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Role of sexual determinism in the genetic structure and diversity in bryophytes: a model based on two sister taxa in the genus *Radula*.

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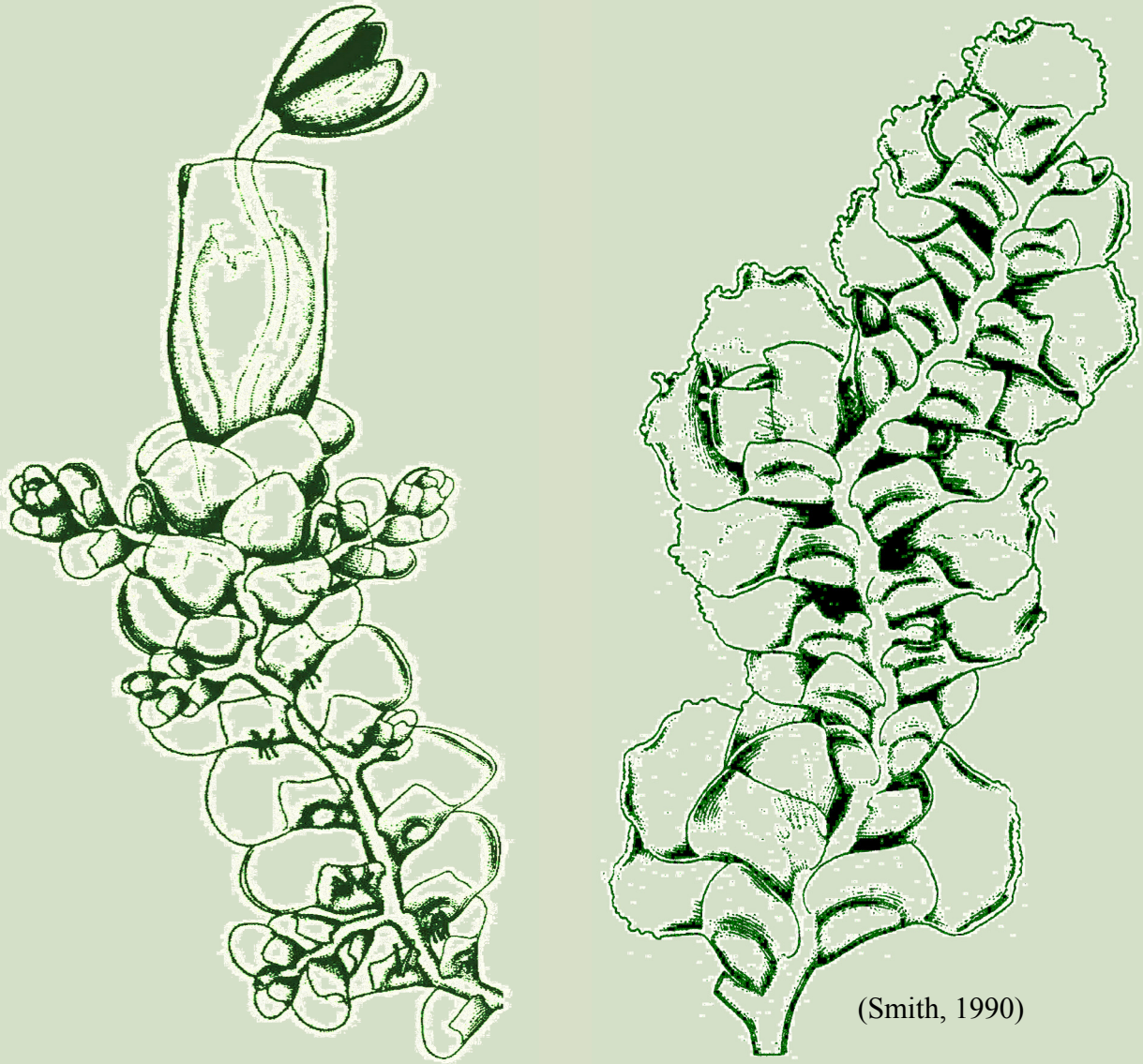

(Smith, 1990)

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Résumé

La capacité d'une espèce à se disperser est un des facteurs majeurs qui détermine son évolution. En effet, il existe un lien entre la dispersabilité d'une espèce et la structure génétique des populations. Les bryophytes, un ensemble de lignées paraphylétiques à la base des plantes terrestres, représentent un modèle de choix pour l'étude de la dispersion car on retrouve parmi ce groupe un très grand nombre de stratégies de dispersion diversifiées inégalé chez les ptéridophytes, les gymnospermes et les angiospermes. Afin d'étudier l'impact du système de reproduction sur la structuration génétique des populations, nous avons utilisé deux espèces sœurs d'hépatique du genre *Radula*, *R. complanata* et *R. lindenbergiana*. Ces deux espèces ne se distinguent que par le système de reproduction, l'une étant monoïque et l'autre dioïque.

Plus précisément, l'impact du syndrome de dispersabilité a été étudié pour revisiter l'une des hypothèses majeures de la biogéographie insulaire, à savoir le caractère relictuel de la flore de Macaronésie. La Macaronésie se compose de l'archipel de Madère, des Canaries, des Açores et du Cap Vert. Cette région floristique est caractérisée par un taux d'endémisme et des radiations spectaculaires dans la flore angiospermique, qui contrastent avec un endémisme qui compte parmi les plus bas en comparaison des autres biota et une absence totale de radiation chez les bryophytes. Trois hypothèses peuvent être avancées pour expliquer ces caractéristiques :

(i) des phénomènes de radiation endémique existent mais ne sont pas suivis d'une différentiation morphologique selon un processus de spéciation cryptique décrit de manière croissant parmi les organismes à morphologie réduite comme les bryophytes ;
(ii) les bryophytes sont des organismes très anciens pourvus d'un potentiel évolutif très amoindri ;
(iii) la très forte dispersabilité des bryophytes entraîne des flux de gènes importants entre les îles et les continents voisins, limitant toute possibilité d'isolement génétique et de spéciation endémique.

Ces trois hypothèses sont revisitées dans un contexte moléculaire sur base d'un échantillonnage couvrant l'entièreté de l'aire de distribution des deux espèces modèles et en utilisant quatre loci chloroplastiques pour inférer leur histoire évolutive à l'aide de statistiques liées à la génétique des populations, à la phylogéographie et à la phylogénie.

Deux différences majeures en termes de diversité et de structuration génétiques ont été mises en évidence entre les deux espèces modèles. Premièrement, aucun signal phylogéographique n'a été décelé chez l'espèce monoïque, indiquant de ce fait que la migration joue un rôle bien supérieur à la mutation dans l'établissement des patterns de distributions génétiques. Au contraire, un signal phylogéographique significatif a pu être mis en évidence dans la variation génétique observée chez *R. lindenbergiana*. Cette différence dans les patrons phylogéographiques de ces deux espèces souligne le rôle prépondérant des spores dans la dispersion à longue distance et la connectivité génétique entre des populations disjointes à l'échelle des continents.

Deuxièmement, la diversité génétique est relativement inférieure chez le taxon monoïque, *R. complanata*, indiquant une histoire évolutive plus récente que *R. lindenbergiana* et une probable diminution importante d'effectif suite aux glaciations quaternaires. Un refuge unique a été identifié en République Tchèque sur base d'une diversité haplotypique supérieure

à celle des autres régions. Par comparaison, *R. lindenbergiana* exhibe une diversité génétique beaucoup plus importante que *R. complanata*. En effet, trois régions ont été identifiées comme des 'hot spots' de diversité génétique à partir desquels la recolonisation post-glaciaire a pu s'opérer.

Parmi les trois régions identifiées comme « hot-spots » de diversité génétique, la Macaronésie se distingue comme la région présentant la diversité la plus élevée et un fort taux d'endémisme haplotypique provenant de deux radiations distinctes. Ce résultat indique que *R. lindenbergiana* se comporte comme un réseau d'espèces cryptiques qui ont subi une radiation imperceptible au niveau morphologique mais soutenue par l'analyse moléculaire. Il offre une explication pour les différences spectaculaires de taux d'endémisme entre la flore angiospermique et bryophytique. Cette radiation peut être attribuée aux nombreuses niches occupées par *R. lindenbergiana* sur les îles, ainsi qu'au dynamisme insulaire propre à ces systèmes qui sont autant de fenêtres d'opportunité pour la diversification d'espèces aussi peu compétitives.

Une méthode récente utilisant les inférences bayesiennes pour reconstruire l'aire de distribution ancestrale d'une espèce a montré que *R. lindenbergiana* était initialement présente en Europe. A partir de ce pool de diversité génétique européen, les résultats suggèrent que l'espèce a colonisé, en parfait accord avec la théorie relictualiste d'Engler, la Macaronésie. L'origine macaronésienne de tous les haplotypes ouest européens qui en sont dérivés suggère de manière non équivoque que les îles de Macaronésie ont servi de refuge lors des cycles glaciaires-interglaciaires du Quaternaire et sont devenus un puit de diversité pour la recolonisation du continent. A l'opposé des théories communément admises selon lesquelles l'évolution de la flore macaronésienne a été totalement découplée de la flore européenne et Nord-africaine pour produire les patrons spectaculaires de biodiversité endémique observés aujourd'hui, le rôle de ces archipels comme refuges glaciaires à partir desquels la recolonisation post-glaciaire de l'Europe a pu être possible ouvre de nouvelles perspectives sur la signification évolutive des îles atlantiques dans la diversification des espèces en Europe.

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Introduction

The dispersal ability of organisms is one of the major factors of evolution. (Gonzalez et al., 1998, Dieckmann et al., 1999, Bohrer et al., 2005). At a fine scale, dispersal limitation plays an important role in the patterning of genetic variation in populations (Baker, 1955, Stebbins, 1957, Holsinger, 2000, Snall et al., 2004). The potential that an organism has to move to a suitable habitat and survive is directly linked to the probability of reaching this habitat and being able to reproduce.

Dispersability is directly linked to diaspores production, either through sexual reproduction with seeds in flowering plants and spores in cryptogams or through asexual reproduction. Seeds and spores are essential for long distance dispersal as they can travel far from the plant with the wind, carried by animals or even transported by water. The evolution of different reproductive strategies is central to the question of species dispersal and evolution of genetic variation.

As a consequence, the mode of reproduction has been one of the major foci in modern biology, and evolution of mating system in plants has been extensively studied (Barrett and Harder, 1996). In plants, two distinct mating systems with, however, many intermediate situations, can be distinguished (Holsinger, 1996). Monoicous plants, exhibit a mode of reproduction wherein both male and female gametangia are produced by the same individual, thereby enhancing the probability of fertilization. On the contrary, in dioicous species, sexes are separated and the probability of reproducing is low because they have to move to find a mate. Dioicous species thus often fail to reproduce sexually. In a study on the reproductive biology of British mosses, Longton (1997) found that 87% of the species, wherein sporophytes are unknown, are dioicous, whereas sporophytes are regarded as occasional to common in 83% of the monoicous species. Fertilization indeed involves that the sperm swims to the archegonia, a process that may be facilitated by micro-arthropods (Cronberg *et al.*, 2006). Fertilization ranges are extremely limited. Antherozoids are capable of swimming as much as 1-2 m at a speed of 100-200 $\mu m\ s^{-1}$. The greatest distance between fruiting plants and the nearest male inflorescence has been recorded at a maximum of 3.8 m in the moss, *Dawsonia superba*. However, fertilization ranges are generally much shorter. Rydgren (2006) found, for example, that 85% of the female shoots with sporophytes were situated within a distance of 5 cm from the nearest male and the longest distance was 11.6 cm

The mode of reproduction is a trade-off between mating success and genetic diversity. Darwin (1876) was the first to propose that selfers posses an advantage over outcrossers because self-fertilization is ensured (Jain, 1976). In outcrossers by contrast, the likelihood of fertilization is much lower than in monoicous plants, but fertilization when it occurs will increases genetic diversity in the outcrossing population. As a consequence, selfers reproduce rapidly but the counterpart of the trade-off associated with mating system is that monoicous species suffer from inbreeding depression (Lande and Schemske, 1985, Charlesworth and Charlesworth, 1990, Charlesworth et al., 1990), while dioicous plants balance the cost of outcrossing by the production of rapid genetic diversity within populations.

Bryophytes, which are a paraphyletic assemblage comprised of the liverworts, mosses, and hornworts, provide ideal biological models for the study of the evolution of mating systems and dispersal mechanisms for two main reasons. First, bryophytes were among the first land plants to make the transition from an aquatic to a terrestrial environment. They are still, however, dependent on water availability for their reproduction (Schuster, 1983-1984)

because the sperm has to swim in an aqueous environment in order to reach the ovule. Second, they exhibit a wide range of reproductive strategies. In bryophytes, and as opposed to seed plants, the gametophyte is dominant and the sporophyte grows on the gametophyte and is parasite in liverworts (Vanderpoorten and Goffinet, 2009). The presence of a dominant gametophytic phase modifies the terminology of monoicous and dioicous described in angiosperms. In bryophyte in fact, monoecy refers to plants wherein male and female gametangia are present on one gametophyte, whereas in angiosperms, the plant itself is the sporophyte.

Finally, in addition to the sexual reproduction mode, bryophytes have developed a variety of mechanisms for production of asexual propagules that is unparalleled among land plants (Duckett and Ligrone, 1992, Schuster, 1983-1984, During, 2007, Shaw and Beer, 1999). However, asexual reproduction only allow for clonal dispersion of the plant.

The production of small spores that are supposedly able to travel across very long distances (Wyatt, 1977, Miles and Longton, 1992, Stoneburner et al., 1992, Van Zanten, 1978) and the production of asexual propagules suggest that bryophytes display high dispersal ability. However, production of spores is rare in bryophyte. Two third of the taxa are indeed unisexual (Schuster, 1983-1984), thereby limiting sexual reproduction. As a consequence, biogeographic theories for bryophyte have traditionally left out the idea of dispersal to explain present distribution patterns. At the species level in fact, bryophytes show much broader distributions than vascular plants (Frahm and Vitt, 1993), and are comparable in distribution to families in angiosperm. In addition, the level of morphological differentiation among bryophyte species is relatively lower than in angiosperm (Shaw, 2001). These arguments have been used to hypothesize that bryophytes have a low evolutionary potential and that their distributions are primarily due to ancient continental drift and past climatic change (Schofield and Crum, 1972).

A reappraisal of dispersal mechanisms in bryophyte biogeography has only arisen recently. Munoz et al. (Munoz *et al.*, 2004) demonstrated for instance a correlation between species distributions and wind connectivity between disjunct islands in the southern hemisphere. Furthermore indirect measurement of long distance dispersal through the use of genetic markers (Snall *et al.*, 2003) shows that dispersal could play a major role in bryophyte distribution (Korpelainen *et al.*, 2005). Unfortunately, direct measures of long distance dispersal are difficult to obtain in experimental studies. In fact, the dispersal ability in those studies are inferred through correlation with wind connectivity (Munoz *et al.*, 2004) or observation of spore viability in cold and dry condition found in the stratosphere (Van Zanten, 1978), but are always indirect measurement of dispersal. The only surveys about propagule survival (Miller and Ambrose, 1976) and diaspore dispersal were made on a small scale (Kimmerer, 1991, Miles and Longton, 1992).

This duality between the “vicariantist” and “dispersalist” theories is not restricted to bryophyte but is part of a much greater debate in evolutionary biology which began with the publication of ‘On the origin of the species’. Darwin said “ the view of each species having been produced in one area alone, and having subsequently migrated from that area as far as its power of migration and subsistence under past and present conditions permitted, is the most probable.” (Darwin, 1859). Darwin was not aware that continents were in movement and believed in a theory afterwards referred to as dispersalist (Humphries and Parenti, 1999). In the 1960s, the reappraisal of Wegener’s plate tectonic theory offered an extraordinary explanation for the disjunct patterns observed in some plants and animals. The current

distributions of biota on earth were the result of land mass separations followed by speciation events due to isolation of populations (Cecca, 2009). This phenomenon, named ancient vicariance, is defined as barriers appearing in a population and creating isolated sub-populations. By a stochastic manner, dispersal events were thought to overwrite patterns established by continental drift. Thus, vicariantists argued that any pattern could be explained by dispersion; as a consequence no pattern at all could be proved to be due to dispersal. Therefore, dispersal was considered as irrelevant noise and observed patterns were best explained by drift (McGlone, 2005).

Recently, the increasing availability of molecular data gave the possibility to revisit those ancient biogeographical questions. In particular, advances in molecular dating offered the possibility to date speciation events and then draw the historical pattern underlying the current distributions of species (de Queiroz, 2005). These new information about evolutionary history of species led to the reappraisal of dispersalism. It is now increasingly acknowledged, as opposed to what was previously thought, that dispersal can create regular distribution patterns (McDowall, 2004) and the fact that continental drift is pre-eminent in shaping distributions is not necessarily true (de Queiroz, 2005). Those recent advances in the field of phylogeography have led to much more dynamic and complex theories that associate vicariance and dispersion.

Our aim in this work is to use recent techniques in molecular biology to test hypotheses that bryophytes have a low dispersal ability and low evolutionary potential. Islands appear as extraordinary natural laboratories (Emerson and Kolm, 2005) that will allow to answer such questions. The Macaronesian archipelagoes in particular, offer all conditions to test those hypotheses. Macaronesia is composed of mid-Atlantic volcanic islands, namely the Azores, Madeira, Selvagems, Canaries and Cape Verde, situated between 15° and 40°N (Hansen and Sunding, 1993). This term was first used by Engler in 1879, and groups the Azores, Madeira and the Canaries into a distinct biogeographical unit based on the similarity in endemics of the angiosperm flora between islands. Later, some authors included the Cape Verde (Dansereau, 1961, Takhtajan, 1969, Bramwell, 1972, Bramwell, 1976) and some enclaves in North Africa and Iberia because they shares similarities in endemics flora (Sunding, 1979). Endemics are accounting for almost 40% of the native Canarian (González Martín and González Artiles, 2001) and Azorean (Schäfer, 2003) floras, respectively. Endemic taxa considered characteristic of the region include several Lauraceae species (e.g. *Laurus azorica, Apollonias barbujana, Persea indica* and *Ocotea foetens*), other taxa that are widespread within the region (e.g. *Dracaena draco* subsp. *draco*), and distinctive Macaronesian endemic groups that have undergone extensive intra-regional radiation, such as the endemic genus *Argyranthemum* and Macaronesian *Echium* (Takhtajan, 1969).

