Assessment of cophylogenetic patterns between the nematode genus *Parapharyngodon spp.* and their reptile hosts in the Canary Islands

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2015

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Acknowledgments

First I would like to thank my supervisor James Harris for giving me the opportunity to accomplish this amazing goal in life. For all the knowledge that you passed on to me, for being always there to help, guide me and clear all my doubts, and for allowing me to go on field work to one of the most marvellous places on Earth. Thank you James for giving the amazing opportunity of being my supervisor.

I would like to thank Ana Perera, my co-supervisor, for all the knowledge and help that you gave me. For always being ready to sacrifice your own time to help me and for always pushing me forward. Ana, I could never thank you enough everything you did for me during this last year. Thank you for being one of the most incredible persons that I had the joy to meet.

To Fátima Jorge, because without you I wouldn't have accomplished ANY of this. Thank you for all the hours, the emails, the help and the knowledge you gave me. And most importantly, I want to thank you for always being *here*, even though you were in the other side of the planet. I am not even sure if there are enough words to describe how grateful I am for everything you did for me. Obrigada.

To Dr. Vicente Roca, for all the help, knowledge and for receiving me in your lab and make sure that I haven't misclassified the specimens. I also want to thank your lovely family for not only welcoming me and taking care of me, but also for making me feel like I was at home and for showing me the wonderful city of Valencia. Muchas gracias por todo.

To all the Ecolab people, Ana, Coelho, Diogo, Isabel, Henrique, Joana, Joao, Kevin, Sandra and Walter, for making my days a little bit brighter. To Daniela, for being always available and for helping me in everything with everything that I needed (you won the best friend competition!). To Beatriz, for answering all my questions (even the stupid ones) and for being the best field work *roommate* ever. To Victoria, you know that this wouldn't have been the same without you and our silliness and shenanigans.

To everyone that accompanied me during field work, for making sure that I did not died in the middle of nowhere and for helping me search for my (and Beatriz) tent when it suddenly decided to become a bird and flew away.

To the *cool* people of CIBIO, for all the productive conversations during lunch and break time and for always giving me a ride.

To my family, for all the time that you dedicated to me. To my mother, Adelaide, *A Laidita*, for making sure that I always eat and for doing the possible and the impossible for me. To my father, Francisco, *O Chico*, for all his philosophies and for the sacrifices he does for me. For everything that you have given up just to guarantee my comfort and well-being and for always making me a better person I want to thank you from the bottom of my being. To Carolina, for having the kindness of not making my life a living hell this last year. Thank you for always being there for me and for being the best ape … I mean … sister that I could have possibly wished for (and for leaving your mark here). To my grandparents, Antonia, Fernando, Antonio and Laurentina, for being proud of my achievements, for investing in me and in my education and for teaching me *que eu tenho que respirar senao morro*. To my uncles and aunts, Anabela, Sara, Jose, Amilcar, Joao, Rita, Fernando and Rosana for all the advices, help and for being always there for me. To my cousins, Miguel, Francisco, Claudia, Margarida, Isabel, Eduardo, Fernando, Joao and Victor, it would not have been the same without you.

To Miriam, for being the greatest friend ever! Even though we only were together maybe five times during this last year. Thank you for always make me laugh, for teaching me how to handle life in a more relaxed way and for all the advices (especially the ones related with the Law) you gave me.

To Rafa. I want to thank you in every aspect that I can. Not only for supporting and helping me unconditionally, but also for always being there for me even when I get crazy and paranoiac. For making me laugh, smile, and sometimes cry… but mainly for allowing me to be myself. You had the worst summer ever because of me *dude*, that really means a lot to me. You are indeed the best living-being in the entire Cosmos and honestly, I could never have done this without you. Also I want to thank to your beautiful family, D. Patrocinia, Sr. Ferreira and Samuel, for being my second home and always take care of me.

To my kittens, for all the love and *purrs* that you gave me during my entire life, and for making sure that this dissertations was at its best by constantly crossing my keyboard and typing the most brilliant thoughts that a cat could have…. *Df I999999999*.

To everybody that walked with me throughout this hard but amazing journey…

Um enorme Obrigada!

