027	-13.769	-16.117	-21.477	Anegada West
386	Anegada West	-10.980	-10.626	-15.647	-20.008	-23.857	Anegada West
387	Anegada West	-14.104	-11.935	-13.222	-13.834	-20.574	Anegada West
388	Anegada West	-14.798	-10.467	-18.274	-14.850	-21.699	Anegada West
389	Anegada West	-13.845	-10.535	-15.883	-15.936	-25.097	Anegada West
390	Anegada West	-14.284	-8.329	-15.234	-13.845	-24.796	Anegada West
392	Anegada West	-15.179	-10.082	-16.411	-18.193	-22.796	Anegada West
393	Anegada West	-16.630	-12.091	-19.204	-18.272	-25.778	Anegada West
395	Anegada West	-11.754	-8.314	-14.212	-15.891	-24.301	Anegada West

Sample ID	Initial Population Assignment	Population log-likelihood values					Population Assignment Post-testing
		Anegada East	Anegada West	Guánica	Ponce	Vieques	
396	Anegada West	-9.116	-5.919	-9.542	-9.698	-17.699	Anegada West
397	Anegada West	-9.272	-9.088	-17.732	-17.728	-19.857	Anegada West
398	Anegada West	-16.707	-14.148	-18.268	-16.045	-21.352	Anegada West
399	Anegada West	-11.832	-10.092	-16.926	-16.793	-16.760	Anegada West
400	Anegada West	-10.112	-7.584	-19.784	-15.450	-24.459	Anegada West
401	Anegada West	-12.392	-8.878	-17.081	-14.943	-23.477	Anegada West
402	Anegada West	-11.110	-9.483	-15.465	-15.046	-20.574	Anegada West
403	Anegada West	-12.532	-10.942	-17.111	-18.225	-24.158	Anegada West
404	Anegada West	-14.685	-6.919	-14.995	-15.930	-23.699	Anegada West
405	Anegada West	-8.990	-7.607	-18.105	-17.608	-25.778	Anegada West
406	Anegada West	-13.260	-8.466	-13.402	-12.938	-25.398	Anegada West
407	Anegada West	-14.389	-7.262	-15.217	-15.658	-22.380	Anegada West
408	Anegada West	-11.968	-7.846	-13.545	-14.055	-17.857	Anegada West
409	Anegada West	-12.004	-7.781	-10.331	-13.474	-23.699	Anegada West
410	Anegada West	-13.305	-7.791	-16.211	-16.146	-24.301	Anegada West
420	Anegada West	-12.584	-6.072	-11.459	-9.238	-18.380	Anegada West
421	Anegada West	-13.417	-6.643	-11.860	-10.198	-20.602	Anegada West
422	Anegada West	-13.938	-6.905	-15.626	-16.275	-21.699	Anegada West
423	Anegada West	-8.848	-6.482	-12.260	-13.697	-20.574	Anegada West
424	Anegada West	-7.018	-5.747	-7.070	-8.301	-13.778	Anegada West
425	Anegada West	-6.582	-5.659	-14.135	-12.299	-20.079	Anegada West
426	Anegada West	-13.704	-7.024	-10.448	-15.045	-18.079	Anegada West
427	Anegada West	-10.646	-6.455	-15.056	-12.247	-21.176	Anegada West
428	Anegada West	-10.100	-5.969	-14.672	-11.077	-20.158	Anegada West
429	Anegada West	-10.446	-6.078	-12.925	-12.887	-13.954	Anegada West
430	Anegada West	-6.565	-4.697	-9.042	-7.241	-16.459	Anegada West
431	Anegada West	-10.547	-7.882	-13.092	-13.012	-19.523	Anegada West
432	Anegada West	-8.740	-6.947	-8.725	-9.319	-11.556	Anegada West
433	Anegada West	-12.705	-8.495	-16.683	-15.440	-19.857	Anegada West
434	Anegada West	-8.362	-6.770	-14.292	-13.927	-18.033	Anegada West