The hypothesis of Engler, later supported, among others, by Takhtajan (Takhtajan, 1969), Bramwell (Bramwell, 1972) and Sunding (Sunding, 1979), is that the actual flora of Macaronesia represents a relict of a formerly widespread subtropical flora that covered southern Europe and North Africa during the Tertiary. In the light of phylogenetic studies, this hypothesis was greatly balanced and the simple Engler refugium model is nowadays untenable (Carine, 2005). The patterns of colonization of those islands are much more complicated then previously thought. There have been, in some groups, repeated colonization and back colonization (Carine *et al.*, 2004) due to the proximity of these islands to the continent, the relatively ancient age of some islands [20Ma on Fuerteventura (Juan *et al.*, 2000)] and their complex volcanism history (Juan *et al.*, 2000).

The angiosperm flora of Macaronesia has thus served as an important focus for biodiversity research since the Enlightenment period and continues to do so (Francisco-Ortega et al. in press), whereas the cryptogamic flora has received much less attention. In part, this is the result of the 'island laboratory' paradigm that has tended to focus the attention, at least of island evolutionary biologists, on those groups that have undergone evolutionary radiations in islands, a phenomenon largely absent from the cryptogamic flora. Nevertheless, cryptogamic plants constitute a significant component of Macaronesian ecosystems, both in terms of numbers and biomass and the region itself is recognised as a globally important centre for both pteridophyte and bryophyte diversity (Vanderpoorten et al, in prep). Furthermore, given the differences in the biology of cryptogams and angiosperms, cryptogamic plants may offer novel insights into the processes underlying the evolution of insular plant diversity.

One of the major differences between cryptogams and angiosperm is the poor level of endemism in the former. In fact, levels of endemism in the angiosperm floras range from 22.5% for Madeira to 44.3% for the Canaries (Vanderpoorten et al, in prep). Among cryptogams, endemism is much lower, ranging from 0.7% in the case of Canary Islands liverworts to 9.7% in the case of Madeiran pteridophytes. The bryophyte flora exhibit very low levels of endemism in comparison with both pteridophytes and angiosperms with, Madeiran bryophytes (1.9%), showing the highest level of endemism among the bryophyte groups. The Canarian liverwort flora also exhibits the lowest level of endemism of the three archipelagos (0.7%). In addition, and as opposed to angiosperms, bryophytes almost completely failed to radiate in Macaronesia. Vanderpoorten & Long (2006) reported, in fact, that 77% of endemics are represented by a single species in their genus.

Several reasons, starting with insufficient taxonomic knowledge, may explain the low level of endemism in bryophytes. In fact, endemic taxa have been discovered during the last twenty years, including *Aloina humilis* (Gallego *et al.*, 1998), *Platyhypnidium torrenticola* (Ochyra *et al.*, 1998), and *Orthotrichum handiense* (Lara *et al.*, 1999) from the Canary Islands; *Frullania sergiae* (Sim-Sim *et al.*, 2000) and *Riccia atlantica* (Sergio and Perold, 1992) from Madeira; and *Thamnobryum rudolphianum* (Mastracci, 2004) from the Azores, suggesting endemism rates might be under-estimated. This trend is, however, counter-balanced by the recent discovery of species previously considered as Macaroneisan endemics on the European and North African continents (*Tetrastichium fontanum*,(Rumsey and Vogel, 1999); *T. virens*, (Gallego *et al.*, 2004); *Thamnobryum madeirensis*, (Jimenez *et al.*, 2000). Furthermore, several endemic species were reduced to synonymy based on recent phylogenetic evidence (*Herbertus azoricus*, (Feldberg *et al.*, 2004); *Platyhypnidium torrenticola*, (Werner *et al.*, 2007); *Tylimanthus azoricus* and *T. madeirensis*, (Burghardt and Gradstein, 2008); *Fissidens luisieri*, (Werner *et al.*, 2009).

Another explanation for the low level of endemism among bryophyte is their high dispersal abilities, which could reduce the impact of geographic barriers and thus, limit the chances of geographic isolation of populations.

Finally, the apparent non-radiation observed among Macaronesian bryophytes might actually result from a lack of morphological differentiation among genetically diverging lineages. Such a process, referred to as 'cryptic speciation', has been increasingly documented in bryophytes (Shaw, 2001).

In this work, we used two sister species of liverworts of the genus *Radula*, namely *R. lindenbergiana* and *R. complanata*, as models to study the evolution of island endemism. This model is interesting because the two species are broadly distributed in the northern hemisphere, are closely related and morphologically similar, but differ in the mating system. In fact, *R. lindenbergiana* is dioicous, whereas *R. complanata* is monoicous. The comparison between these two sister species allows us to test the following hypotheses: Does the monoicous species, *R. complanata*, have an advantage in terms of dispersal ability? Do they show a similar pattern of genetic structure in areas where they both occur, especially in terms of refugia and post-glacial re-colonization patterns? In particular, we aim at examining if the lack of island endemism in those species is reflected at the molecular level or if it is, conversely, a result of repeated dispersion between archipelagos and continent or due to evolutionary stasis in this species.

An unexpected result of the phylogeographic investigation was the discovery of chloroplast heteroplasmy. Heteroplasmy reflects the presence of two plastid genotype in one individual. The results of this discovery is presented in annex, in a short article consisting in a general explanation of the phenomenon followed by the presentation of the evidence in *Radula* and finally discussion and perspectives about the impact of such a finding on phylogeny.

Materials and methods

The use of model in biology is the most popular way to investigate global questions. The species used as model are themselves interesting for taxonomic related questions but their evolutionary history is thought to be representative of a much broader assembly and help to answer more general issues in evolutionary biology. In this section, I present the two model species in terms of taxonomy and ecology in order to document their position in the tree of life. Then, I briefly explain the lab work necessary to obtain the DNA sequences, from which inferences about the evolutionary history of those species will be made. Finally, all the performed analyses are explained with a link to the goal of each of the statistical treatment.

1. Species as models

In this study, two sister species of liverworts in the genus *Radula* (N. Devos, pers. Comm.) were selected as models. Using sister taxa allow to (i) compare the history of recently diverging species which, in this case, have different mating strategies, then (ii) to give insights into the advantages and disadvantages of both strategies and their impact on the distribution patterns of the two species.

The genus *Radula* is part of the Marchantiophyta, or liverworts, which comprise approximately 5,000 species. The liverworts, which represent the first lineage of land plants, are characterized by thalloïd or leafy forms. (Crandall-Stotler *et al.*, 2009). They produce a single sporangium at the top of the sporophyte that grows primarily by cell extension. The sporangium typically dehisces along four valves, allowing for the release of spores and elaters. The latter are modified cells which, through their hygroscopic movements, promote the dispersal of spores. Spores develop into a single gametophyte, which is the dominant form of the bryophyte life cycle. In extreme cases, species are only known from the gametophytic stage because of the lack of sporophyte production owing to the geographical and/or ecological segregation among sexes (McLetchie and Puterbaugh, 2000) or extinction of one of the two sexes (Schuster, 1983-1984).

The two species used in this work, *R. complanata* and *R. lindenbergiana* cannot be distinguished when sterile(Smith, 1990). They are leafy liverworts, composed of a stem with widely spreading, imbricated leaves. The size of the shoot never reaches more than 2 cm long and leaves are on average 1050 µm long and 850 µm wide. The two species form prostrate patches with a yellow-green colour and are often found with other mosses and liverworts such as *Porella* or *Frullania*. They either grow directly on barks with host tree specificity or on rocks, but are never reported growing on bare soil. They are quite tolerant to desiccation compared to other bryophyte species and their distribution range encompasses nearly the whole northern hemisphere (Soderstrom *et al.*, 2008).

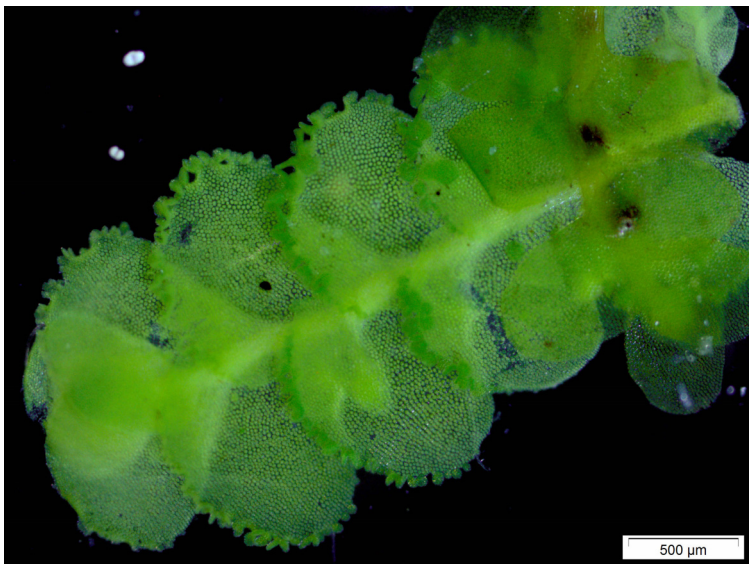
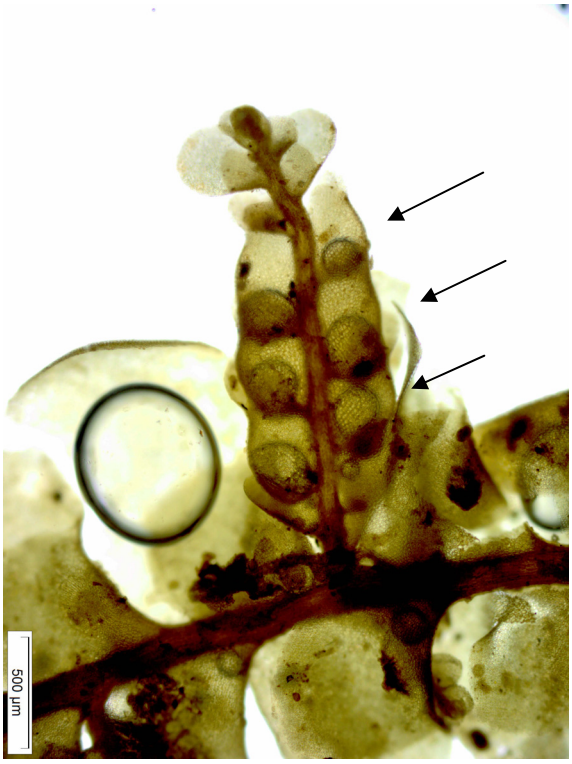


Fig 1: detail of R. complanata under binocular microscope, note the presence of many gemmae at the margin of leaves. (photo: B. Laenen)

Fig 2: Radula complanata (top) and R. lindenbergiana (bottom left male and right female), fertile shoots. For R. complanata, arrows indicate the gynoecium and the male bracts (two pairs are visible). For R. lindenbergiana, three pairs of male bracts are indicated by arrows on the left illustration. On the right, an arrow indicates the gynoecium. Note the absence of inflated male bracts at the bottom of the female structure. (photo: B. Laenen)



The mode of reproduction is different in both species, but they produce asexual propagules called gemmae (***Fig 1***). Those gemmae act as an efficient way of dispersal as they are produced in great number. However, they can only give raise to new colony genetically identical to the mother plant.

Radula complanata is paroicous, i.e. male and female reproductive structures are on the same branch, (***Fig 2***) and the species is almost always fertile. The gynoecium is typically at the top of a long branch and surrounded at the base by female bracts. The male bracts that cover the antheridia (difficult to observe as they flood out) are found in one, two or three pairs and are inflated at the bottom (***Fig 2***) (Smith, 1990).

Radula lindenbergiana is a dioicous species, meaning that male and female are on separated plants. The gynoecium is similar to the one of *R. complanata* except that male bracts are not found below the gynoecium. The male plant has thin, elongated branches that bear small imbricated male bracts.

2. Sampling

A total of 127 samples of *R. complanata* was obtained from different herbarium collections, among which a selection of 63 samples covering essentially Europe, North Africa and two samples from the northwest American coast, was made in order to include material collected at least twenty years ago (see detailed table in annex). The species range also encompasses China and Japan, but it was, however, not possible to obtain sufficiently recent collections from those areas for DNA extraction. Each sample was checked under the dissecting scope in order to characterize its sexual condition and was marked as monoicous, dioicous or sterile. Out of the 63 selected samples, ten turned out to be sterile.

The total number of specimens of *R. lindenbergiana* collected and used for DNA extraction is 118. The sampling almost covers the entire range of the species, including Eastern and Western Europe, North Africa, South Africa, Caucasus, Russia and Macaronesia. The same problem for Asiatic samples was encountered for the sampling of *R. lindenbergiana*. After microscopic examination, 42 samples were considered as sterile.

The sampling for Macaronesia was completed during a field trip in January to the Azores. The Azores is an archipelago composed of nine islands divided in three sectors, the oriental, the central and the occidental. We have visited five islands distributed in the three

Fig 3: Typical waterfall habitat in the island of Flores. (photo: B. Laenen)

sectors, namely Sao Miguel from the oriental sector, Faial, Pico and Terceira from the central sector and Flores from the occidental one. A total of 17 *R. lindenbergiana* specimens were collected during the trip. Samples were identified every evening and divided in two. One part was kept in Eppendorf® tube with silica gel for future DNA extraction and another part was kept for making herbarium voucher.

Fig 4: Micro-environment near warm spring due to volcanic activities reveals species with more tropical affinities such as Fissidens sp. and some Sematophyllaceae (photo: B. Laenen)

The original laurisylva forest of all the islands was degraded and the presence of many non-indigenous and invasive species was noticed, notably *Pittosporum undulatum,* originating from Australia, *Cryptomeria japonica*, which was intensively planted and now regenerates naturally, and dense formation of *Edychium gardneranum* and *Sphaeropteris cooperi* respectively a rapidly invasive Zingiberaceae and an Australian tree fern. The endemic heather *Erica scoparia subsp. azorica* was found on all islands in all vegetations stages but was really dominant at high altitude. The most western island, Flores, was the less affected by human activities but the ancestral biota is remarkably scarce and found in sites that are difficult to access due principally to extensive cattle grazing and introduction of alien species. Despite the impoverishment of the vascular flora, the Azores reveal a rich and diversified bryophyte flora with several species of *Radula*, *Rhynchostegiella* and endemics such as *Echinodium* and *Leptoscyphus azoricus.* Micro-environment such as warm spring due to volcanic activity, include reveals species with more tropical affinities such as *Fissidens sp*. and some Sematophyllaceae (***Fig 4***). At higher altitudes, it is possible to distinguish a bryophyte flora with more boreal affinity with species such as *Lophozia incisa, Polytrichum strictum* and *Sphagnum rubellum.*

Radula lindenbergiana was expected to occur in natural habitats such as tree barks in laurisylva or wet rocks. Surprisingly, however, the species was almost always found in secondary habitat, especially walls made of volcanic stones at low elevation (***Fig 5***), or even in botanical gardens. When found on bark in the forest, the species was not dominant contrary to the situation on the continent, but was overwhelmed by other *Radula* such as *R. wichurae* and *R. carringtonii*.

Fig 5: Volcanic stone walls among low elevation pastures on the island of Pico represent the preferred habitat for R. lindenbergiana on the Azores, where it co-occurs with Mediterranean (Ptychomitrium nigrescens) and Atlantic (P. crispulum) species, as well as the Macaronesian endemic R. wichurae and the eastern Atlantic endemic R. carringtonii. (photo: A. Désamoré).

3. Lab work

Each sample consists of several individuals from one patch, which is thought to comprise only clones given the prime importance of gemmae that are produced in great quantity in both species. Samples were rapidly frozen using liquid nitrogen and ground with a Genogrinder 2000. DNA extraction was performed with the DNeasy Plant Minikit from Quiagen with a diminution of the total volume in the elution phase in order to obtain more concentrated DNA.

In order to obtain suitable data for phylogeographic inference, non-coding cpDNA has been chosen. In fact, these loci have shown enough variability to infer phylogenetic relationships at various taxonomic levels (Taberlet et al., 1991, Gielly and Taberlet, 1994) and their routine amplification is facilitated by the existence of universal primer in flanking conserved regions (Taberlet *et al.*, 1991). Six loci that have shown infra generic variations in *Radula* (N. Devos, pers. comm.) were tested and the four most variable loci were chosen. These loci are the *atp*B-*rbc*L intergenic spacer, t*rn*G region, t*rn*L region and *rps*4 gene. Universal primers as described by Shaw et al (2003a) were initially used - (***Table 1***), but a new primer pair was designed for *trn*L from available sequences obtained using universal primers with the online software (http://www.sigma-genosys.com/calc/DNACalc.asp) to overcome amplifications difficulties with less recent herbarium materials.

Table 1: List of primer used to amplify the four cpDNA loci in the phylogeography of R. complanata and R. lindenbergiana.