Abstract

Parasites affect some of the most important biological traits at the level of the host representing models of great interest to study coevolutionary patterns. *Parapharyngodon* is a genus of nematodes characterized by small bodies, sexual dimorphism and a direct life-cycle, depending entirely on their hosts to disperse. However, there is an ongoing discussion in the scientific world where some authors argue that *Parapharyngodon* species should be taxonomically classified as belonging to *Thelandros,* while others consider this a sister genus of *Parapharyngodon*. In order to assess the taxonomic status of *Parapharyngodon spp.* an integrative taxonomic approach, using both morphologic and genetic data, was carried. Phylogenetic analyses were performed using both 18S and 28S rRNA nuclear DNA sequences and combined with morphometric statistic tests in order to infer the relationships between *Parapharyngodon* species and the ones of *Thelandros*. Two *Thelandros* species and various *Parapharyngodon spp.* appeared as well-differentiated clades, potentially corroborating the generic status of *Parapharyngodon*. However, "*Thelandros" galloti* was estimated to be a sister species to *Parapharyngodon echinatus,* indicating the need of reassess the generic classification of this species. Other "*Thelandros*" species may also actually belong to *Parapharyngodon*, so the morphological characters used to delimit these groups also need to be redefined. Unexpectedly, *Parapharyngodon micipsae* is most likely a morphotype of *P. echinatus* and *P. galloti* rather than a distinct separate species. Again, this highlights the difficulty of delimiting species of these nematodes using only morphological characters. Although much is known about the morphological and ecological traits of *Parapharyngodon spp.,* little attention has been paid to the phylogeny of this group, or the potential for cospeciation within their hosts. In the Canary Islands *Parapharyngodon* species have been recorded to infect all three extant endemic lizard genera from this islands (*Gallotia*, *Chalcides* and *Tarentola*). DNA sequences from both18S and 28S rRNA nuclear markers were used to estimate the phylogeny of these parasites, which could then be compared to the well-known phylogenetic estimates of the reptile hosts. Two emerging different lineages were revealed, one from the most eastern islands of Lanzarote, Fuerteventura and Gran Canaria and the other comprising the more western islands of La Palma, La Gomera, Tenerife and El Hierro. Concerning the colonization patterns, it seems that this parasites colonized the Canary Islands in multiple independent events possibly partially related to the ones of *Tarentola* ancestral.

Unexpectedly, since the hosts are all endemic to the islands, one sample from a gecko from Morocco forms part of one lineage, again demonstrating the complex nature of the model system. Gran Canaria harbours two sister lineages, one specific to *Tarentola* hosts and the other parasitizing *Gallotia* and *Chalcides* individuals. However, in general it is difficult to relate the estimates of genetic relationships with morphological differentiation, with hosts or even with geographic locations. Still, more studies using faster-evolving mitochondrial markers are needed to better understand *Parapharyngodon* phylogeny and then more accurately infer host-parasite interactions.

Keywords

Canary Islands, *Chalcides*, colonization, *Gallotia*, host-parasite interactions, morphology, *Parapharyngodon*, phylogeny, *Tarentola.* 18S rRNA, 28S rRNA

Resumo

Os parasitas afetam diversos e importantes aspetos biológicos ao nível do hospedeiro, representando, dessa forma, modelos de grande interesse para estudar padrões coevolutivos. *Parapharyngodon* é considerado um género de nemátodos caracterizados pelos seus tamanhos reduzidos, dimorfismo sexual e ciclos de vida direto, dependendo inteiramente no seu hospedeiro para dispersarem. No entanto uma atual discussão no mundo científico tem vindo a questionar o estatuto taxonómico de *Parapharyngodon* onde alguns autores argumentam que as espécies de *Parapharyngodon* devem ser classificadas como pertencentes ao género *Thelandros,* enquanto outros discordam*. De* forma a compreender o estatuto taxonómico de *Parapharyngodon* spp, análises filogenéticas foram elaboradas utilizando os genes nucleares 18S e 28S rRNA e combinado testes estatísticos de morfometria, de forma a inferir quais as relações evolutivas entre as diferentes espécies de *Parapharyngodon* e *Thelandros.* Duas espécies de *Thelandros* e as de *Parapharyngodon* apareceram como clades bem diferenciadas, potencialmente corroborando o estatuto de género relativo a *Parapharyngodon. "Thelandros" galloti* revelou ser uma linhagem "irmã" de *Parapharyngodon*, dessa forma evidenciando a necessidade de um redefinição dos caracteres morfológicos que permitem a delimitação entre *Parapharyngodon* e *Thelandros*. Adicionalmente, outras espécies de "*Thelandros"* podem dessa forma pertencer ao género *Parapharyngodon*, reforçando assim a urgência em reconsiderar a classificação taxonómica destes grupos. *Parapharyngodon micipsae* é possivelmente um morfotipo de *P. echinatus* e *P. galloti* e não uma espécie separada, validando a dificuldade de classificar estes grupos de nemátodos considerando apenas características morfológicas. Apesar do grande output de informação relacionados com as características morfológicas e ecológicas dos indivíduos de *Parapharyngodon spp.* pouca atenção tem sido prestada aos padrões evolutivos destes parasitas e às forças aderentes aos seus hospedeiros que podem causar coespeciação. Nas Ilhas Canárias diferentes espécies de *Parapharyngodon* infetam os diferentes lagartos endémicos destas ilhas (*Gallotia*, *Chalcides* e *Tarentola*). O uso de sequências de DNA relativas aos genes nucleares 18S e 28S rRNA permitiu a inferência da filogenia deste parasita, podendo sequencialmente ser comparados às dos seus hospedeiros. Os resultados revelaram a distinção entre duas linhagens (uma das ilhas mais a este Lanzarote, Fuerteventura e Gran Canaria e a outra das ilhas mais a oeste La Palma, La Gomera,