435	Anegada West	-8.629	-6.074	-15.752	-13.960	-18.158	Anegada West
436	Anegada West	-8.404	-5.648	-11.676	-12.819	-19.176	Anegada West
437	Anegada West	-13.162	-7.459	-14.364	-14.324	-20.158	Anegada West
438	Anegada West	-7.299	-5.521	-11.175	-12.554	-14.158	Anegada West
439	Anegada West	-13.389	-10.337	-16.349	-19.111	-17.255	Anegada West
440	Anegada West	-11.264	-7.021	-16.921	-12.295	-20.158	Anegada West
441	Anegada West	-11.753	-8.451	-11.504	-13.640	-21.398	Anegada West
442	Anegada West	-13.793	-8.509	-15.616	-15.228	-22.000	Anegada West
443	Anegada West	-8.280	-7.043	-15.107	-16.714	-18.079	Anegada West
444	Anegada West	-9.344	-6.790	-10.372	-6.296	-12.459	***Ponce***
445	Anegada West	-12.099	-6.909	-15.084	-13.506	-20.760	Anegada West
446	Anegada West	-11.582	-6.565	-13.079	-11.224	-19.778	Anegada West
447	Anegada West	-9.010	-11.020	-9.394	-10.534	-13.010	***Anegada East***
448	Anegada West	-9.794	-8.330	-15.025	-14.762	-20.334	Anegada West
449	Anegada West	-12.193	-8.929	-16.178	-13.493	-23.255	Anegada West
450	Anegada West	-9.246	-7.189	-17.041	-12.640	-20.875	Anegada West
451	Anegada West	-12.054	-9.859	-15.087	-15.725	-22.875	Anegada West
452	Anegada West	-8.146	-7.910	-11.719	-12.744	-17.556	Anegada West
530	Anegada West	-13.521	-8.675	-14.241	-13.691	-24.000	Anegada West
80	Guánica	-13.675	-13.336	-10.248	-14.690	-19.380	Guánica
89	Guánica	-13.907	-16.883	-6.666	-12.903	-17.937	Guánica
90	Guánica	-14.204	-11.528	-8.430	-8.494	-19.903	Guánica
91	Guánica	-14.037	-14.244	-6.467	-10.351	-22.000	Guánica

Sample ID	Initial Population Assignment	Population log-likelihood values					Population Assignment Post-testing
		Anegada East	Anegada West	Guánica	Ponce	Vieques	
92	Guánica	-14.400	-18.741	-5.807	-14.594	-17.044	Guánica
93	Guánica	-16.722	-16.741	-8.541	-13.776	-23.699	Guánica
94	Guánica	-12.663	-13.896	-5.498	-13.782	-17.727	Guánica
95	Guánica	-11.565	-16.935	-5.526	-12.590	-11.771	Guánica
96	Guánica	-14.097	-16.881	-5.055	-14.338	-20.551	Guánica
97	Guánica	-12.026	-14.289	-5.833	-12.734	-17.949	Guánica
98	Guánica	-9.034	-11.295	-6.523	-12.051	-14.584	Guánica
99	Guánica	-17.536	-18.725	-6.591	-13.182	-21.505	Guánica
100	Guánica	-13.782	-14.564	-7.530	-11.570	-18.255	Guánica
132	Guánica	-16.010	-15.450	-7.285	-7.317	-22.982	Guánica
133	Guánica	-12.541	-16.540	-7.378	-13.507	-19.061	Guánica
134	Guánica	-12.460	-11.229	-7.733	-12.936	-19.857	Guánica
135	Guánica	-11.991	-11.992	-6.824	-9.608	-17.556	Guánica
136	Guánica	-11.830	-14.893	-5.085	-9.392	-15.061	Guánica
140	Guánica	-11.830	-13.939	-5.200	-9.994	-16.283	Guánica
141	Guánica	-14.563	-16.793	-6.701	-12.881	-20.857	Guánica
142	Guánica	-11.529	-13.551	-5.453	-9.091	-15.982	Guánica
143	Guánica	-11.796	-12.908	-5.309	-8.254	-16.505	Guánica
144	Guánica	-12.061	-14.048	-4.801	-10.356	-17.602	Guánica