Loci	Primer forward		Primer reverse	
*trn*G	*trn*G_F	ACCCGCATCGTTAGCTTG	*trn*G_R	GCGGGTATAGTTTAGTGG
*rps*4	*rps*5 F	ATGTCCCGTTATCGAGGACCT	*trna*5R	TACCGAGGGTTCGAATC
*Atp*B-*rbc*L	*atp*B672_F	TTGATACGGGAGCYCCTCTWAGTGT	*atp*B910_R	TTCCTGYARAGANCCCATTTCTGT
*trn*L	*trn*F	ATTTGAACTGGTGACACGAG	*trn*C	CGAAATCGGTAGACGCTACG
*trn*L (own)	Ben_*trn*L_F	TCAGGGAAACCTAGGGTGAA	Ben_*trn*L_R	CCGGCAATTTTTGTTTCTGT

Amplification of DNA fragments was performed by polymerase chain reaction (PCR) using the following settings for one reaction; 6.775 µl RNase free H_2O, 1.5 µl buffer 10X supplied with the Taq polymerase enzyme, 2.4 µl of a solution containing each nucleotide (1mM each), 0.6 µl $MgCl_2$ 50 mM, 0.75 µl of each primer (10 µm), 1.125 µl of BSA and 0.3 µl of Taq polymerase. One µl of DNA was added for a total of 15 µl per sample. Higher concentration of $MgCl_2$ was used for old herbarium specimens. The PCR included one cycle of denaturation at 95° for 2 min; 35 cycles of 30s denaturation at 95°, 45s of annealing at 50°, 2 min of extension at 72° followed by 7 min at 72°. 5µl of the PCR product was run on a 1% agarose gel and stained with ethidium bromide, gel green or Sybersafe to check for the presence of the amplified product. Amplification fragment were either sent to Macrogen for sequencing or purified with a solution of 0.2 µl Exonuclease, 0.2 µl Phosphatase and 2.4 µl H_2O for a total of 3 µl per sample. Then, sequencing reaction using BigDye included 2 min at 96°, 25 cycles of 15s at 96°, 10s at 50°, 4 min at 60°.

4. Analysis

Sequence editing and alignment.

Sequences were aligned automatically using the contig option on Sequencher 3.1 and gaps were inserted when necessary to conserve homology among sequences. The alignment was then verified visually and sequences where edited based on the electropherogram. Every variable site was checked and ambiguous symbols conform to IUPAC code, were inserted when the signal was unclear.

Haplotypic composition and distribution.

All individuals displaying identical sequences across the four loci were assigned to each of 21 haplotypes for *R. lindenbergiana* and 8 for *R. complanata*. When haplotype could not be determined, for example when only one locus could be amplified, or when multiple base calls blur the signal (see annex), individuals were removed from the analysis.

A haplotype map was constructed using the location of each individual and the corresponding haplotypes. However, individual belonging to the same haplotype and found in the same region were not illustrated because the resolution of the map is trans-continental and genetically identical individual separated from less than 50 km could not be distinguished. The representation of haplotypes on a map is an easy way to analyse at first sight a dataset and draw possible patterns that need to be further tested. Haplotype occurrences were inserted into a data matrix, with 8 and 4 biogeograhic regions for *R. lindenbergiana* and *R. complanata* respectively. These regions correspond to those defined by Van der Wijk & Mardagant (1969) with the following modifications: (i) Macaronesia was split into the Canary islands, Madeira, and the Azores; the Iberian Peninsula was individualized from the rest of Europe; and Asia was considered as a single unit (***Table 2***).

Table 2: Geographic , modified from van der Wijk & Mardagant (1969), used to partition genetic variation across four cpDNA loci in R. lindenbergiana (left) and R. complanata (right). Number of individuals per region is indicated.

	R. lindenbergiana	nb of individual		*R. complanata*	nb of individual
1	Canary islands	24	1	North America	2
2	Madeira	5	2	Central Europe	14
3	Azores	10	3	Mediterranean region	12
4	Iberian peninsula	8	4	Caucasus	1
5	North Africa	4			
6	Central Europe	20			
7	South Africa	4			
8	"Caucasus and Asia"	9			

Genetic diversity analysis.

They are many ways to characterize the diversity of an assemblage of populations. Two approaches were investigated here to provide a representation of the genetic diversity among all populations in *R. lindenbergiana*. The first one is a molecular analysis of variance (AMOVA) (Weir, 1996, Weir and Cockerham, 1984). This analysis is based on pairwise comparisons between haplotypes using an Euclidian distance matrix. The variance is calculated among and within regions and permutation tests are used to test the significance of the inter-regional differentiation. The results are expressed as the percentage of variance explained by intra- and inter region differentiation and give an appreciation of the general genetic pattern describing the data.

The second approach involved two genetic diversity indexes, namely haplotypic and nucleotidic diversity based on haplotype frequencies. Haplotypic diversity describes the relative diversity of haplotypes considering their frequencies, while nucleotidic diversity shows the divergence existing among haplotypes. Those indexes were calculated for each region and are given by the formula below:

Haplotypic diversity:

$$H = \frac{n}{n-1}(1-\sum_{i=1}^{k} pi^2)$$

n = number of gene copies in the region
k = number of haplotypes
pi = haplotype frequency

Nucleotidic diversity:

$$\pi = \Sigma pi.pj\,\pi ij$$

pi, pj = frequencies of the ith and jth haplotype
πij = number of mutations between haplotype i and j.

Both analyses were performed with the program Arlequin version 3.1 (http://cmpg.unibe.ch/software/arlequin3)**.**

Population differentiation.

The presence of a geographic structure in the *R. lindenbergiana* and *R. complanata* dataset was estimated with F-statistics. First, a global Fst (Weir and Cockerham, 1984) was calculated across all populations. The Fst can be defined as the probability to find identity in homology between haplotypes in a region compared to this probability if there were no delimited regions. Then, pairwise Fst between regions were calculated. In this case the probability of identity between the two regions is compared to the probability to find the same allele if the two regions are considered together. The significance of the Fst was tested by constructing the distribution of the null hypothesis (Fst=0) by random permutations of individuals among regions. A total of 999 permutations per Fst value were performed and p-values were obtained by comparing the proportion of simulated Fst values with the observed one. A global Fst significantly different from zero means that there is a genetic structure in the data, and a significant Fst between two regions means that the two regions are genetically differentiated. The absolute Fst values can be therefore, but with caution, interpreted as an indirect measure of dispersion between populations, with high values corresponding to more structured pattern and hence, less dispersion.

In order to test for the presence of phylogeographic signal in the data, Fst was compared to Nst (Pons et Petit,1996, Burban et al. 1999) as implemented by SpaGedi (Hardy and Vekemans, 2002). In fact, the classical Fst estimation is only based on allele or haplotypes frequencies without taking into account the relatedness of the haplotypes. As defined by Pons and Petit (Pons and Petit, 1996), the Nst is similar to a Fst but is weighted using a genetic distance among haplotypes. A value of Nst>Fst indicates the presence of phylogeographic signal in the data. This means that the mutation rate is higher than the dispersal rate, and hence, that closely related haplotypes tend to occur within the same geographical region, whereas distantly related haplotypes do not occur in sympatry. Indeed, individuals accumulate mutations before migrating into another area. The distribution of the null hypothesis (Nst=0) is constructed in the same way as Fst. The hypothesis that Nst>Fst is tested by permuting the row and column of the distance matrix in order to generate a random association between pairs of haplotypes.

Phylogenetic analyses

A network analysis was performed with the TCS program (Clement *et al.*, 2000). This is a parsimony based method that represents a minimum spanning networks, which involves that the minimum number of mutations is required to build the network. Each node represents a mutational step. A poly-T segment in *R. complanata*, which is a chloroplast microsatellite region, was discarded from the analysis because those regions are known to evolve faster than the rest of the sequence (Lee *et al.*, 2007) and are highly prone to homoplasy.

Bayesian reconstructions of haplotype relationships were also performed in order to assess the support for the branches of the network and obtain branch lengths which are an essential feature for ancestral character state reconstruction (see below), from an explicit nucleotide substitution model. Sequences of *R. wichurae* and *R. caringtonii* (supplied by N. Devos) were used as outgroup due to their close relation with *R. lindenbergiana* and *R. complanata* (N. Devos, pers. comm.). A nucleotide substitution model was selected using the program Modeltest (Posada and Crandall, 1998), which uses a likelihood ratio test to determine which model provides the best fit to the data. A model is composed of parameters

that are the mutation rates, proportion of nucleotides, proportion of invariable sites and a gamma distribution which models the heterogeneity in mutation rates among sites.

The Bayesian analyses were performed using the program MrBayes. The principle of the method is to sample trees and model parameters by means of a Monte Carlo Markov chain (hereafter, MCMC), which visits the space of trees and model parameters. At each iteration of the chain, the topology, branch lengths or model parameters are perturbated. The likelihood of the new combination of tree and rate parameters is calculated and this new condition of the chain is accepted according to the Metropolis-Hastings term. This process is reiterated many times, so that likelihoods reach a plateau, meaning that the chain has reached stationary. The ascending period before the plateau is called the burnin and is discarded. After the burnin, trees and model parameters are sampled at regular intervals to form their posterior probability distributions. Ten millions generations were conducted and trees were sampled every 1000 generations. After burnin removal, a fifty percent majority-rule consensus tree is created based on all the trees sampled. The robustness of a branch is assessed through its posterior probability, which corresponds to the proportion of trees wherein the branch in question is resolved.

In order to test the monophyletic origin of Macaronesian haplotypes, we performed a constrained Bayesian analysis wherein Macaronesia was forced to be monophyletic. Then, we used the Bayes factors, which are approached by twice the difference in the harmonic mean of the log-likelihood between the constrained and unconstrained analysis. Threshold values of the Bayes factors of 2, 5 and 10 are considered as evidence, strong evidence, and very strong evidence for a hypothesis over another (Pagel and Meade, 2004).

Ancestral distribution reconstruction

In order to retrace the evolution of the distribution in *R. lindenbergiana*, a character reconstruction method using a Bayesian inference was used (Pagel, 1999). Haplotypes were grouped according to their distribution into four character states, namely, haplotypes occurring only in Macaronesia (1), only in Europe (2), only in South Africa (3) and haplotypes occurring in more than one region, which we refer to combined distributions (4). We then used a model employing forward and backward transition rates among each pair of character states to reconstruct ancestral range distributions onto the phylogeny. We then used the Monte Carlo Markov-chains implemented by BayesTraits 1.0 (Pagel and Meade, 2006) to sample trees from the MrBayes analysis and rate parameters. In the absence of information on rate parameters, the latter were sampled from flat, uniform distribution priors ranging between 0 and 100. The combination of a tree and rate parameters was accepted or rejected depending on the Metropolis-Hastings term. The MCMCs were run for 50,000,000 generations and sampled every 10,000 generation. The trees and rate parameters sampled from the posterior probability distribution were finally used to reconstruct, at each node of interest, the probability of occurrence within each of the four geographic areas. In order to circumvent the issues associated with the fact, that not all of the trees necessarily contain the internal nodes of interest, reconstructions were performed using a 'most recent common ancestor' approach that identifies, for each tree, the most recent common ancestor to a group of species and reconstructs the state at the node, then combines this information across trees (Pagel and Meade, 2004).

Results

1. Data description

The final dataset consists of 84 and 29 individuals of *R. lindenbergiana* and *R. complanata* respectively. The genetic variation in *R. lindenbergiana* consists of 21 parsimony informative sites, five singletons and five insertion/deletion events among haplotypes. Altogether, variable characters represent in total 1.36% of the four cpDNA loci. The relative variation at the same loci for *R. complanata* was comparatively very low with a total 0.26% of variation distributed at a single parsimony informative site, four singletons and two insertion/deletion events (see details in ***Table 3***). As a result, 21 and 8 haplotypes were identified within *R. lindenbergiana* and *R. complanata,* respectively.

Table 3: Nucleotide polymorphism at four non-coding cpDNA loci in a sample of 84 and 29 specimens of R. lindenbergiana and R. complanata from their entire distribution range

R.indenbergiana/R,complanata	*atp*B-*rbc*I	*rps*4	*trn*G	*trn*L	Total
Sequence length (bp)	420	610	475	411	1916
Variable sites	5/1	5/1	11/1	5/2	26/5
Parsimony informative sites	4/0	5/0	7/0	5/1	21/1
Insertion deletion events	0/0	0/0	4/2	1/0	5/2

2. Haplotypes distribution.

Radula complanata

There are three widespread haplotypes (***Fig 6***): 4, 5 and 6, where 5 and 6 are separated by one mutation; 4 and 5 by two indels and one mutation; and 4 and 6 by two indels of one base pair. Haplotype 3 is restricted to Corsica, haplotype 2 to the Caucasus, haplotype 1 to Southern France and haplotype 7 to the Czech Republic. In North America, only haplotype 6 has been found, but the sampling consists only of two individuals. The region containing the highest number of haplotypes is Eastern Europe with Slovakia, Czech Republic and Switzerland where haplotypes 4, 5, 6 and 7 occurs. Two haplotypes, 6 and 5, are present in the UK. The Pyrenees, Spain and Morocco are occupied only by haplotype 4.

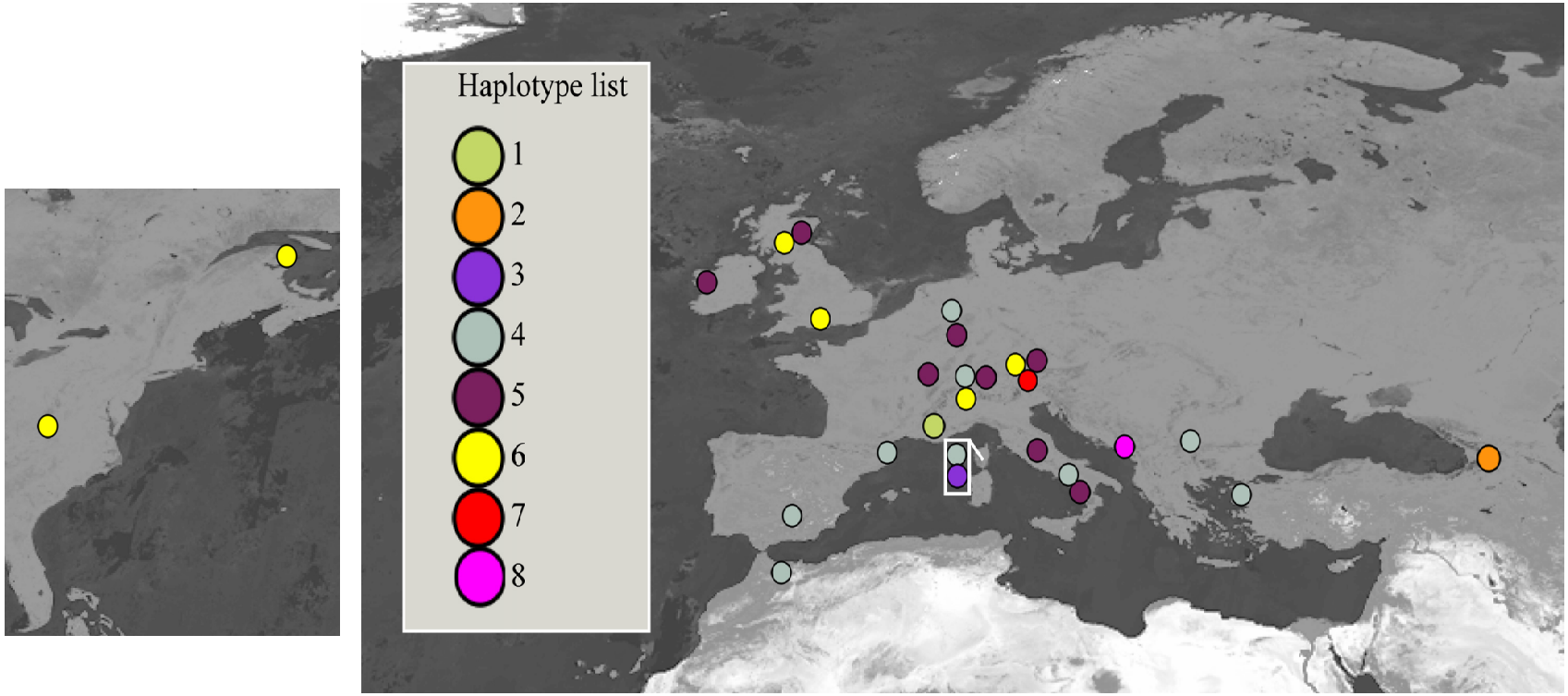


Figure 6: Distribution of haplotypes inferred from cpDNA in R. complanata. Each colour corresponds to one haplotype number listed in the haplotype list in the left box. The figure on the left represents the distribution in North Eastern America.

Radula lindenbergiana

There are four widespread haplotypes that can be divided in two groups (***Fig 7***), an Eastern and a Western group. The Eastern group comprises haplotype 21, which is present in France, Belgium, Czech Republic and extends to Caucasus and Asia. The Western group comprises haplotypes 3 from Spain, South-eastern France, the UK, the Azores, Madeira and the Canaries; haplotype 2 from Western Spain, Northern Morocco and the Azores; and haplotype 1 from Spain, Majorca, Morocco and the Canaries. Two endemic haplotypes, 11 and 4, are found in Scandinavia, one in Scotland (hap 6), one in Tenerife (hap 7), two in Madeira (hap 18 and hap 12), one in Ireland (hap 20) and one in Gran Canaria (hap 15). Haplotype 5 is present in Portugal and Gran Canaria while haplotypes 8 and 10 are only present in the Canary Islands where they respectively occur on Gran Canaria, La Gomera, El Hierro (hap 8) and La Palma, Fuerteventura (hap 10). Finally, haplotype 13 is shared between Sao Miguel (Azores) and La Palma (Canaries).