Tenerife e El Hierro). Analisando os padrões de colonização das Ilhas Canárias parece que estes parasitas colonizaram estas ilhas em eventos múltiplos e independentes, possivelmente, e parcialmente, relacionados com os dos ancestrais de *Tarentola*. Ainda assim, uma amostra recolhida num gecko em Marrocos integra a linhagem de Fuerteventura e Lanzarote, reforçando a natureza complexa deste sistema. Gran Canaria alberga duas linhagens "irmãs", uma especifica de *Tarentola* e a outra especifica de parasitas encontrados em *Gallotia* e *Chalcides*. No entanto, mais estudos utilizando genes com uma maior taxa de mutação (genes mitocondriais) são necessários para uma melhor compreensão da filogenia de *Parapharyngodon* e dessa forma compreender melhor as diferentes interações hospedeiro-parasita.

Palavras-chave

Chalcides, colonização, filogenética, *Gallotia*, interações hospedeiro-parasita, Ilhas Canárias, morfologia, *Parapharyngodon,* parasita, *Tarentola,* 18S rRNA, 28s rRNA

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List of Abbreviations

Km: Kilometre

Mya: Million years ago

rRNA: ribossomal RNA

RNA: Ribonucleic acid

DNA: Deoxyribonucleic acid

BI: Bayesian inference

NJ: Neighbor-joining

MP: Maxium Parsimony

ML: Maximum likelihood

MCMC: Markov Chain Monte Carlo

BL: Body lengt

BW: Body width

TL: Tail length

NR: Nervous ring

OBL: Oesophageal bulb length

OBW: Oeshophagael bulb width

OL: Esophagus length

OW: Esophagus width

LAL: Lateral alae length

LAW: Lateral alae width

TW: Tail width

SS: Spicule shape

Spi: Spicule length

SW: Spicule width

VL: Vagina length

Vu: Vulva position

EL: Egg length

Ela: Egg average length average

EW: Egg width

EWa: Egg average width

General Introduction

Parasite-host interaction as models of coevolution

In a strict and more conventional definition a parasite is a living being that spend a significant amount of time depending on a given specie to feed and live (Poulin and Morand, 2004). Although parasites have great impact at vary function levels of the biosphere (Combes, 2001) its importance is usually neglected and the recorded scientific studies concerning parasite usually have the ultimate goal of eradicating this species (Poulin and Morand, 2004). However parasites usually affect important traits at the level of the host (Combes, 2001), representing exciting models to understand ecological and evolutionary processes not only at the level of the parasite itself but also at the level of the host. Parasitism has evolved in a way where the outcome cost-benefit resulting from an inter-species biological interaction is favourable to the parasitic living-form, and where parasite benefit directly from its host specific life traits. In general parasitic organisms need their hosts to fulfil their needs in at least one of the following aspects: habitat, motility or energy (Combes, 2001). Although the host-parasite interaction may be advantageous to one of the involved forms on the other hand this interaction may result in disadvantages to the host, even ultimately causing its death. However host organisms, per se, are equipped with mechanisms that that play an important role minimizing parasite infection – for example the immune system. In addition, parasites to survive also need to respond and adapt to other host characteristics: host discontinuity in space (host abundance) and time (hosts mortality), and host evolution (Combes, 2001; Huelsenbeck et al., 2003). This process of long term durable interaction leads the parasite to evolve in an "arms race" with their host resulting in a coevolutionary process (Page, 2003).

Coevolutionary forces were first noticed and documented by Charles Darwin in his book "Fertilisation of Orchids" (Darwin, 1877), and nowadays coevolution represents a subject of high interest in the scientific world including thousands of publications in a panoply of topics that cover biological studies, methodological developments and reviews on specific issues (e.g. Ehrlich and Raven, 1964; Janzen, 1966; Taper and Chase, 1985; Dietl, 2003). Coevolution is a widely studied topic and can occur in the form of a (i) mutualistic or symbiotic interaction, where both parties' gain advantages from the association, (ii) prey-predator model or, in this case, (iii) host-parasite interaction.