145	Guánica	-13.730	-14.957	-6.107	-10.224	-16.125	Guánica
146	Guánica	-13.392	-15.409	-5.158	-11.419	-16.329	Guánica
147	Guánica	-13.392	-15.409	-5.158	-11.419	-16.329	Guánica
148	Guánica	-11.888	-13.585	-6.310	-7.911	-16.635	Guánica
149	Guánica	-14.841	-17.333	-5.771	-12.335	-20.459	Guánica
184	Guánica	-14.736	-13.795	-8.074	-10.532	-24.903	Guánica
185	Guánica	-11.675	-10.966	-7.264	-9.943	-19.176	Guánica
186	Guánica	-9.886	-12.171	-9.811	-16.489	-17.061	Guánica
187	Guánica	-12.327	-14.310	-5.794	-9.196	-17.204	Guánica
188	Guánica	-11.301	-13.692	-6.004	-10.370	-17.158	Guánica
189	Guánica	-13.239	-12.586	-6.380	-8.941	-18.158	Guánica
190	Guánica	-13.709	-12.733	-7.677	-12.642	-20.857	Guánica
191	Guánica	-12.010	-13.364	-6.450	-11.161	-17.903	Guánica
192	Guánica	-13.877	-13.800	-6.252	-11.535	-19.857	Guánica
193	Guánica	-11.469	-13.881	-4.958	-12.235	-16.283	Guánica
194	Guánica	-12.034	-14.439	-5.316	-12.380	-17.982	Guánica
195	Guánica	-11.673	-14.042	-7.138	-13.290	-18.158	Guánica
196	Guánica	-11.469	-13.159	-7.120	-7.848	-16.937	Guánica
197	Guánica	-10.857	-13.446	-4.744	-11.714	-15.727	Guánica
198	Guánica	-12.372	-14.341	-5.564	-11.271	-16.635	Guánica
199	Guánica	-12.372	-14.341	-5.564	-11.271	-16.635	Guánica
200	Guánica	-11.592	-13.972	-5.556	-14.487	-19.824	Guánica
201	Guánica	-7.611	-9.986	-7.342	-9.884	-12.238	Guánica
202	Guánica	-17.746	-19.271	-5.815	-14.284	-23.061	Guánica
203	Guánica	-13.053	-15.480	-7.378	-12.269	-18.477	Guánica
204	Guánica	-12.071	-12.785	-5.609	-10.743	-17.539	Guánica
205	Guánica	-13.744	-15.811	-7.416	-13.032	-20.158	Guánica
206	Guánica	-13.443	-16.201	-7.621	-13.974	-20.760	Guánica
207	Guánica	-16.683	-17.390	-5.483	-11.791	-19.840	Guánica
208	Guánica	-11.469	-13.343	-6.492	-12.980	-16.937	Guánica
209	Guánica	-8.877	-13.835	-5.070	-11.113	-12.919	Guánica
210	Guánica	-11.097	-13.092	-6.678	-9.982	-18.556	Guánica
232	Guánica	-14.604	-13.121	-10.400	-10.486	-21.556	Guánica
233	Guánica	-10.505	-12.173	-7.277	-11.871	-15.982	Guánica

Sample ID	Initial Population Assignment	Population log-likelihood values					Population Assignment Post-testing
		Anegada East	Anegada West	Guánica	Ponce	Vieques	
234	Guánica	-12.578	-15.569	-7.431	-14.223	-13.311	Guánica
235	Guánica	-15.657	-15.157	-7.193	-15.613	-22.602	Guánica
236	Guánica	-14.740	-13.499	-6.074	-13.916	-20.903	Guánica
237	Guánica	-14.831	-12.665	-7.209	-12.755	-18.903	Guánica
238	Guánica	-15.657	-15.157	-7.193	-15.613	-22.602	Guánica
239	Guánica	-15.657	-15.157	-7.193	-15.613	-22.602	Guánica
282	Guánica	-16.843	-17.488	-7.716	-14.278	-23.681	Guánica
283	Guánica	-15.366	-18.603	-8.180	-17.647	-22.079	Guánica
284	Guánica	-14.877	-13.891	-9.334	-10.875	-21.061	Guánica
285	Guánica	-13.833	-12.221	-5.420	-10.310	-20.602	Guánica
286	Guánica	-14.369	-15.308	-9.277	-11.099	-21.954	Guánica