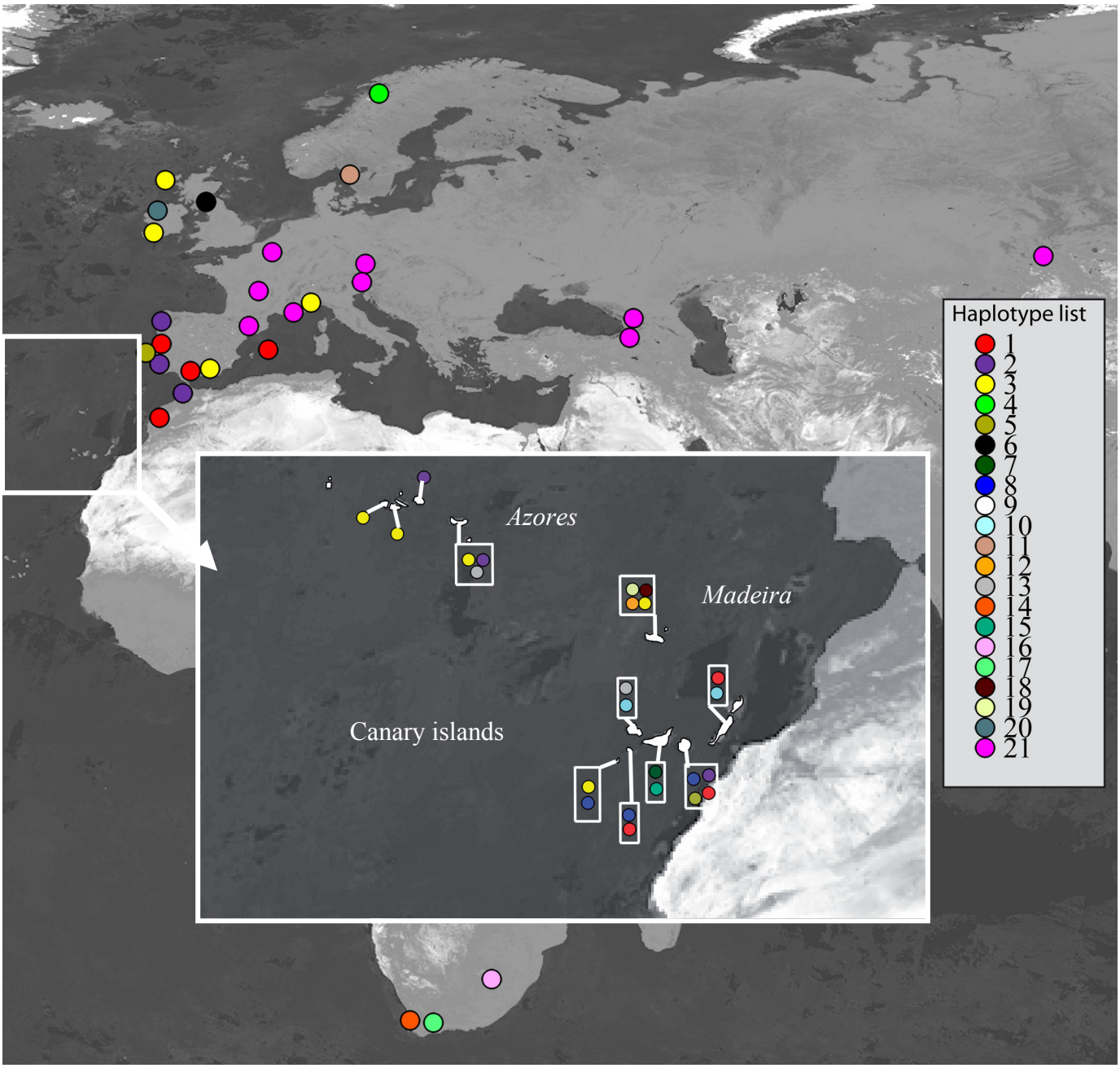


Fig 7 : Distribution of haplotypes inferred from cpDNA in R. lindenbergiana. Each colour corresponds to one haplotype number listed in the haplotype list in the right box. The central frame is a zoom on the Macaronesian archipelagos.

3. *Genetic diversity analyses.*

The two index of genetic diversity used, haplotypic and nucleotidic, provide complementary information. The haplotypic diversity shows the proportional diversity of haplotypes present in a region, while the nucleotidic diversity informs about the relative mutation difference between haplotypes. Three regions are characterized by a haplotypic diversity >0.8, namely Madeira (0.90), the Canary Islands (0.89) and South Africa (0.83). Then, Iberian Peninsula has a haplotypic diversity of 0.75 and is followed by the Azores (0.64) and Central Europe (0.62). The two regions with the lowest haplotypic diversities are North Africa (0.50) and Caucasus plus Asia, with a haplotypic and nucleotidic diversity of 0.0 because only one haplotype is found in that region. Madeira exhibits the highest nucleotidic diversity (0.0031 +/- 0.0021). It has to be noted that as for haplotypic diversity, North Africa and Caucasus and Asia have the lowest values (0.002 and 0) which represent the paucity of genetic diversity. The other regions show similar values of nucleotidic diversity with, however, fewer differences among haplotypes in the Iberian Peninsula (0.0009) and South Africa (0.0013).

Table 4: Haplotypic and nucleotidic diversity of the geographical regions with their corresponding standard deviation in Radula lindenbergiana. Values are sorted decreasingly by their haplotypic diversity.

	Sample size	Nb of Haplotype	Haplotypic diversity	sd	Nucleotidic diversity	sd
Madeira	5	4	0,90	0,16	0,0031	0,0021
Canary island	24	10	0,89	0,04	0,0018	0,0010
South Africa	4	3	0,83	0,22	0,0013	0,0010
Iberian peninsula	8	4	0,75	0,14	0,0009	0,0007
Azores	10	4	0,64	0,15	0,0016	0,0010
Central Europe	20	6	0,62	0,11	0,0022	0,0013
North Africa	4	2	0,50	0,27	0,0002	0,0003
Caucasus and Asia	9	1	0,00	0,00	0,0000	0,0000

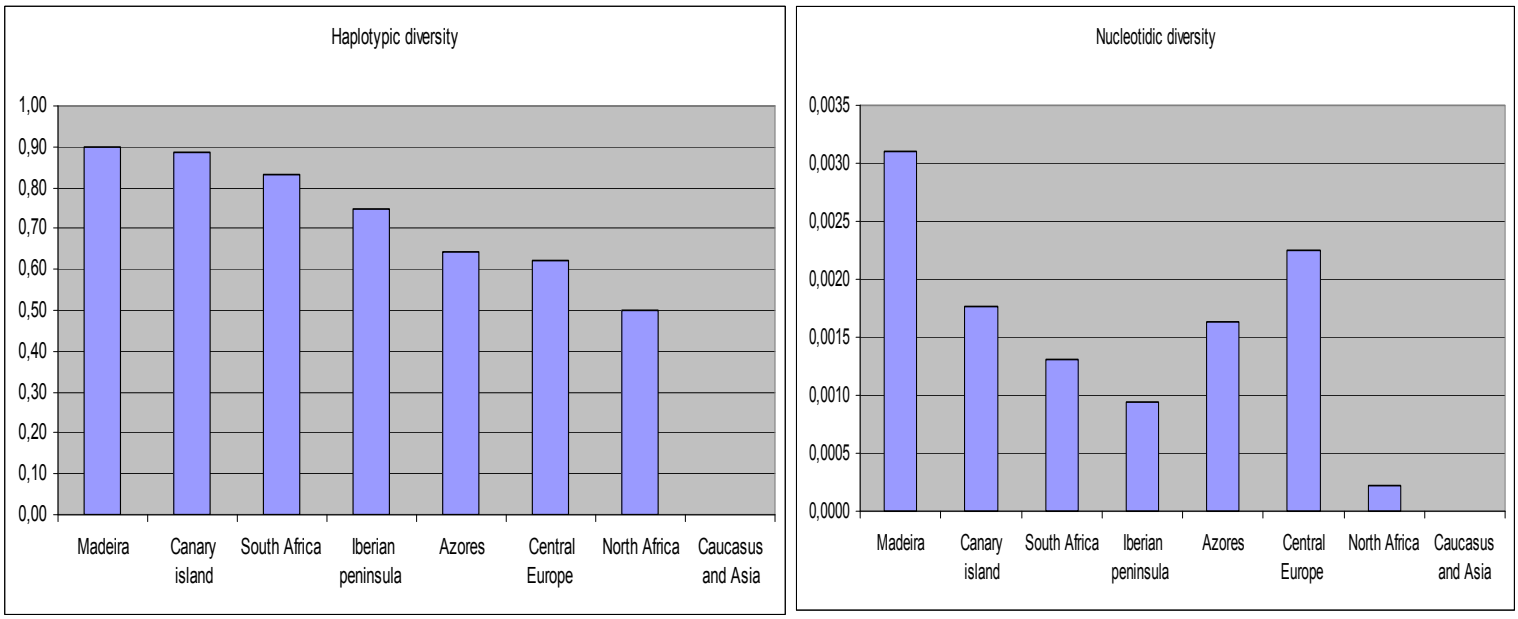


Fig 8: Geographic partition of haplotypic and nucleotidic diversity inferred from variation at four cpDNA loci in Radula lindenbergiana

The genetic diversity was also characterized by an AMOVA to determine if the genetic variation is essentially found among or inside the regions. The result of this analysis (***Table 5***) shows, despite significant differences were found among regions, that the genetic variation is located principally within region (72.46%) which can be due to the numerous endemics alleles in Canary Islands, Madeira and Central Europe representing differentiation within regions but common haplotypes still occur in many regions and decrease the variability among regions.

Table 5: Molecular variance analysis in Radula lindenbergiana from the variation observed at four cpDNA loci among a sample of specimens spanning the entire distribution range of the species. The p-value represents the result of a test consisting 1023 permutation. d.f. = degree of freedom.

Source of variation	d.f.	Sum of Squares	Variance components	Percentage of variation	P-value
Among regions	7	11.09	0.1272	27.54	**<0.0001**
Within regions	76	25.43	0.3347	72.46	
Total	83	36.52	0.4618		

4. Population differentiation.

Inter-regional genetic differentiation was assessed through the Fst global index. In *R. lindenbergiana*, the South African region did not share any haplotype with the other regions, which may thus artificially raise the Fst and Nst values. As a consequence, analyses were performed with and without the South African region. The results show that the global Fst (Table 6) values are significant regardless of the inclusion of South Africa. Pairwise Fst's (Table 7) provide more insight to draw a general population differentiation pattern. A significant Pairwise Fst indicates that the two regions can be considered as two differentiated genetic entities, whereas non-significant values indicates that the Fst is not different from zero and regions are similar from a genetic point of view. The majority of pairwise Fst has a significant value but some comparison have a Fst not different from zero, notably the relation between the Canaries, the Iberian peninsula and North Africa that shows an interesting homogeneity regarding Fst comparisons. Furthermore, Madeira and the Azores are not significantly differentiated and low values (0.10-0.16) are found between Macaronesian islands and between islands and Iberian Peninsula. Madeira and the Azores show more differentiation with North Africa, with respective values of 0.28 and 0.32. The Caucasian region has the highest Fst values with all the other regions except Central Europe. This result is not surprising accounting that Caucasus and Asia have only one haplotype also present in Europe but absent in all the other regions. The Central Europe region is well differentiated (0.24-0.41) from the Macaronesian islands, Iberian Peninsula and North Africa, resulting in a West/East separation of populations. Surprisingly, some comparisons with South Africa, which shares no haplotypes with other regions, did not show significant values. However, this is only an artefact because the sampling in South Africa is too small, but the isolation of South Africa is further supported by very high values of Nst (0.770-0.897).

The global Nst values is 0.678 with South Africa and 0.487 without it but in both cases, Nst is significantly higher that Fst, indicating that a significant phylogeographic signal is present in the data. Such a signal means that genetically closely related haplotypes are found within the same region. Nst pairwise comparisons reveal that a phylogeographic signal exists between South Africa and all the other regions and also that the Canaries and the Iberian Peninsula are phylogeographically differentiated from Caucasus and the Asian region (0.836; 0.922).

The global Fst value in *R. complanata* as a value of 0.25 and is marginally significant (p-value = 0.02) indicating that a weak albeit significant genetic structure exists in the studied area. However, the Nst index was not significant (p-value=0.186) rejecting the hypothesis of a phylogeographic signal in *R. complanata*.

Table 6: Population differentiation in R. lindenbergiana and R. complanata represented by global Fst and Nst values with p-values corresponding to the result a permutation test. Result of Nst>Fst test is indicated at the right.

Global Fst and Nst value in *R. lindenbergiana*					Test Nst>Fst>0
	Fst	P-value	Nst	P-value	P-value
With Sth Africa	0.222	**<0.0001**	0.678	**<0.0001**	**<0.0001**
Without Sth Africa	0.230	**<0.0001**	0.487	**<0.0001**	**0.03**

Global Fst and Nst value in *R. complanata*			
Fst	P-value	Nst	P-value
0,25	**0,02**	0,16	**0,186**

Table 7: Pairwise Nst and Fst comparison between pairs of region. P-value is obtained from a permutation test. N.S= non significant value.

Name i	Name j	Nst	P-value	Fst	P-value
Canary islands	Caucasus and Asia	**0.836**	**0.0250**	**0.44**	**<0.0001**
Iberian peninsula	Caucasus and Asia	**0.922**	**0.0350**	**0.64**	**<0.0001**
Canary islands	Central europe	*N.S.*	*N.S*	**0.24**	**<0.0001**
Azores	Caucasus and Asia	*N.S*	*N.S*	**0.66**	**<0.0001**
Iberian peninsula	Central europe	*N.S*	*N.S*	**0.31**	**0.0000**
Madeira	Caucasus and Asia	*N.S*	*N.S*	**0.66**	**0.0010**
North Africa	Central europe	*N.S*	*N.S*	**0.41**	**0.0010**
Canary islands	Azores	*N.S*	*N.S*	**0.14**	**0.0010**
Azores	Central europe	*N.S*	*N.S*	**0.28**	**0.0020**
South Africa	Caucasus and Asia	*N.S*	*N.S*	**0.74**	**0.0020**
North Africa	Caucasus and Asia	*N.S*	*N.S*	**0.85**	**0.0020**
Central europe	South Africa	**0.770**	**0.0090**	**0.31**	**0.0088**
Canary islands	Madeira	*N.S*	*N.S*	**0.10**	**0.0176**
Madeira	Central europe	*N.S*	*N.S*	**0.25**	**0.0186**
Azores	South Africa	**0.776**	**0.0100**	**0.29**	**0.0205**
Iberian peninsula	South Africa	**0.866**	**0.0020**	**0.22**	**0.0293**
Canary islands	South Africa	**0.829**	**<0.0001**	**0.13**	**0.0293**
Madeira	North Africa	*N.S*	*N.S*	**0.28**	**0.0352**
Madeira	Iberian peninsula	*N.S*	*N.S*	**0.16**	**0.0459**
Azores	North Africa	*N.S*	*N.S*	**0.32**	**0.0469**
Madeira	*Azores*	*N.S*	*N.S*	*N.S*	*N.S*
Azores	*Iberian peninsula*	*N.S*	*N.S*	*N.S*	*N.S*
Central europe	*Caucasus and Asia*	*N.S*	*N.S*	*N.S*	*N.S*
Canary islands	*North Africa*	*N.S*	*N.S*	*N.S*	*N.S*
North Africa	*South Africa*	**0.897**	**0.0410**	*N.S*	*N.S*
Madeira	*South Africa*	**0.788**	**0.0040**	*N.S*	*N.S*
Canary islands	*Iberian peninsula*	*N.S*	*N.S*	*N.S*	*N.S*
Iberian peninsula	*North Africa*	*N.S*	*N.S*	*N.S*	*N.S*

5. Phylogenetic analysis

In the fifty percent majority-rule consensus tree with both *R. complanata* and *R. lindenbergiana* (***Fig 9***) *R. lindenbergiana* appears as a monophyletic group (pp=0.9) while *R. complanata* is represented as a polytomy at the base of the tree. The branches leading to the *R. complanata* haplotypes are relatively short compared with *R. lindenbergiana*. Four major clades (I, II, III, V) are recognized within the *R. lindenbergiana* clade (***Fig 10***). Clade I, which is sister to the remainder of the other clades, is composed of the widespread haplotype 21, which occurs only in Europe, Caucasus and Asia, and haplotype 4, which is endemic to Norway. Clades II, III and IV are clustered together with a posterior probability of 0.84. Clade II (pp=1) is exclusively composed of South African haplotypes (14, 16 and 17) and has very long branches compared to the rest of the tree. Clade III (pp=0.89) includes many haplotypes among which six are endemic (18, 19 Madeira; 7, Gran Canaria; 15 Tenerife; 11, Sweden; 20 Ireland), two are more widespread (1,2, Canary islands, Azores, North Africa and Iberian peninsula), one is found both in the Canary Islands and Iberian peninsula (15) and one occurs in Madeira and the Azores (13). Within clade III, clade IV, supported with a p.p. of 0.74, is formed by haplotypes 20, 11, 7 and 2. Finally, within clade IV, haplotype 12 from Madeira is sister to another sub-clade supported at 0.98 and composed of haplotypes 8, 9 and 10, which are restricted to the Canary Islands, and haplotype 3, which is widespread and found in the Azores, Canary islands, Madeira, Spain, South France, and the UK.

The haplotype network of *R. lindenbergiana* has a similar structure to the tree based on Bayesian inference, but provides more information about clade relationships. In the network (***Fig 11***), haplotypes 18 and 19 from Madeira cluster together are the link between clade II from South Africa and clade III inside which clade IV is recognisable but without haplotype 11. Clade I, III and IV are derived from haplotype 6 from Scotland

The phylogenetic reconstruction in *R. complanata* is characterized by a comparatively low polymorphism and the presence of many autapomorphies (***Table 13***) that lead to a star like topology (***Fig 12***). Haplotype 6, which is present in Europe and North America, occupies a central position in the network from which all the other haplotypes evolved.