Host-parasite interactions represent an exciting model to study coevolutionary patterns (Page, 2003). Associations between a given host and their parasite might arise by direct heritage from ancestral species (association by descent) or by host-switching events (association by colonization; Brooks and McLennan, 1991). However, perfect phylogenetic matches between host and parasite are rarely found and congruent coevolutionary patterns between a given host and its parasite is not the rule but the exception (Vienne et al., 2013). Indeed, parasite phylogeny rarely mirror the one of their host since the parasite might switch from one host to another, speciate independently, go extinct, fail to colonize all descendants or fail to speciate (Figure 1).

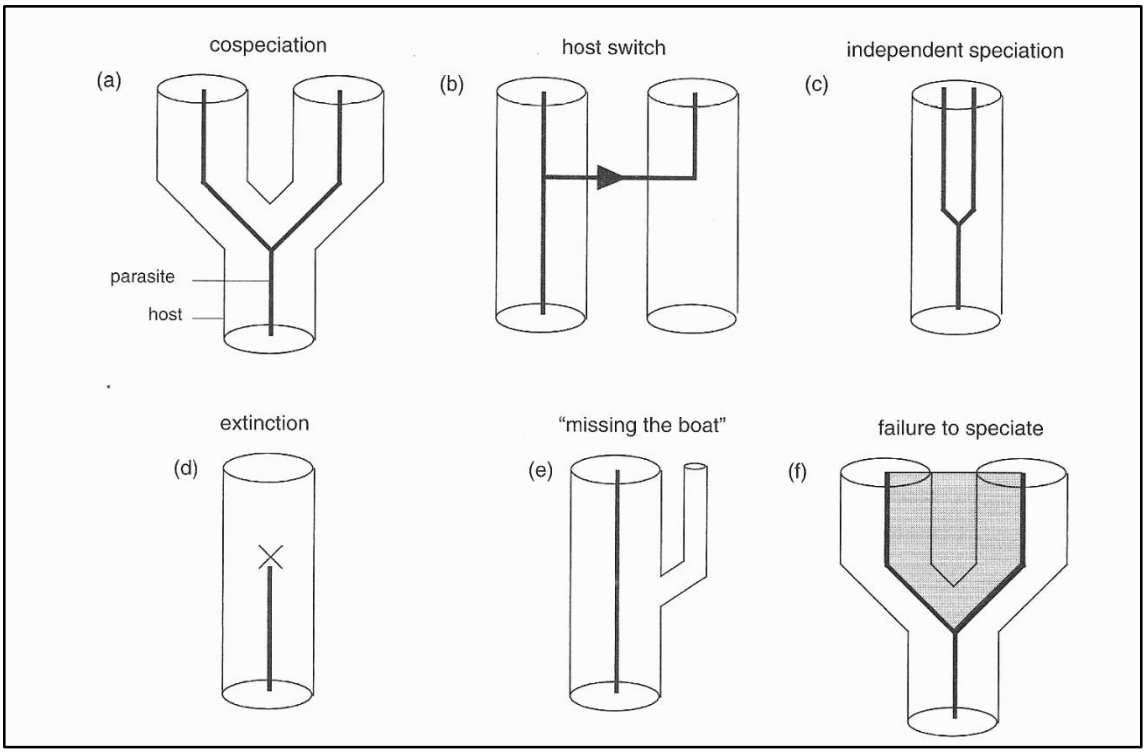

Figure 1 – Processes in host-parasite association. The different scenarios represent cospeciation between the host and parasite (a), host-switching (b), independent speciation of the parasite within the same host (c), extinction of the parasite (d), absence of a parasite in a host lineage (e) and host speciate independently from the parasite (f) (From Page, 2003).

The study of cophylogenies combine species associations, molecular systematics and historical biogeography to infer the level of congruence between tightly associated organisms – e.g. parasite-host cophylogeny – (Balbuena et al., 2013). However, scientists still debate which are the most reliable techniques to properly analyse cophylogenies. Cophylogenetic analysis can be classified in event-based methods and global-fit methods (Desdevises, 2007). Event-based methods consist in

finding the most likely coevolutionary pattern of the related taxa and several approaches such as Brooks' Parsimony Analysis (Brooks, 1981), PACT (Wojcicki and Brooks, 2005) and TreeMap (Charleston and Page, 2002) have been proposed. However, event-based methods are very computationally demanding and require full resolved phylogenies and additional data – e.g. node ages and geological history - that may represent a challenge to obtain (Balbuena et al., 2013). On the other side, global-fit methods have the potential to quantify the congruence between two phylogenies although they do not evaluate, directly, evolutionary scenarios (Balbuena et al. 2013). Methodologies such as PACo (Balbuena et al., 2013), ParaFit (Legendre et al., 2002) and HCT (Hommola et al., 2009) represent some examples of global-fit methods.