287	Guánica	-16.122	-15.059	-10.729	-15.586	-23.824	Guánica
288	Guánica	-14.505	-15.897	-5.507	-12.030	-18.681	Guánica
289	Guánica	-15.037	-16.800	-5.778	-12.326	-21.454	Guánica
299	Guánica	-16.878	-15.513	-14.033	-15.478	-21.255	Guánica
243	Ponce	-11.991	-10.236	-8.970	-3.758	-16.760	Ponce
244	Ponce	-14.572	-14.761	-10.153	-6.987	-18.477	Ponce
245	Ponce	-11.523	-10.093	-7.758	-5.358	-18.079	Ponce
246	Ponce	-14.532	-11.545	-10.533	-4.139	-19.380	Ponce
247	Ponce	-13.292	-13.374	-10.042	-6.295	-18.158	Ponce
248	Ponce	-12.699	-11.611	-7.172	-4.676	-18.079	Ponce
249	Ponce	-14.000	-11.276	-10.433	-3.481	-17.459	Ponce
250	Ponce	-17.031	-15.654	-9.333	-5.582	-19.079	Ponce
251	Ponce	-15.833	-13.451	-13.930	-3.914	-19.556	Ponce
252	Ponce	-16.435	-15.242	-12.737	-4.425	-19.556	Ponce
253	Ponce	-17.037	-16.900	-12.304	-4.766	-19.556	Ponce
254	Ponce	-16.736	-15.934	-14.789	-4.927	-21.255	Ponce
255	Ponce	-15.833	-13.854	-12.103	-4.993	-19.255	Ponce
256	Ponce	-16.134	-13.228	-13.240	-3.843	-18.459	Ponce
257	Ponce	-14.134	-12.065	-10.159	-6.003	-20.477	Ponce
258	Ponce	-14.806	-13.309	-9.095	-4.991	-18.857	Ponce
259	Ponce	-12.372	-11.655	-8.922	-4.924	-17.857	Ponce
260	Ponce	-15.871	-13.782	-8.722	-6.825	-18.459	Ponce
261	Ponce	-15.204	-14.840	-8.176	-5.580	-16.238	Ponce
262	Ponce	-18.332	-16.183	-10.851	-6.660	-19.255	Ponce
263	Ponce	-12.196	-12.471	-9.182	-5.587	-18.158	Ponce
264	Ponce	-14.657	-12.807	-12.975	-5.779	-18.556	Ponce
265	Ponce	-11.228	-11.883	-8.258	-7.083	-15.238	Ponce
266	Ponce	-14.134	-11.814	-9.249	-4.256	-19.079	Ponce
267	Ponce	-14.099	-13.355	-8.621	-5.238	-18.760	Ponce
268	Ponce	-15.178	-13.619	-9.447	-5.861	-20.760	Ponce
270	Ponce	-11.866	-10.170	-9.404	-6.290	-20.158	Ponce
271	Ponce	-12.469	-12.142	-10.822	-7.185	-18.459	Ponce
272	Ponce	-12.469	-9.483	-9.337	-4.631	-20.459	Ponce
273	Ponce	-12.928	-8.942	-9.456	-4.635	-20.158	Ponce
274	Ponce	-14.065	-11.672	-10.174	-6.522	-18.937	Ponce
275	Ponce	-12.469	-9.483	-9.337	-4.631	-20.459	Ponce
276	Ponce	-13.731	-13.606	-8.415	-6.060	-19.238	Ponce
277	Ponce	-14.275	-13.311	-8.644	-6.181	-18.158	Ponce
278	Ponce	-17.037	-15.667	-13.225	-7.683	-20.602	Ponce
279	Ponce	-14.736	-12.937	-10.233	-5.899	-20.903	Ponce
280	Ponce	-12.469	-11.727	-8.565	-4.877	-16.459	Ponce
281	Ponce	-17.552	-17.853	-15.499	-10.803	-17.988	Ponce

Sample ID	Initial Population Assignment	Population log-likelihood values					Population Assignment Post-testing
		Anegada East	Anegada West	Guánica	Ponce	Vieques	
7	Vieques	-19.244	-19.708	-21.062	-19.254	-4.396	Vieques
8	Vieques	-16.536	-17.699	-16.656	-18.922	-2.317	Vieques
9	Vieques	-19.661	-20.956	-20.232	-18.295	-5.225	Vieques
10	Vieques	-13.526	-17.017	-16.656	-18.922	-1.965	Vieques
12	Vieques	-13.253	-15.484	-14.665	-17.028	-2.141	Vieques
305	Vieques	-12.049	-15.444	-14.966	-17.329	-2.266	Vieques