Constrained analysis reveals that forcing Macaronsesia to be monophyletic result in a significant increase in likelihoods as reveal by Bayes factors higher than 2. Results are summarized in ***Table 8***;

Table 8: Harmonic means of the log-likelihood derived from two successive Bayesian analyses of a sample of specimens of R. lindenbergiana across its entire distribution range and genotyped at four cpDNA loci. In the first analysis, the MCMC's were unconstrained whereas in the second one, only topologies that are compatible with a monophyletic Macaronesian concept were visited. The Bayes factors, which are approached by twice the difference in the harmonic mean of the log-likelihood between the two runs, measure the significance of the difference in log-likelihood imposed by the constraint and values >2, 5 and 10 are considered as significant, highly significant, and very highly significant (Pagel et al. 2004).

	likelihood harmonic means
Macaronesia constrained	-3033.78
Unconstrained	-3031.35
bayes factors (- 2 **Δ**)	**4.86**

Fig 9: 50% majority-rule consensus tree from the trees sampled from the posterior probability distribution derived from a Bayesian analysis of four chloroplast regions in a sample of R. complanata and R. lindebergiana across their entire distribution range, with R. carringtonii and R. wichurae used as outgroups. Branch lengths were averaged across the trees of the posterior probability distribution. Labelled clades are described in the text. Support for the branches is provided by their posterior probabilities.

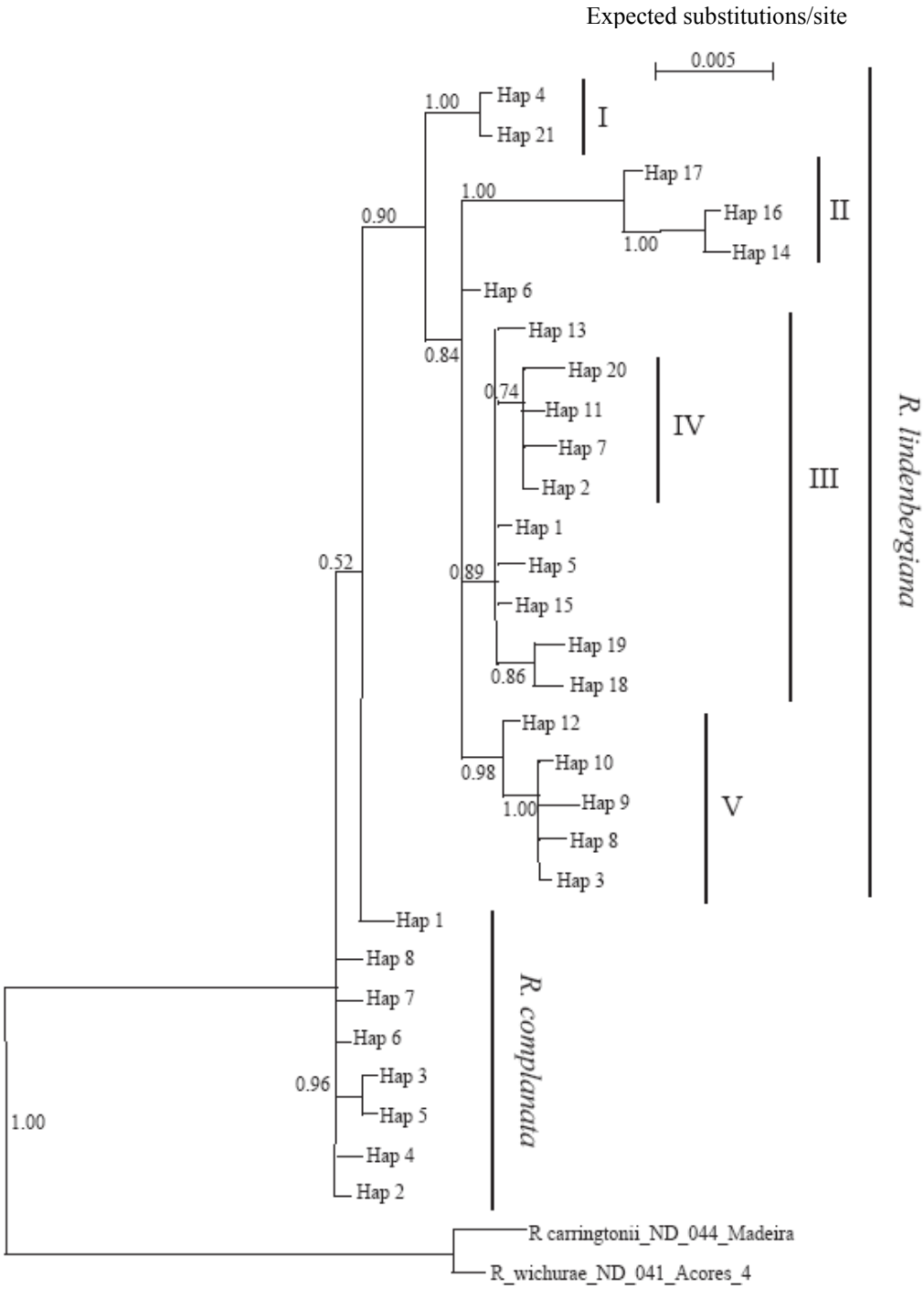


Fig 10: Focus on the R. lindenbergiana clade from Fig 9. Haplotypes are represented by pie diagrams whose size is proportional to the haplotype frequency across the whole sampling and coloured areas (legend bottom left) provide information on the haplotype frequency distribution across all the geographic regions where it occurs.

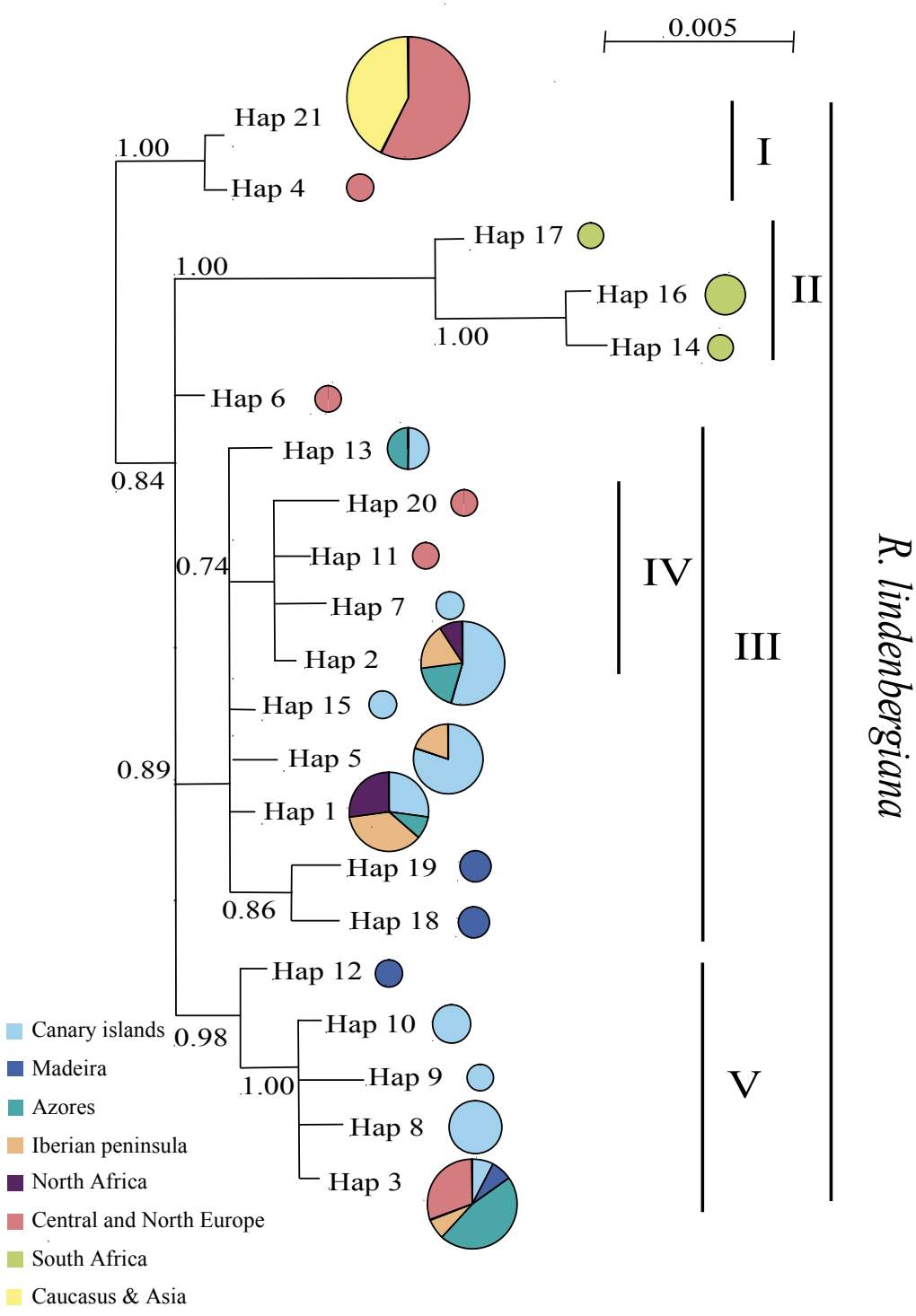
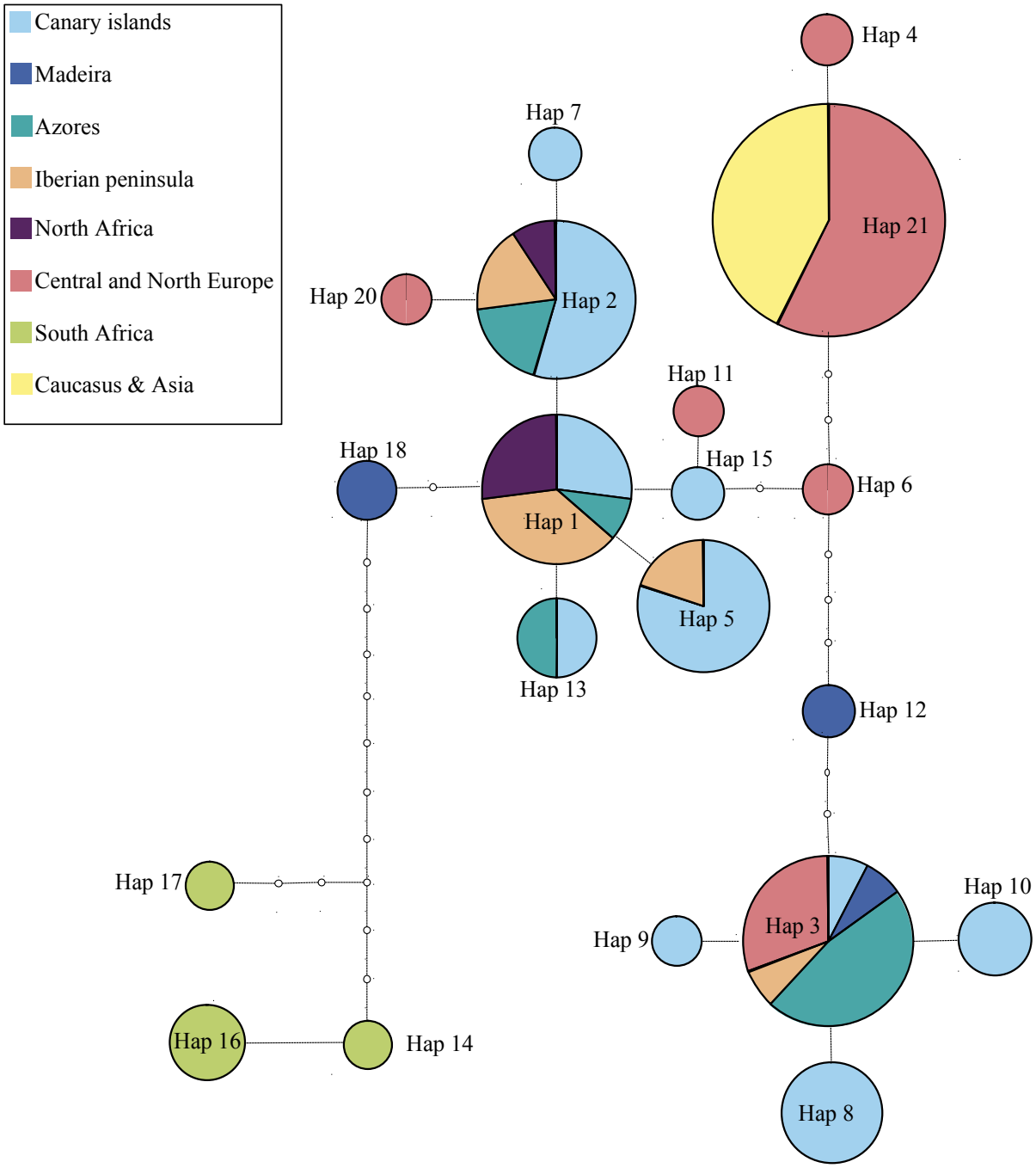


Fig 11: Haplotype network of R. lindenbergiana derived from the variation of a sample of specimens covering the entire distribution range of the species at four cpDNA loci. Each step corresponds to a mutation and white circle to non-sampled or extinct haplotypes. Haplotypes are represented by pie diagrams whose size is proportional to the haplotype frequency across the whole sampling and coloured areas (legend bottom left) provide information on the haplotype frequency distribution across the entire geographic region where it occurs.



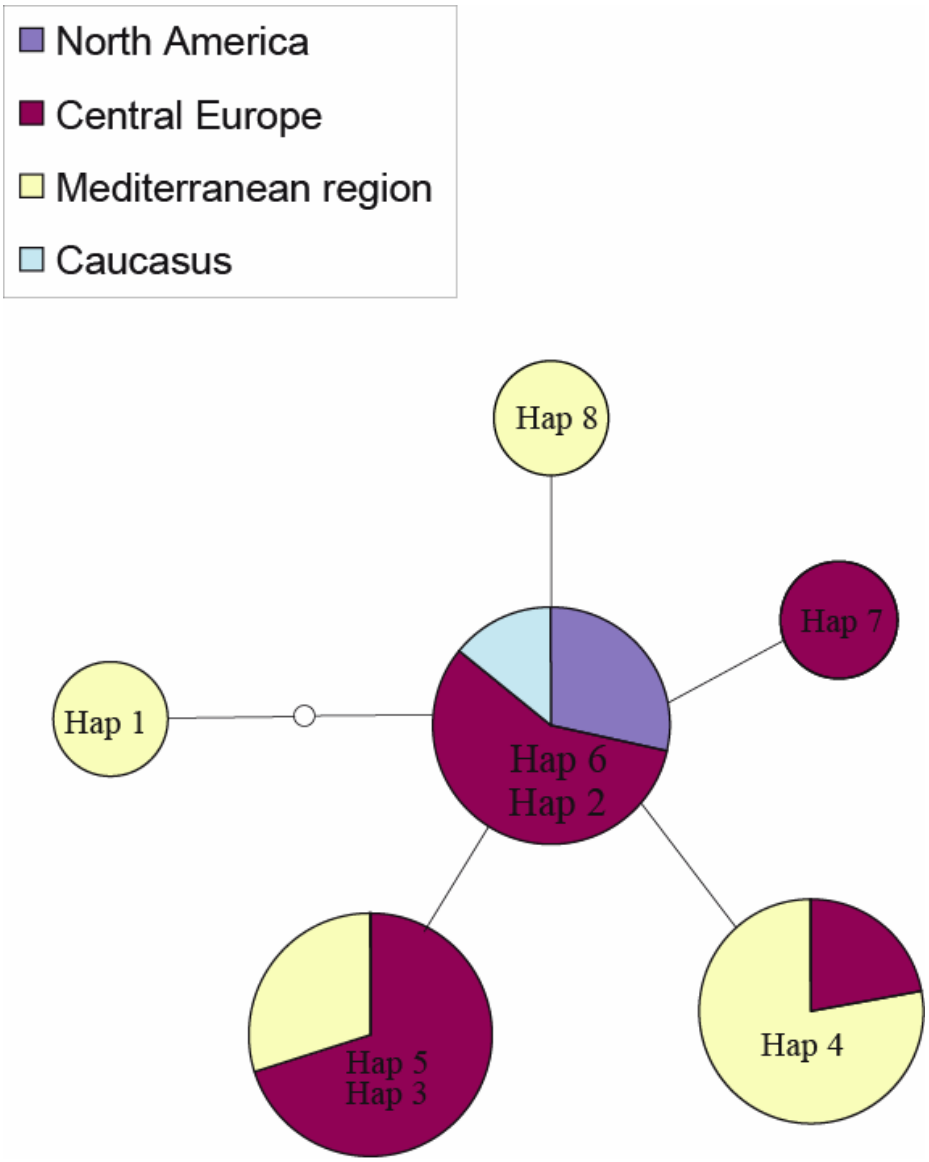
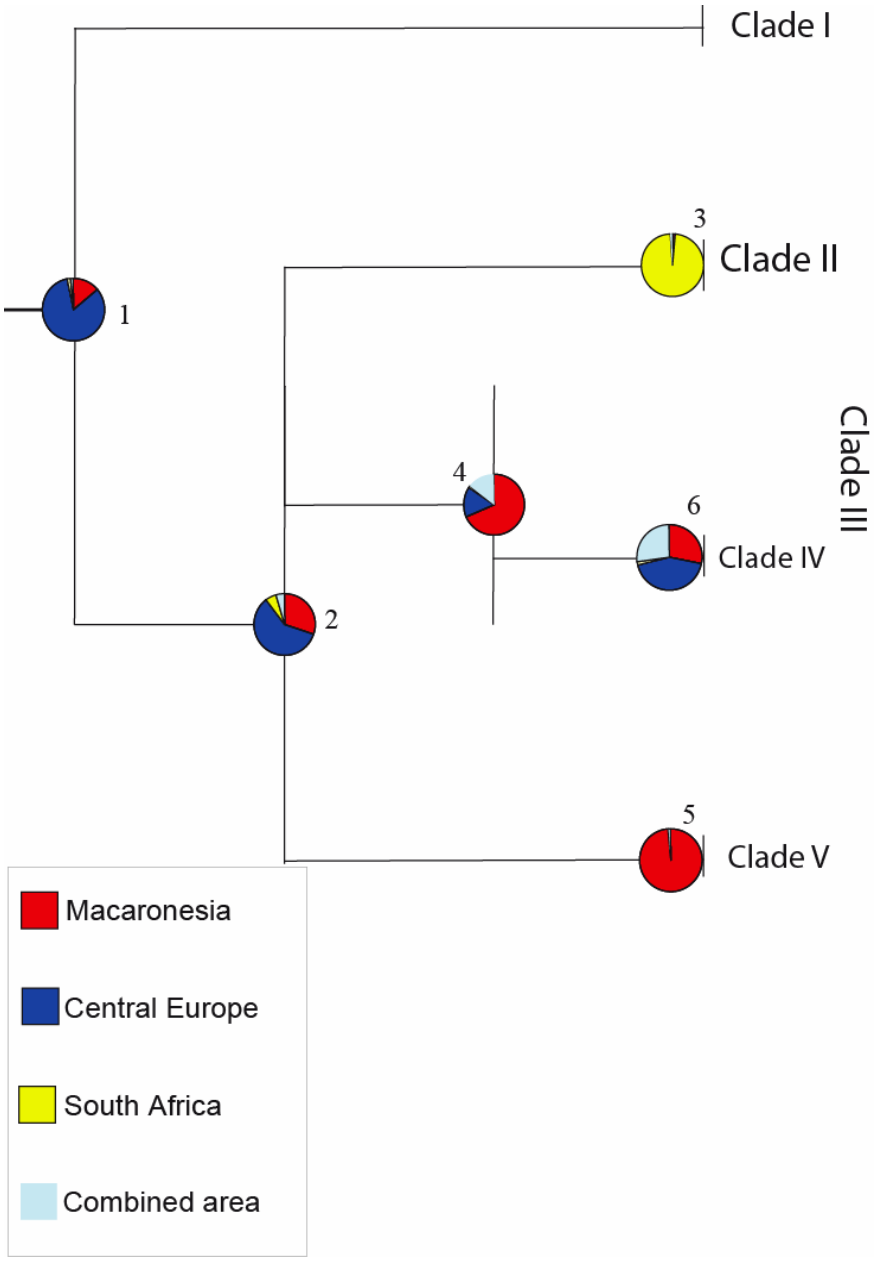