Although scientists have been mostly focused on the analysis of a given host phylogeny and its parasites, little attention has been paid to the coevolution of a single parasite species on multiple hosts (Banks and Paterson, 2005). Parasites infecting multiple hosts are relatively common and several explanations have been proposed in order to clarify this phenomena (Figure 2): cryptic parasite species (two species of parasites that are actually classified as a single species because there were not found morphological differences between populations), parasites morphological convergence (different parasites species that are erroneously classified as a single one due to morphological similarity), recent or ancient host switching, incomplete host switching and parasite inertia (when a parasite does not speciate when a host does) (Banks and Paterson, 2005). Also, misclassified hosts might lead to such patterns of multi-host parasitism. Multi-host parasites represent a challenge for analysis given that most cophylogenetic methods cannot deal with such interactions (Banks and Patterson 2005). However alternative approaches such as the creation of *dummy lineages* (Brooks et al., 2004) or the use of parsimony principle approaches (Hugot et al., 2001) can be helpful to unravel this problem, especially in cases of cryptic speciation. Moreover, the recognition of the processes that are causing an organism to parasitize several hosts is crucial to understand the parasite distribution in terms of host (Banks and Paterson, 2005).

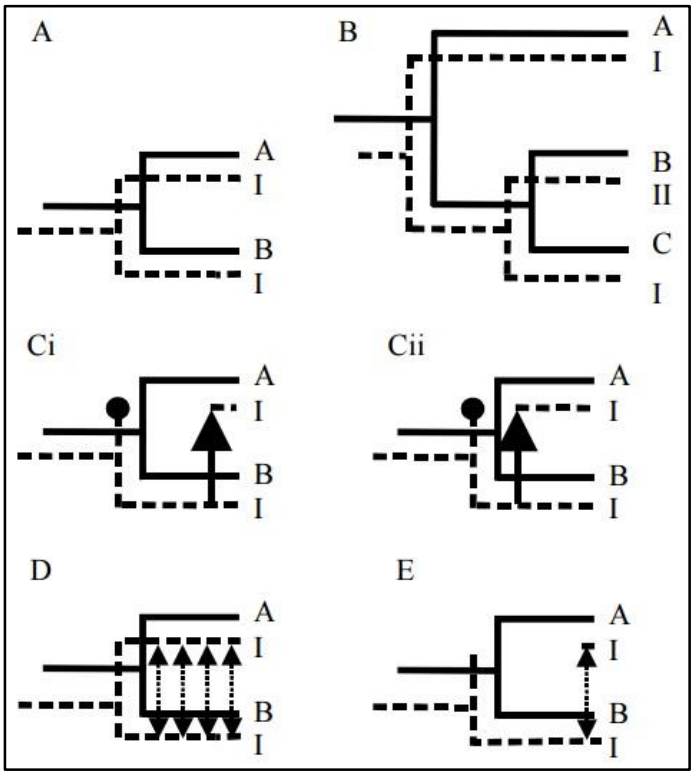

Figure 2 - Phylogenies for the host - solid lines - and parasite - broken lines - representing the processes that can produce multi-host parasitism. The different scenarios represent cryptic speciation (A), morphological convergence (B), recent (Ci) and ancient (Cii) host switching, failure to speciate (D) and incomplete host switching (E) (From Banks and Paterson, 2005).

Cophyogenetic analysis might also provide important clues in resolving the evolutionary history of the host (Rannala and Michalakis, 2003). Using parasites as a proxy to reveal evolutionary patterns of the host is especially useful when data from the host show high ancestral polymorphism or lack population structure (Nieberding and Olivieri, 2006). However this methodology has shown to be more effective when the generation time and parasite population size is smaller than the one observed in the host (Nieberding and Oliveri, 2006). Furthermore, inference of the host phylogeny is stronger when using genetic data from parasites that transmit vertically rather than horizontally (Whiteman and Parker, 2004). Although some works have been published using this approach, studies using several parasites that infect a given host are also needed in order to assess congruent patterns that may clarify important historical and evolutionary events occurring at the host level (Nieberding and Olivieri, 2006).

Canary Islands

Islands represent a useful model to study species evolution because of their distinct geological processes that originated isolated environments where the water surrounding them acts as strong barriers to typical nonvolant terrestrial species dispersal, interrupting gene flow. Moreover, in many cases, the diversity of habitats resulting from geological history makes islands the perfect scenarios for the occurrence of endemic species (Emerson, 2002).

The Canary Islands are one of the best-studied island system in the world, both in term of their geological history, but also concerning the origin of its biodiversity (Sanmartín et al., 2008). This archipelago is part of the Macaronesian islands, a group of archipelagos of volcanic origins. It is located approximately 110 km northwest from the African coast, surrounded by the Atlantic Ocean and is comprised by seven main islands: El Hierro, La Palma, La Gomera, Tenerife, Gran Canaria, Fuerteventura and Lanzarote (Figure 3). Except Lanzarote and Fuerteventura (that are separated by shallow waters with less than 200 meters depth; Fernández-Palacios and Anderson, 1993; Sanmartín et al., 2008), these islands are separated by deep oceanic platforms and have never been connected to the mainland, although Lanzarote and Fuerteventura would probably have been connected at some point due to the shallower sea levels between them (Sanmartín et al., 2008). The islands constituting the Canary archipelago have different origins according to a temporal gradient from East to West where the eastern islands are older than the western ones. According to the estimates, the oldest islands(Lanzarote and Fuerteventura) emerged about 20 million years ago, while the youngest islands of La Palma and El Hierro are only a little over 1 million years old (Guillou et al., 2004; Ancoechea et al., 2006; Sanmartín et al., 2008). The estimated island historical ages can be seen in Figure 3.

Formation of the Canary Islands is however controversial. While it is mostly accepted the theory stating that these islands were formed because of the slowly north-east movement of the African Plate over a volcanic hotspot in the Atlantic Ocean (Carracedo et al, 1998; Guillou et al. 2004), some authors have proposed alternative formation scenarios; according to some authors this archipelago could in fact have been originated by a mantle thermal anomaly revived by a propagating fracture from the Atlas mountains and further amplified by tectonic forces (Anguita and Hernán, 2000), or that

its genesis could be consequence of tectonic-controlled volcanism with a history of irregular orogenic pulses (Ancoechea et al. 2006).

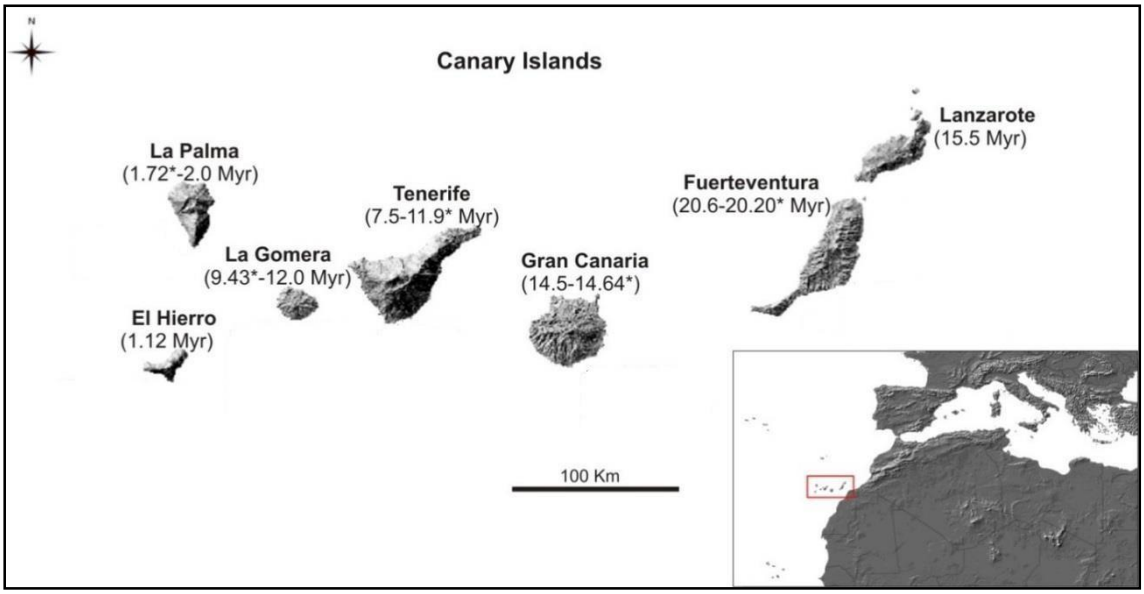

Figure 3 - Map of the Canary Islands archipelago with the indication of the age of the seven different island (Myr) (Ages with * belong to Guillou et al., 2004 and ages without * are from Carracedo et al., 1998; Adapted from Jorge, 2009).

Despite the fact that most authors agree on an east to west geological origin, there seems to be an open discussion about the concise historical age of each island. Indeed, the Canary Islands seems to have particular features that do not relate to other specificities found in volcanic archipelagos causing some controversial in islands time estimation (Anguita and Hernán, 2000; Sanmartín et al., 2008). Lanzarote and Fuerteventura were connected in the Pliocene and although they are no longer in contact with the volcanic hotspot they still show volcanic activity (Coello et al., 1992; Fernández-Palacios and Anderson, 1993). Moreover, although Lanzarote is more distant to the volcanic hotspot its formation seems to be more recent than Fuerteventura oogenesis (Anguita and Hernán, 2000). The same pattern is found in La Gomera and Tenerife islands where although La Gomera is closer to the hotspot, Tenerife formation seems to have happened before than La Gomera genesis (Anguita and Hernán, 2000. Also, the island of Tenerife arose from the connection of three independent shield volcanoes (Roque del Conde, Teno and Anaga) while La Gomera ascended from a single edifice prior to the subaerial growth of Teno and Anaga edifices which does not corroborate an east to west origin (Ancochea et al., 2006). Finally, La Palma and El Hierro Islands seemed to have a contemporary formation (La Palma is slightly older than El Hierro) which may indicate that the east-west formation trending line have been disrupted after

La Gomera formation and is now following a north-south geological dual line (Carracedo et al., 2001).