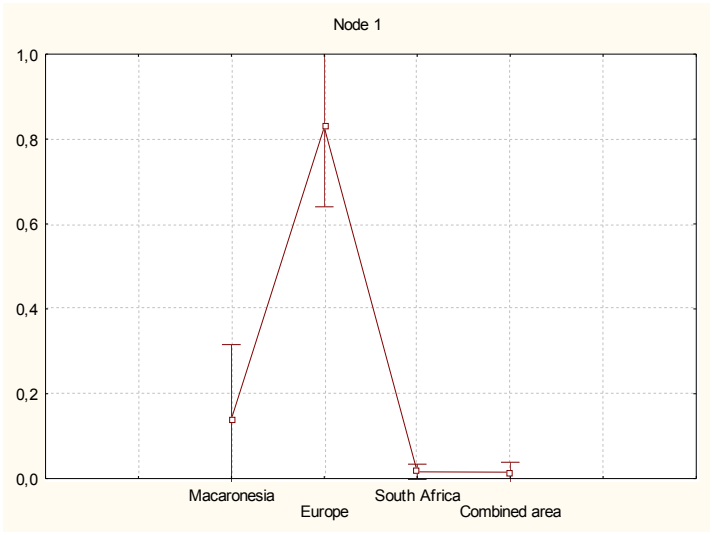
Fig 12: **Haplotype network of R. complanata derived from the variation of a sample of specimens covering the entire distribution range of the species at four cpDNA loci. Each step corresponds to a mutation and white circles to non-sampled or extinct halotypes. Haplotypes are represented by pie diagrams whose size is proportional to the haplotype frequency across the whole sampling and coloured areas (legend top left) provide information on the haplotype frequency distribution across all the geographic regions where it occurs.**

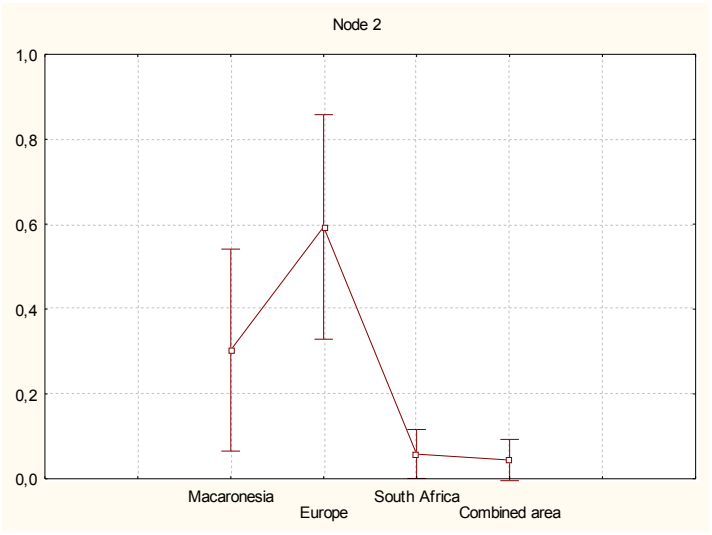
Ancestral distribution reconstruction.

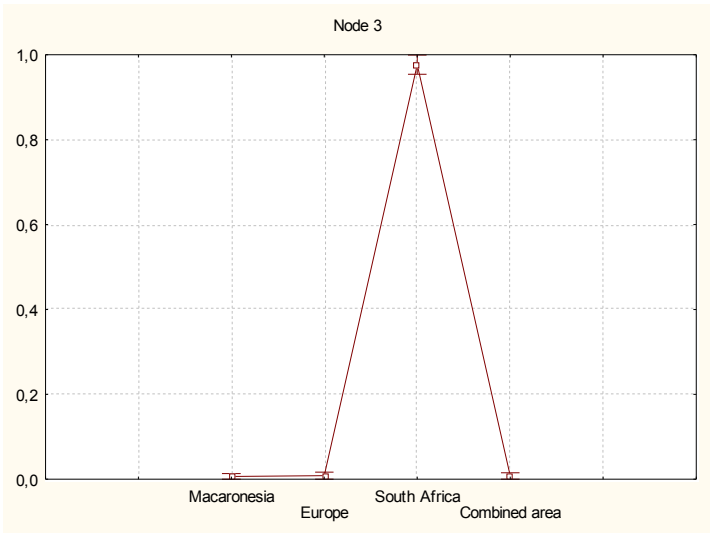
The reconstruction shows that the most probable ancestral area of distribution at the root of the tree is Europe with a posterior probability of 83%. The most recent common ancestor of clades II, III and V is reconstructed as mainly European but with less support (59%). The ancestral distribution area of clade II and V are unambiguously recognized as South African (97%) and Macaronesian (98%), respectively. Clade III is similarly reconstructed as Macaronesian in origin, but with a lower posterior probability of 68%.. The reconstruction at clade IV is ambiguous and involves three areas with similar posterior probabilities.

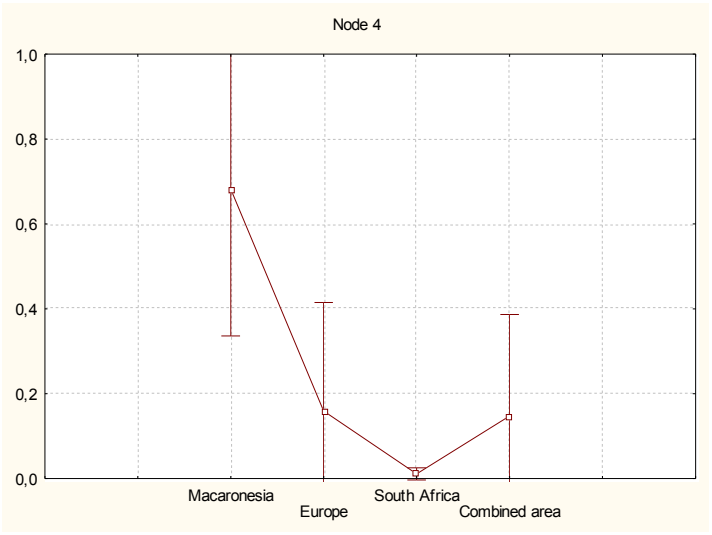
Fig 13: Ancestral area reconstruction represented on a simplified tree from Fig 10. Mean posterior probabilities of geographic range (see colour labels) at internal nodes are indicated by a pie diagram

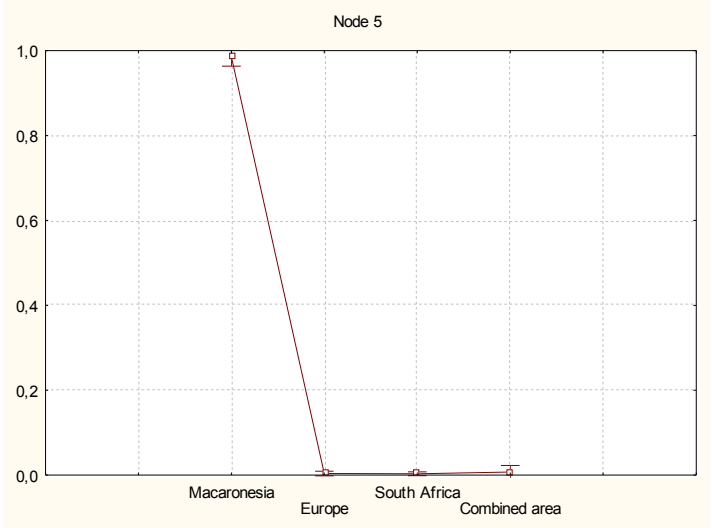












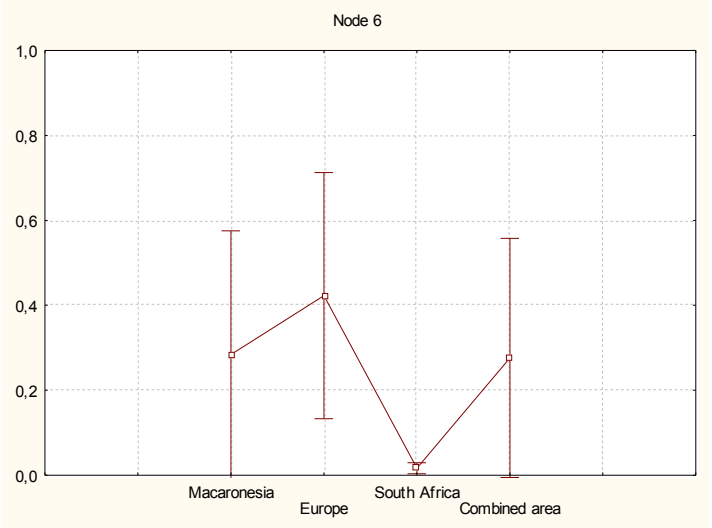


Fig 14: Posterior probability distribution of ancestral distribution ranges at selected nodes of the phylogeography of R. lindenbergiana. Dots represent means and intervals represent the 95% intervals of confidence. Node numbers correspond to those in Fig 13.

Discussion

Significance of the mating system in the structure and genetic diversity of R. complanata and R. lindenbergiana

The present comparative study of two *Radula* species gives some insight into the genetic structure of two bryophytes with different reproductive strategies but similar distribution ranges and ecological requirements. Two main differences in genetic diversity and structure were observed between the two species.

The first main difference between *R. complanata* and *R. lindenbergiana* is the weakness of the geographic signal present in the data in the former, as evidenced by the marginally significant global Fst, and the absence of any phylogeographic signal. Due to the presence of male and female gametangia on the same plant, the production of sporophytes is indeed very frequent in *R. complanata*, which is a crucial feature in the dispersal ability of the species. In fact, whilst both species produce masses of gemmae, which mostly support the growth of local populations, spores are abundantly produced only by *R. complanata*. The spores are involved in long-distance dispersal, as evidenced by both correlative investigation between species dispersal ranges and spore viability (Van Zanten, 1978) and in-situ experiments (Kimmerer, 1993, Kimmerer, 1994). The high potential for long-distance dispersal of *R. complanata* may account for its wide-ranging haplotypic distribution pattern. In particular, frequent events of dispersion are likely to erase the phylogeographic structure in the species as evidenced by the non-significant Nst value and the star-shape phylogeography. No signature of range disjunction was even detected among trans-Atlantic disjunct populations. This contrasts with the marked genetic differentiation previously found among North American and European populations of most trans-Atlantic disjunct bryophyte species (Shaw et al., 2003b, Huttunen et al., 2008). The absence of any trans-oceanic genetic differentiation in *R. complanata* is suggestive of intense long-distance dispersion, as already observed in cosmopolitan, weedy species (e.g., *Tortula muralis* (Werner and Guerra, 2004); *Ceratodon purpureus* (Mc Daniel and Shaw, 2005) or species with many, small spores such as in the Polytrichaceae (Van der Velde and Bijlsma, 2003), which are characterized by the absence of any structure in their global phylogeography.

In the dioicous *R. lindenbergiana* by contrast, spatial patterns of genetic variation are completely different and suggestive of dispersal limitations by distance. For instance, the global Nst is significantly higher than the global Fst, which suggests that mutation rates are higher than dispersal rates in the species. The presence of a phylogeographic signal is evident upon examination of the haplotype distribution map. The most striking transcontinental range disjunction in *R. lindenbergiana*, i.e. the Eurasian/South African disjunction, is paralleled by complete haplotypic differentiation. In fact, the South African region shares no haplotype with the other regions. South African haplotypes form a fully supported monophyletic group resulting in high and significant Nst values as compared to all other biogeographic regions. In the haplotype network, the South African clade is linked to haplotype 18 from Madeira, but with many mutational steps in-between. This position on the network therefore appears as indicative of an ancient vicariance event between Macaronesia and South Africa. Similar patterns of disjunction between Macaronesia and Southern Africa have been reported in angiosperms. For example, the Macaronesian endemic genus *Phyllis* is nested within an African clade (Anderson *et al.*, 2001) and the Macaronesian endemic species *Ocotea foeten*s is sister to the South African *O. bullata*, *O. grayi* and *O. malcombery*. This ancient disjunction can be attributed to two main factors. First, the separation between South Africa

and Macaronesia may represent a relict of a Tertiary flora that was more widespread and has undergone range contraction when the climate became cooler and drier (Chanderbali et al., 2001, Frahm, 2005). Second, the isolation of South African haplotypes can be the result of an ancient long distance dispersal event. In the absence of fossil record of *R. lindenbergiana*, however, these two scenarios are impossible to tell apart. Within Eurasia, a sharp haplotypic differentiation is evident along an East-West gradient. This differentiation, which occurs along the geographical barrier of the Pyrenees, is supported by high Fst values between Central Europe and all of the western regions

In a previous investigation on the evolution of mating system in *Radula* (Devos, pers. comm.), the most recent common ancestor of *R. complanata* and *R. lindenbergiana* was reconstructed as being dioicous. This indicates that the monoicous condition of *R. complanata* is derived, thereby supporting Schuster's hypothesis of a general evolutionary trend from dioecy to monoecy. In the case of *R. complanata*, the gain of a monoicous condition seems to have been paralleled by an increase in dispersal ability associated with a sharp increase in sporophyte production. Thus, although the significance of the evolution of mating systems in bryophytes would require additional comparative phylogeographies between monoicous and dioicous species pairs, the results presented here suggest that innovations in the mating system provided to the recently evolved monoicous species an adaptive advantage in terms of dispersability.

The second major difference between the genetic patterns of *R. complanata* and *R. lindenbergiana* is the strikingly lower diversity of the former. In fact, only eight haplotypes were found in *R. complanata*, vs. 21 in *R. lindenbergiana*, which indicates a lower genetic diversity in the monoicous taxon. Since nucleotide substitution rates are believed to be highly autocorrelated among sister species, which is one of the major assumptions behind Sanderson's popular non-parametric rate smoothing for molecular dating (Sanderson, 1997), the observed difference in genetic diversity between the two species must be attributed to differences in their evolutionary history, i.e., a more recent origin of modern haplotypes of *R. complanata.*

In fact, it has been shown that the pattern of present plant distributions in Europe is essentially due to the quaternary glacial-interglacial cycles (Hewitt, 1999, Medail and Diadema, 2009). During the glacial period, the range of species shifted southwards into the Mediterranean region (Petit *et al.*, 2003) within suitable areas such as protected valleys. When the climate became warmer, species began to re-colonize the northern part of Europe from the southern refugia, which are typically characterized by a high genetic diversity. In fact, re-colonization occurs through founder events, which implies that migrants represent a small and random sample from a more diversified population (Petit *et al.*, 2003). Northwards re-colonization principally occurred along three pathways from the southern refugia, as summarized by Hewitt (2000). Those routes from southern European peninsulas are known as the hedgehog, grasshopper and bear re-colonization patterns. However, the position of refugia and the re-colonization routes differ considerably from one species to another, depending notably on their dispersal ability. In bryophyte, northern refugia have been detected in several taxa, with a possible refuge in south England (Natcheva and Cronberg, 2003, Van der Velde and Bijlsma, 2003).