The Canary Islands show great diversity of habitats including laurisilva, volcanic lava cages, pine forests, lowland scrublands and open xeric environments (Juan et al. 2000). This habitat diversity, combined with geological isolation, interspecific competition, and adaptive radiation is responsible for the considerable endemic biodiversity found in this archipelago (Sanmartín et al., 2008). Some of the Canary Island taxa seem to follow a step-by-step colonization pattern that is then followed by concomitant or within-island speciation; also, some taxa seem to follow a different approach where taxa follow an inter-island colonization but only between similar habitats (Sanmartín et al. 2008). On the other hand, several Canary endemic groups seem to have colonized this archipelago in multiple independent events resulting in non-monophyletic taxa groups (Sanmartín et al., 2008). The four major colonization patterns found in the Canary Islands are discussed in Figure 4. Furthermore several phylogenetic studies have shown that the majority of the Canary Islands closest taxa are original from North Africa, Iberian Peninsula and from other Macaronesia islands such as Madeira and Cape Verde (Carine et al., 2004).

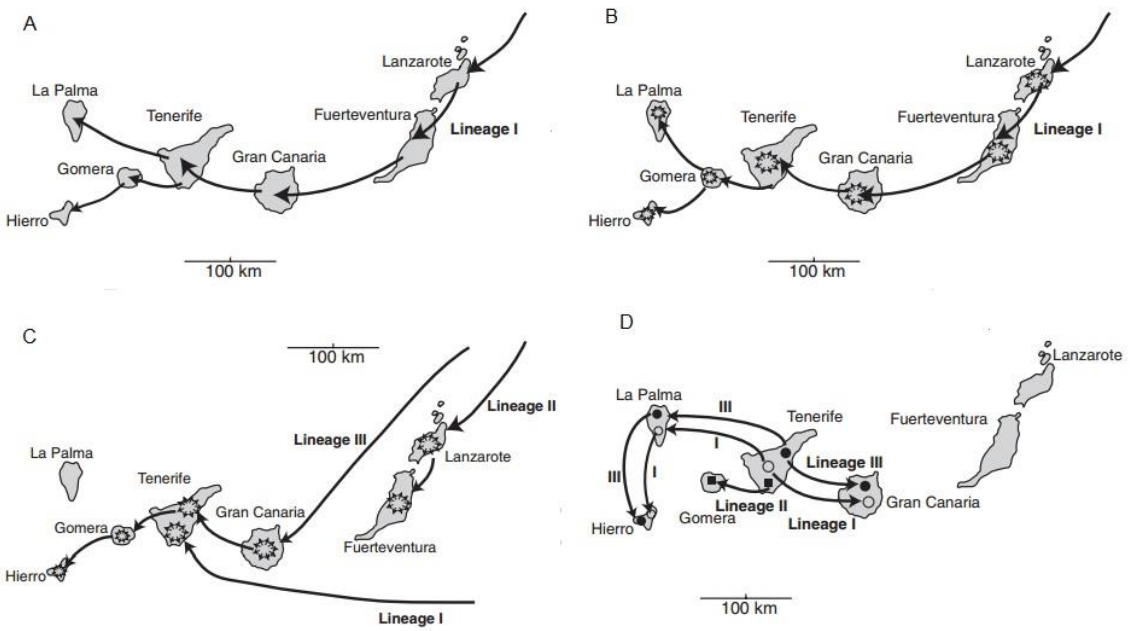

Figure 4 – Four major colonization models of the Canary Islands. Model A: Stepwise colonization with concomitant speciation; Model B: Stepwise colonization followed by within-islands speciation; Model C: Multiple colonization followed by within-island speciation; Model D: Inter-island colonization between similar habitats (From Sanmartín et al., 2008).

Regarding extant endemic reptiles, the Canary archipelago include representatives of three families: Lacertidae, Scincidae and Phyllodactylidae. All representatives from these families are endemic to these islands with the exception of *Tarentola boettgeri* that also habits the Selvages Islands.