In *R. complanata*, only a single putative refugium was identified in East-Central Europe (Czech Republic) based on its higher haplotype diversity than the other European regions. Willis and Niklas (2004) indeed found fossils of broad-leaf trees (*Fagus sylvatica,*

Ulmus, Populus, Salix and Betula) in the Czech republic, which are precisely amongst the preferred phorophytes of the species. By contrast, examination of the patterns of genetic diversity in *R. lindenbergiana* across its distribution ranges reveals three 'hot-spots' of diversity and haplotypic endemism, namely Macaronesia, the Iberian Peninsula, and South Africa. The presence of more refugia that are genetically more diverse in *R. lindenbergiana* than in *R. complanata* suggests that the former experienced less drastic reductions in population size during the glaciations, resulting in a present higher recovery of the species in terms of genetic diversity.

Cryptic speciation: hidden diversity on islands.

Among the tree hot-spots of genetic diversity identified in *R. lindenbergiana*, Macaronesia is, with nucleotidic diversity levels of 0.89 and 0.90 in the Canaries and Madeira, respectively, the most prominent. In fact, despite the lack of any apparent morphological differentiation between insular and continental populations of *R. lindenbergiana*, the molecular data presented here unambiguously point to the evolution of multiple endemic haplotypes in Macaronesia. The striking star-shape of clade V on the haplotype network is consistent with the interpretation that the group underwent a rapid radiation, much similar to what has been recurrently reported amongst the angiosperm flora (Emerson and Kolm, 2005). The situation is comparable for clade III, which is the second main lineage of Macaronesian endemic radiation of the species. This diversification pattern, which is at first sight consistent with the expectations of Engler's refugium model, offers one explanation for the apparent lack of radiation amongst Macaronesian bryophytes: that is, bryophytes exhibit reduced morphologies as compared to angiosperms and their diversification is not necessarily paralleled by morphological differentiations. This phenomenon, known as cryptic speciation, has increasingly been reported in bryophytes (Shaw, 2001).

As opposed to what has been observed among many angiosperms, wherein the bulk of endemics are restricted to a single island (Vanderpoorten et al, in prep), the Canarian endemic haplotypes of *R. lindenbergiana* occur across several islands. This observation, along with the very low Fst among islands, but also with North Africa and the Iberian Peninsula, which are suggestive of intense dispersion, contradict the hypothesis that Macaronesian bryophytes failed to radiate owing to their high dispersability (Vanderpoorten *et al.*, 2007). The evolution of multiple Canarian endemic lineages within *R. lindenbergiana* can be interpreted as a consequence of island dynamism and the presence of a high number of niches (Emerson and Kolm, 2005). In fact, islands are dynamic entities, where volcanism and perturbations can create new opportunities for species to diversify. Local extinction, followed by re-colonization leading to vicariance event, has been demonstrated in many Hawaiian biota (Roderick and Gillespie, 1998). The dynamic nature of oceanic island habitats might be a crucial feature for the diversification of a pioneer species with low competitive ability such as *R. lindenbergiana*. The species is, furthermore, amongst the most common leafy liverworts in the Canaries, where it can be found across a very wide range of habitats, from dry, xeric exposed lowland rock outcrops within sub-desertic woody *Euphorbia* vegetation, epiphytic or even epiphyllous in the laurel forest, to the highest vegetation belts. Such a wide ecological range might have promoted the evolution of several strains, as recently demonstrated in the aquatic moss *Platyhypnidium riparioides* at the landscape scale (Hutsemekers *et* al., in press).

Within the Azores, by contrast, no endemic haplotype of *R. lindenbergiana* was identified, which is consistent with the observation that radiations in that archipelago are, as opposed to the Canaries and Madeira, almost absent (Carine and Schaefer, (in press)). One interpretation for the lack of endemic radiation on the Azores is that their colonization occurred more recently. As a matter of fact, we only systematically observed *R. lindenbergiana* on secondary habitats in the Azores, e.g. on volcanic rock walls among pasture or even in botanical gardens, but never in laurel forests, where the species is amongst the most dominant leafy liverworts in the Canaries and on Madeira. Furthermore, despite targeted field prospections, we did not find *R. lindenbergiana* on the island of Flores, which is the westernmost island and arguably the one characterized by the lowest levels of human disturbance. These observations are consistent with the idea that *R. lindenbergiana* has colonized the Azores very recently, where it was perhaps accidentally introduced, and did not diversify because all niches were already occupied by other *Radula* species, especially *R. carringtonii* and *R. aquilegia*, which are particularly abundant on the archipelago.

The biogeographic history of R. lindenbergiana: revisiting Engler's model

Present-day genetic diversity in *R. lindenbergiana* is partitioned between a South-western group comprised of the Iberian Peninsula, North Africa and Macaronesia, and a second, widespread group comprised of the remaining of Europe from the Pyrenees to the Caucasus and Asia. This separation is supported by high and significant Fst between the two groups. A phylogeographic study between two peat mosses in the genus *Sphagnum* (Szovenyi et al., 2007, Szovenyi et al., 2006) reveals the same pattern of disjunction between the Atlantic coast and the eastern part of Europe. This shows that the separation between an Atlantic fringe and a more continental group in bryophytes may be a recurrent pattern of distribution, as evidenced by the significance of the hyper-Atlantic element within the European bryophyte flora (Hill and Preston, 1998, Rothero, 2005).

In *R. lindenbergiana*, the separation between the two groups occurs at the base of the tree and is characterized by many mutation steps, suggesting an ancient vicariance event. The most widespread group is, by comparison with the South-western one, genetically depauperate with only two haplotypes, suggesting that populations from this area underwent severe bottlenecks during the glaciations. In fact, only a single haplotype is dominant from France to Asia, suggesting survival of a much reduced population within a refugium with subsequent fast post-glacial expansion that resembles the fast radiation of the monoicous *R. complanata*. In this regard, the dual mating of *R. lindenbergiana*, which produces both vegetative gemmae and spores, might be significant to explain the ability of the species to disperse as such fast rates when no oceanic barrier involving long-distance dispersal occurs.

The South-western group is genetically much more diversified. This high diversity, which culminates in Macaronesia and in fact includes many endemic haplotypes to the region, is consistent with the expectations of Engler's refugium model. Such a hypothesis is further supported by the reconstruction of the ancestral distribution range of *R. lindenbergiana* as European at the root, in full agreement with a European origin for more than 90% of Macaronesian endemic angiosperms (Carine *et al.*, 2004). The resolution of two independent clades of Macaronesian haplotypes suggests that Macaronesia was colonized at least twice. In fact, the reconstruction of clade III and V shows that the ancestor of both clades was exclusively present in Macaronesia and the network shows that Macaronesian haplotypes do not have a monophyletic origin. Furthermore, constrained analysis demonstrates that

Macaronesian haplotypes are not monophyletic but derive from an ancestral European gene pool.

The high diversity found among Macaronesian haplotypes, together with the Macaronesian origin of all the haplotypes found in Western Europe, suggests that Macaronesian archipelagos could have served as a potential refugium during the Quaternary glaciations and as a potential sink for re-colonization of Europe. Together with the case of the angiosperm genus *Convolvulus* (Carine et al., 2004), which shows an identical pattern of back-colonization from Macaronesia to Europe, this is the first time that Macaronesian islands are considered as a potential refugium during the Quaternary glaciations, from which the re-colonization of Europe occurred. In fact, the evolutionary histories of the Macaronesian and European floras have traditionally been thought as being uncoupled, with spectacular radiations amongst some elements of the Macaronesian flora leading to the unique patterns of diversity observed today.

The results of the present analyses suggest that Europe has been colonized from Macaronesia twice along different routes, one by a member of clade V and the other by members of clade III. Haplotype 3 from clade V colonized the Mediterranean coast of Spain and France with however, a disjunctive presence in the UK. This disjunction could be explained by a long dispersal event. Within clade III, there have been three different colonizations, demonstrating the high connectivity between Macaronesia and the European Atlantic coast, as revealed by the Fst analysis. Haplotype 1 and 2 are both present in Northern Morocco and West Spain, which is indicative of a similar colonization pathway. It is noteworthy that haplotype 2 gave raise to an endemic haplotype (20) in Ireland. This is characterized by only a single microsatellite insertion-deletion event. Since microstatellite regions are assumed to evolve at a fast rate (Lee *et al.*, 2007), this further suggests a recent origin of the back-colonization to Europe from Macaronesia. Finally, a last colonization of Spain was achieved by haplotype 5, which is shared between Spain and Gran Canaria. Those multiple colonizations from Macaronesia emphasise the major role of Atlantic islands as sinks of biodiversity for the post-glacial re-colonization of Europe. Caujapé-Casteel (2004) founds similar results in the angiosperm genus *Androcybium*, which persisted within a glacial refugium in the Canaries during the Pliocene before re-colonizing North Africa. However, as opposed to *R. lindenbergiana*, there was only one re-colonization limited to North Africa. Many bryophyte species exhibit a disjunct hyper-Atlantic distribution pattern (e.g., the moss *Myurium hochstetteri*; the liverworts *Radula carringtonii* and *R. holtii*) between Macaronesia and the westernmost fringe of Europe (Western Scotland, Ireland and Portugal), and this suggests that the significance of Macaronesia as a major refuge for the European bryophyte flora that allowed its post-glacial re-colonization might have been completely ignored.

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Annex I

First report of chloroplast heteroplasmy in bryophyte: mixing stories of two liverworts in the genus Radula.

Introduction

Despite of its small size (150 kbp (Clegg et al., 1994)) and lower evolution rates (Clegg, 1993, Clegg et al., 1994), the chloroplast genome has been widely used for reconstructing molecular phylogeny in plants. (Soltis et al., 1999, Shaw et al., 2005). Some reasons explain the pre-valence of the chloroplast genome on the mitochondrial and nuclear one, even if the later represent a much more source of information in regard to its larger size (Bennett et al., 2003, Bennett and Leitch, 2005, Leitch et al., 2005, Soltis, 1998) and wider range of gene and non-coding regions. However, working on the nuclear genome raises the risk to deal with multiple gene copies, often paralogous (Page, 2000, Page and Charleston, 1997), which can lead to contradictory pattern in gene trees compared to species trees. (D'Erchia et al., 1996). The doubt about the utility of nrDNA in phylogenetic studies (Alvarez and Wendel, 2003, Bailey et al., 2003, Razafimandimbison et al., 2004) is also supported by the discovery of gene duplication event resulting in the formation of pseudogene copy. That pseudogene can have experienced incomplete homogenization that can blur the phylogenetic signal in the data. The use of nuclear low-copy was argued to counter the restriction about utilization of nrDNA in phylogeny. However, the level of polymorphism is not constant among plants and when a locus gives sufficient information for one group; it is relatively not variable for another. (Sang, 2002)

Consequently, most of the current phylogenies are based on cpDNA which is easily amplified by PCR reactions and where universal primers are available for all groups of plants *(Taberlet et al., 1991, Demesure et al., 1995, Dumolin-Lapegue et al., 1997).* Chloroplast genome is thought to be mostly composed of unicopy gene (Soltis, 1998) and two assumptions dominated the scientific community about cpDNA. Firstly, the chloroplast is considered to be uniparentaly inherited in land plants *(Birky, 1995, Corriveau and Coleman, 1988)* and no recombination occurs within (Palmer et al., 1988, Doyle, 1992, Vogl et al., 2003, Clegg, 1993, Wolfe and Randle, 2004). These two assumptions have been widely accepted as they simplify questions about uses of cpDNA sequences in phylogeny.

Biparental inheritance seems to occur in one third of the angiosperms but mechanisms which avoid the coexistence of two different chloroplasts in one individual (heteroplasmy) have been proposed to explain the maintain of homoplasmy (Wolfe and Randle, 2004). These mechanisms are selection against deleterious mutations, vegetative sorting, or differential disintegration of organelles or organellar DNA in the zygote.

However, a growing amount of evidence counterbalances these two hypotheses homoplasmy (Wolfe and Randle, 2004). For example, many case of mitochondrial heteroplasmy have been reported homoplasmy (Wolfe and Randle, 2004) while chloroplastic heteroplasmy has been reported in some instances in flowering plants including Silene, Medicago, Coreopsis and Cynomorium, and in a conifer (*Chamaecyparis*) (Wolfe and Randle, 2004).

The non-recombinant nature of the chloroplast has also been challenged by in vitro somatic cell fusion in *Nicotinia* (Medgyesy et al., 1985). Furthermore, indirect calculation of recombination in natural population has been reported for two conifers (Huang et al., 2001, Marshall et al., 2001) and a rearrangement within cpDNA in an Asteraceae was also observed (Vijverberg et al., 1999). Strong evidence for heteroplasmy and recombination in *Erica arborea* (unpublished) suggest that this phenomenon is maybe more common than previously thought.

The assumption concerning the chloroplast of vascular plants seems to have been expanded to the bryophyte without any convincing evidence. For example, a search on ISI web of knowledge for "heteroplasmy and bryophyte", "heteroplasmy and moss" and "heteroplasmy and liverworts" gives no result, emphasing the fact that the questions about heteroplasmy in bryophytes have not been the subject of great interest. Evidence for uniparental inheritance in the allopolyploid moss *Plagiomnium curvatulum* represents one of the few attempts to prove the inheritance of the chloroplast in bryophyte. As a consequence, few is known about the occurrence of biparental inheritance in bryophyte and nowadays no example of heteroplasmy was discovered in this group.

During a study on a comparative phylogeography between two sisters taxa in the genus *Radula* (liverworts) based on cpDNA, we found evidence for heteroplasmy in multiple loci. This first report of heteroplasmy in bryophyte might indicate that heteroplasmy could be more frequent than previously thought.

Materials and methods

The sampling consists of 113 individuals with 84 and 29 respectively for *Radula lindenbergiana* and *Radula complanata*. The sampling cover nearly the whole range of each species but it was impossible to get material from China and South-East Asia.

DNA was grinded from pool individuals from a dried patch in liquid nitrogen using a Genogrinder2000 (Duke Lab). Extraction was performed on column using a Quiagen column minikit for plants (*QIAGEN*®) and following manufacturer's protocol.

Four loci were used to genotyped all individuals and are namely, *atp*B-*rbc*L, rps4, *trn*G and *trn*L. These loci were selected because of their polymorphism among the Radula genus (N. Devos., pers. comm.). Universal primers as described by Shaw et al (2003) were initially used. Due to the difficulties encountered with the amplification of *trn*L in several accessions, a specific set of primers was designed within conserved regions at the 5' and 3' ends of the molecule. The new primers are *trn*L_F 5'TCAGGGAAACCTAGGGTGAA3' and *trn*L_R 5'CCGGCAATTTTTGTTTCTGT3'.

Polymerase chain reactions (PCR) were carried out in 15 µl volumes reaction using 1.5 µl of 10x reaction buffer, 2.4 µl of dNTPs mix (1 mM each), 0.6 µl of 50 mM MgCl2, 0.75 of each primer (10 µM), 1.125 µl of BSA, 0.3 µl of taq DNA polymerase and 1 µl of DNA. Each of the 35 PCR cycles comprised denaturation at 95° for 5 min; 35 cycles of 30s denaturation at 95°, 45s of annealing at 50°, 2 min of extension at 72° followed by 7 min at 72°. This recipe was used for all genes with some modifications depending on the age of samples and amplification easiness.

Sequences were edited using Sequencher 3.1. In some instances, roughly equal peak height of two nucleotide states suggested superposition of several gene copies, making it necessary to use cloning techniques prior to sequencing. PCR products were cloned using the TOPO-TA kit.

Contigs were constructed from single-stranded forward and reverse sequences using Sequencher 3.1. Sequences were aligned manually using Se-al.2.0a11 (Rambaut, 1995) and gaps were inserted where necessary to preserve positional homology.

Result

Basing on the four cpDNA loci, we identified 21 haplotypes for *R. lindenbergiana* (L1-L21) and 8 for *R. complanata* (C1-C8). Multiple base calls in the chromatographs of the *trn*L sequences were found in sixteen individuals ***(Fig 1)*** in *trn*L and cloning confirmed that multiple copies were present in the concerning samples. In order to verify that multiple alleles were present within individual and not the result of pooled populations, re-extraction was performed on only one shoot to ascertain that only one individual is involved. Sequence additivity was found in all the four loci and the provenance of the two copies was realized by recognition of parental haplotypes. Three different situations were brought to light, first a mix between haplotype L1 and C1 exists in nine individuals and the sequential additivity is found at each of the 12 segregating sites across the four loci. The second case is a mix between haplotypes L2 and C1 found in six individuals and sequential additivity appears at all segregating sites between the two haplotypes, in this case seven positions in *trn*L and *rps*4. The last case involves only one individual and shows a shift in the reading frame due to the presence of both an insertion and deletion event of forty bases pairs in *trn*L. This is also supported by three segregating sites between L1 and L3 in *atp*B-*rbc*L. All the information are summarized in ***Table 1***.

Table 1: Summary of polymorphic sites where multiple base calls occurs.

	TrnL									atpB-rbcL				rps4			TrnG	
	T	T	T	T	A	A	A	G	No Deletion	A	A	G	A	G	T	C	G	C
R. lindenbergiana 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
R. complata 1	A	A	A	A	.	.	.	A	.	G	G	.	G	A	C	.	T	T
Hybrid 1	**W**	**W**	**W**	**W**	.	.	.	**R**	.	**R**	**R**	.	**R**	**R**	**Y**	.	**K**	**Y**
R. lindenbergiana 2	.	.	.	.	T	.	.	A	.	G	.	.	G	.	C	T	T	T
Hybrid 2	**W**	**W**	**W**	**W**	**W**	.	.	A	.	G	.	.	G	**R**	C	**Y**	T	T
R. lindenbergiana 3(39 madeira)	.	.	.	.	.	.	.	.	40BP deletion	G	.	A	G	.	.	.	?	?
Hybrid 3(46)	.	.	.	.	.	.	.	.	########	**R**	.	**R**	**R**	.	.	.	?	?

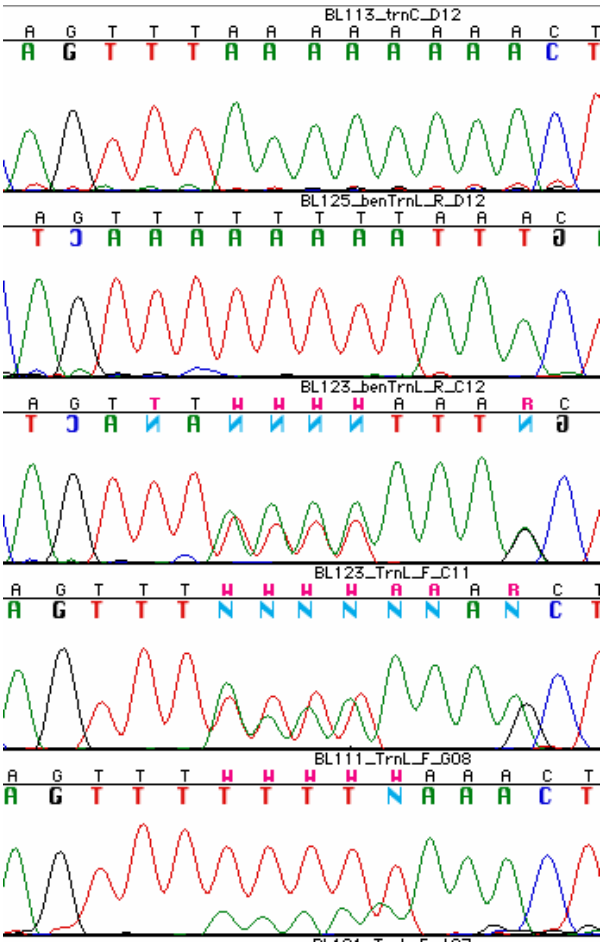

Fig 1: Example of double base calls on electropherogram. The presence of double peaks is indicative of multiple genes in one individual.

Discussion

The results indicate that multiple copies of cpDNA occur in single plants of *Radula*, which raises the questions about the process leading to such a situation. This intra-individual cpDNA polymorphism can be attributed to gene duplication followed by a transfer to the nucleus or to the presence of more than one chloroplast population per individual, namely heteroplasmy. This phenomenon is less uncommon than previously thought and many cases of transfer from organelle to the nucleus has been reported, especially in the mitochondrion (Bensasson et al., 2001) (Martin and Herrmann, 1998) and involves most often regulatory gene relocated in the nucleus (Shadel and Clayton, 1997). Cases of transfer from chloroplast to nucleus have also been reported in plants with for example the holoparasitic *Orobanche cumana* where the *rbc*L gene is present in both the chloroplast and nucleus (Delavault and Thalouarn, 2002). The presence of three cytoplasmic compartments in plants increases the potential transfer between these compartments (Hoch et al., 1991, Stern and Lonsdale, 1982). However, the hypothesis of a transfer to the nucleus suggests that the nuclear copy will follow an independent evolution from the one in the chloroplast resulting in random mutations. The fact that the two copies present in the chloroplast can be attributed to specific haplotypes of *R. lindenbergiana* and *R. complanata* rules out the possibility of a gene transfer to the nucleus and therefore enhances the hypothesis for heteroplasmy in these species of liverworts.

Heteroplasmy raises the question of biparental inheritance in bryophyte, which has never been recorded. The occurrence of biparental inheritance in angiosperm appears to be in one third of the cases. Nevertheless, mechanisms of sorting have been invoked to maintain only one parental copy of chloroplast in the mature plants. (Birky, 1983, Smtih S.E., 1986, Mogensen, 1996). Reports of stable heteroplasmy from biparental inheritance have been shown in *Passiflora* (Hansen et al., 2007), *Oenothera* (Chiu et al., 1988) and *Erica* (unpublished data) and pointed out that sorting out mechanisms are maybe not as efficient than previously thought. In bryophytes, there is no broad scale survey on many taxa on parental inheritance and only examples of maternal inheritance have been demonstrated in the *Plagiomnium curvatulum*. There is no evidence of equivalent sorting out mechanisms in bryophytes and we can therefore hypothesize that biparental inheritance through paternal chloroplast leakage during fecundation, can exist and lead to stable heteroplasmy. Presence of one chloroplast in the sperm of bryophyte was shown by Renzaglia (1987). The organelle shows the reduction of its content, notably thylakoids, in electronic microscopy but remains present until the fecundation and nothing is known about the potential functionality of the chloroplast after sperm germination. In this case, the two cases of heteroplasmy detected need a hybridization event between the two species and the transmission of two parental chloroplasts to the progeny.

Finally, the focus is on the potential common occurrence of chloroplast heteroplasmy throughout the whole land plants group and the possibility that signs of heteroplasmy have not been recognized by phylogenetists because of the widely acceptance of the two assumptions of maternal inheritance and non recombining nature of the chloroplast. This case of heteroplasmy in *Radula* should raise the attention of people working with cpDNA and pointed that multiple base calls in the electropherogram are not necessary ‘noise’ due to Taq polymerase replication error during PCR (Gunther et al., 1998, Jacobs et al., 1999, Tanabe et al., 2002) but could be a sign for the detection of heteroplasmy. Further investigations are needed in order to distinguish an isolate case of heteroplasmy in *Radula* from a more common phenomenon in bryophytes.

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Annex II

Table describing specimen herbarium vouchers, the reproduction system and the number of loci sequenced.

DNA	Genus	Species	Country	Collection Number	Colector	Reproduction system	Nb of amplified loc
3	*Radula*	*complanata*	France	*	A. Vanderpoorten	monoique	4
47	*Radula*	*complanata*	Switzerland	25474	A. Lawalree	monoique	4
49	*Radula*	*complanata*	Scotland	6039	S.L. Jury	monoique	4
59	*Radula*	*complanata*	Ireland	13,096	Theo Arts	monoique	4
64	*Radula*	*complanata*	Switzerland	97/3462	Juul Slembrouck	monoique	4
66	*Radula*	*complanata*	Germany	99/4039	Juul Slembrouck	sterile	4
69	*Radula*	*complanata*	France	8902	Herman Stieperaere	monoique	4
70	*Radula*	*complanata*	Caucasus	120132	V. Vasak	monoique	4
73	*Radula*	*complanata*	Corsica	COR2007/63	A. Vanderpoorten	monoique	4
75	*Radula*	*complanata*	Corsica	COR2007/151	A. Vanderpoorten	monoique	4
85	*Radula*	*complanata*	Germany	5455	R. Düll and R. Ma Ros	monoique	4
91	*Radula*	*complanata*	Spain	14890	R. Ma Ros and R. Monreal	monoique	3
113	*Radula*	*complanata*	Italy	E00286630	D.G. Long	monoique	4
115	*Radula*	*complanata*	Italy	E00286632	D.G. Long	monoique	4
117	*Radula*	*complanata*	Italy	E00286634	D.G. Long	monoique	4
119	*Radula*	*complanata*	France	E00286636	D.G. Long	monoique	4
120	*Radula*	*complanata*	England	E00286629	D.G. Long	monoique	4
121	*Radula*	*complanata*	Scotland	E00108689	D.G. Long	monoique	4
148	*Radula*	*complanata*	Czec Republic	9060	J. Kucera	dioique	4
171	*Radula*	*complanata*	Slovakia	871	J. Kucera	monoique	4
173	*Radula*	*complanata*	Slovakia	515	J. Kucera	monoique	4
180	*Radula*	*complanata*	Austria	4589	J. Kucera	monoique	4
182	*Radula*	*complanata*	Czec Republic	1116	J. Kucera	monoique	4
232	*Radula*	*complanata*	Turkey	48101/h	Papp,B	sterile	4
239	*Radula*	*complanata*	Bulgary	49150/h	Papp,B	monoique	2
249	*Radula*	*complanata*	Montenegro	49200/h	Papp,B	monoique	4
253	*Radula*	*complanata*	Morocco	3096	L. Draper	monoique	2
286	*Radula*	*complanata*	USA	*	N. Devos	monoique	3
306	*Radula*	*complanata*	Canada,	94885	W.B. Schofield, R.J.Belland	monoique	3
1	*Radula*	*lindenbergian*	France	*	A, Vanderpoorten	dioique	4
2	*Radula*	*lindenbergian*	France	*	A. Vanderpoorten	dioique	3
5	*Radula*	*lindenbergian*	France	*	A. Vanderpoorten	sterile	4
6	*Radula*	*lindenbergian*	Gran Canaria	TFCBry 15.283	D.M. Gonzalez-Mancebo, Julio Leal	dioique	4
7	*Radula*	*lindenbergian*	Gran Canaria	TFCBry 15.282	D.M. Gonzalez-Mancebo, Julio Leal	dioique	4
8	*Radula*	*lindenbergian*	Gran Canaria	TFCBry 15.281	D.M. Gonzalez-Mancebo, Julio Leal	dioique	4
12	*Radula*	*lindenbergian*	Gran Canaria	TFCBry 15.304	D.M. Gonzalez-Mancebo, Julio Leal	dioique	4
13	*Radula*	*lindenbergian*	Gran Canaria	TFCBry 15.303	D.M. Gonzalez-Mancebo, Julio Leal	dioique	4
14	*Radula*	*lindenbergian*	Gran Canaria	TFCBry 15.306	D.M. Gonzalez-Mancebo, Julio Leal	sterile	4
15	*Radula*	*lindenbergian*	Gran Canaria	TFCBry 15.307	D.M. Gonzalez-Mancebo, Julio Leal	dioique	4
17	*Radula*	*lindenbergian*	Gran Canaria	TFCBry 15.302	D.M. Gonzalez-Mancebo, Julio Leal	dioique	4
18	*Radula*	*lindenbergian*	Gran Canaria	TFCBry 15.227	D.M. Gonzalez-Mancebo, Julio Leal	dioique	4
19	*Radula*	*lindenbergian*	Gran Canaria	TFCBry 15.279	D.M. Gonzalez-Mancebo, Julio Leal	dioique	4
22	*Radula*	*lindenbergian*	Sweden	39585	Tomas Hallingbäck	dioique	3
24	*Radula*	*lindenbergian*	Madeira	B119391	Lars Hedenäs and Irene Bisang	dioique	4
25	*Radula*	*lindenbergian*	Madeira	B119392	Lars Hedenäs and Irene Bisang	dioique	3
28	*Radula*	*lindenbergian*	South Africa	RSA 03/06	Theo Arts	sterile	4
30	*Radula*	*lindenbergian*	South Africa	RSA 09/32	Theo Arts	dioique	4
31	*Radula*	*lindenbergian*	South Africa	RSA 08/23	Theo Arts	dioique	4
32	*Radula*	*lindenbergian*	South Africa	RSA 18/29	Theo Arts	dioique	4
33	*Radula*	*lindenbergian*	Caucasus	189028	V. Vasak	dioique	3
35	*Radula*	*lindenbergian*	Gran Canaria	189030	V. Vasak	dioique	4
36	*Radula*	*lindenbergian*	Caucasus	120133	V. Vasak	sterile	4
37	*Radula*	*lindenbergian*	Caucasus	88414	V. Vasak	dioique	4
39	*Radula*	*lindenbergian*	Madeira	15646	Theo Arts	dioique	3
43	*Radula*	*lindenbergian*	Madeira	16147	Theo Arts	dioique	2
46	*Radula*	*lindenbergian*	Madeira	B119395	Lars Hedenäs and Irene Bisang	dioique	3
52	*Radula*	*lindenbergian*	Portugal	20,039	Theo Arts	monoique	4
62	*Radula*	*lindenbergian*	Ireland	12,882	Theo Arts	dioique	4
74	*Radula*	*lindenbergian*	La Palma	PALM1526	A. Vanderpoorten	sterile	4
76	*Radula*	*lindenbergian*	Morocco	2812	I. Draper, F. Lara and V. Mazimpaka	dioique	4
78	*Radula*	*lindenbergian*	Spain	P-0200194/303	B. Albertos, R. Garilleti and F.Lara	dioique	4
79	*Radula*	*lindenbergian*	Morocco	*	I. Draper, F. Lara and V. Mazimpaka	sterile	4
96	*Radula*	*lindenbergian*	Morocco	13767	Albertos, Cano, Loy, zimpaka and Ros	dioique	4
97	*Radula*	*lindenbergian*	Morocco	21048	Draper, Lara and Mazimpaka	dioique	4
98	*Radula*	*lindenbergian*	Spain	6396	J. Guerra	sterile	4
101	*Radula*	*lindenbergian*	Spain	10075	J. Guerra	sterile	4
104	*Radula*	*lindenbergian*	France	*	*	dioique	4
107	*Radula*	*lindenbergian*	Majorca	E00286642	D.G. Mann	sterile	4
109	*Radula*	*lindenbergian*	Portugal	E00286644	F. Sales and S. Neves	sterile	4
112	*Radula*	*lindenbergian*	Norway	E00286639	D.G. Long, D. Schill, L. Söderström	dioique	4
116	*Radula*	*lindenbergian*	Portugal	E00286633	D.G. Long	dioique	4
122	*Radula*	*lindenbergian*	Scotland	E00286622	D.G. Long	dioique	2
125	*Radula*	*lindenbergian*	Scotland	E00286607	D.G. Long	dioique	4
126	*Radula*	*lindenbergian*	Scotland	E00286604	D.G. Long	dioique	3
128	*Radula*	*lindenbergian*	Majorca	E00286647	D.G. Long	sterile	4
129	*Radula*	*lindenbergian*	La Palma	E00286649	D.G. Long	dioique	3

DNA	Genus	Species	Country	Collection Number	Colector	Reproduction system	Nb of amplified loc
129	*Radula*	*lindenbergian*	La Palma	E00286649	D.G. Long	dioique	3
130	*Radula*	*lindenbergian*	Belgium	j43414	A. Sotiaux	sterile	4
131	*Radula*	*lindenbergian*	Ireland	1,4298611111	W.Labeij	sterile	4
134	*Radula*	*lindenbergian*	Tenerife	24194	Theo Arts	sterile	3
137	*Radula*	*lindenbergian*	La Gomera	24114	Theo Arts	dioique	4
139	*Radula*	*lindenbergian*	Andorre	81p9477	M. Onraedt	dioique	3
150	*Radula*	*lindenbergian*	Austria	9267	J. Kucera	sterile	4
151	*Radula*	*lindenbergian*	Austria	9417	J. Kucera	sterile	4
154	*Radula*	*lindenbergian*	Czec Republic	8514	J. Kucera	dioique	4
183	*Radula*	*lindenbergian*	Czec Republic	39631	F. Müller	dioique	4
186	*Radula*	*lindenbergian*	La Gomera	k244	F.Mueller	sterile	4
188	*Radula*	*lindenbergian*	La Gomera	k117	F.Mueller	sterile	4
196	*Radula*	*lindenbergian*	El Hierro	TCFBry 17103	D.M. Gonzalez-Mancebo, Julio Leal	dioique	3
197	*Radula*	*lindenbergian*	El Hierro	TCFBry 17104	D.M. Gonzalez-Mancebo, Julio Leal	dioique	4
201	*Radula*	*lindenbergian*	El Hierro	*	D.M. Gonzalez-Mancebo, Julio Leal	sterile	4
202	*Radula*	*lindenbergian*	Fuerteventura	TCFBry 17099	D.M. Gonzalez-Mancebo, Julio Leal	sterile	4
203	*Radula*	*lindenbergian*	Fuerteventura	TCFBry 17098	D.M. Gonzalez-Mancebo, Julio Leal	sterile	4
204	*Radula*	*lindenbergian*	Russia	g1d1152	Nadya Konstantinova	dioique	2
205	*Radula*	*lindenbergian*	Caucasus	k544/3-05	Nadya Konstantinova	sterile	4
207	*Radula*	*lindenbergian*	Caucasus	k525/5-07	Nadya Konstantinova	dioique	4
210	*Radula*	*lindenbergian*	Caucasus	k433/1-08	Nadya Konstantinova	dioique	4
211	*Radula*	*lindenbergian*	Caucasus	k392/2-08	Nadya Konstantinova	dioique	4
212	*Radula*	*lindenbergian*	Russia	102802	Nadya Konstantinova	sterile	4
214	*Radula*	*lindenbergian*	Açores	*	A. Vanderpoorten, A.Désamoré et B.	sterile	4
216	*Radula*	*lindenbergian*	Açores	*	A. Vanderpoorten, A.Désamoré et B.	dioique	4
217	*Radula*	*lindenbergian*	Turkey	48674/h	Papp,B	sterile	4
218	*Radula*	*lindenbergian*	Turkey	48498/h	Papp,B	dioique	2
219	*Radula*	*lindenbergian*	Turkey	48190/h	Papp,B	sterile	4
257	*Radula*	*lindenbergian*	Açores	11/5	A. Vanderpoorten, A.Désamoré et B.	sterile	4
258	*Radula*	*lindenbergian*	Açores	11/7	A. Vanderpoorten, A.Désamoré et B.	sterile	4
259	*Radula*	*lindenbergian*	Açores	11/12	A. Vanderpoorten, A.Désamoré et B.	sterile	4
260	*Radula*	*lindenbergian*	Açores	11/13	A. Vanderpoorten, A.Désamoré et B.	sterile	4
261	*Radula*	*lindenbergian*	Açores	11/15	A. Vanderpoorten, A.Désamoré et B.	sterile	4
267	*Radula*	*lindenbergian*	Açores	9/1	A. Vanderpoorten, A.Désamoré et B.	sterile	4
269	*Radula*	*lindenbergian*	Açores	9/4	A. Vanderpoorten, A.Désamoré et B.	dioique	4
270	*Radula*	*lindenbergian*	Açores	9/13	A. Vanderpoorten, A.Désamoré et B.	dioique	4
278	*Radula*	*lindenbergian*	GranCanaria	GC8	A. Vanderpoorten	dioique	4
282	*Radula*	*lindenbergian*	GranCanaria	GC12	A. Vanderpoorten	dioique	4