Lizards represent a very diverse vertebrate group either in terms of anatomy, ecology and diet. When compared to other animal groups, for example mammals and birds, lizards do not have the ability to disperse much. In the Canary Islands this is not the exception, where the different described genera have distinct behaviours, diets and vagilities (see the chapters relative to *Gallotia* spp., *Chalcides* spp. and *Tarentola* spp.). Still, all the three genera are parasitized by the same parasite genus, *Parapharyngodon* spp. The phenomena that are shaping Parapharyngodon species evolution in these lizard genus remain unknown and therefore the use of these hosts as models to infer coevolucionary interactions are of great interest to understand patterns of colonization, host-switching and maybe cryptic speciation in the Canary archipelago.

Gallotia spp.

The Lacertidae family is divided in two sub-families: Lacertinae and Gallotiinae. While the first one includes 14 genera, widely distributed, the second is represented by the genus *Gallotia*, endemic to the Canary Islands, and by the genus *Psammodromus* present in south-west Europe and north-west Africa (Harris et al., 1998; Harris, 1999). *Gallotia* is endemic to the Canary archipelago and its former ancestor colonized these islands once in the Miocene, between 9 and 12.5 Mya (Arnold et al. 2007).

Within *Gallotia* there are seven recognized extant endemic species to these islands: *G. galloti*, *G. caesaris*, *G simonyi*, *G. bravoana*, *G. intermedia*, *G. stehlini* and *G. atlantica* (Maca-Meyer et al. 2003; Figure 5). *Gallotia* is a monophyletic group where phylogenetic inferences show that *G. stehlini* - from Gran Canaria - is basal to the other *Gallotia* species, and *G. atlantica* - from the eastern islands - originates from the subsequent node (González et al., 1996; Cox et al., 2010; Maca-Meyer et al., 2003). According to this, *Gallotia* species would have colonized the Canary Islands in an east-west pattern, following the geological ages of the islands. However Gran Canary would have been the first island to be colonized (by *G. stehlini* ancestral) where the western

Gallotia lineages would originate from *G. atlantic*a ancestral, rather than the one of *G. stehlini* (Cox et al. 2010).

Gallotia species have very distinct body sizes, which allows an easy identification of the different species. In fact two distinctive groups can be distinguished: a group including small and medium, and another grouping giant lizards. Regarding the first one, species belonging to this group are *G. atlantica*, *G. galloti* and *G. caesaris*; *G. atlantica* is present in Lanzarote and Fuerteventura islands and inhabits coastal sandy areas, scrublands, open dry forests and anthropogenic modified areas ranging from sea level up to 670 meters of altitude in Lanzarote and 800 meters in Fuerteventura; *G. galloti* can be found in Tenerife and La Palma and lives in open, rocky and scrubland areas; *G. caesaris* is present in La Gomera and El Hierro and lives in scrubland and cultivated and urban areas (Valido and Nogales, 1994; Márquez and Mateo,2002; Baéz, 2002a; Mateo and Péres-Mellado, 2002; Valido and Nogales, 2003). The second group is formed by giant lizards and includes: *G. stehlini*, *G. intermedia*, *G. bravoana* and *G. simonyi*, where all species – excluding *G. stehlini* – have restricted distributions and are classified as endangered (Mateo, 2002a; Mateo, 2002b; Mateo and Márquez, 2002; Rando, 2002) *G. stehlini* is endemic to Gran Canaria and can be found in open areas, scrublands and rocky and humid areas; *G. intermedia* is actually restricted to volcanic massif area (Teno massif) in Tenerife; *G. bravoana* is now restricted to dry cliffs with sparse vegetation in La Gomera island; *G. simonyi* is endemic to El Hierro and is now confined to small number of cliffs (Gonzáles et al., 1996; Salvador, 2015a).

Additionally three extinct giant lizards could have been once observed in the Canary Islands: *G. goliath*, *G. maxima* and *G. auaritae*. The first two species were present in Tenerife Island, while *G. auaritae* was found in La Palma. Although little is known about this species, authors have been putting a lot of effort telling the story of this giants. In fact, *G. goliath* fossils were genetic analysed and results showed that this this specie was a member of the *G. simonyi* clade (Maca-Meyer et al. 2003); moreover and despite be fact that no genetic material could be extracted from *G. maxima* remains, several authors had proposed a synonymy between *G. maxima* and *G. goliath* both belonging to *G. simonyi* group, based of morphological, behavioural and evolutionary traits (Barahona et al., 2000). *G. auaritae* was first recognized as a sub-specie *of G. simonyi* (Mateo et al., 2001) but it was later classified as a single specie (Afonso and Mateo, 2003); however no genetic analysis were performed to corroborate this classification. Moreover, several recent studies have proposed that this lizard is not

extinct and a small population of *G. auaritae* can be found in the north of La Palma (Mínguez et al. 2006; Miras et al., 2009); still, despite the exciting news that may be synonymous of more genetic and environmental information on this specie, caution is never the less because more studies are needed to evidence that this specie is not in fact extinct (Mateo, 2009).

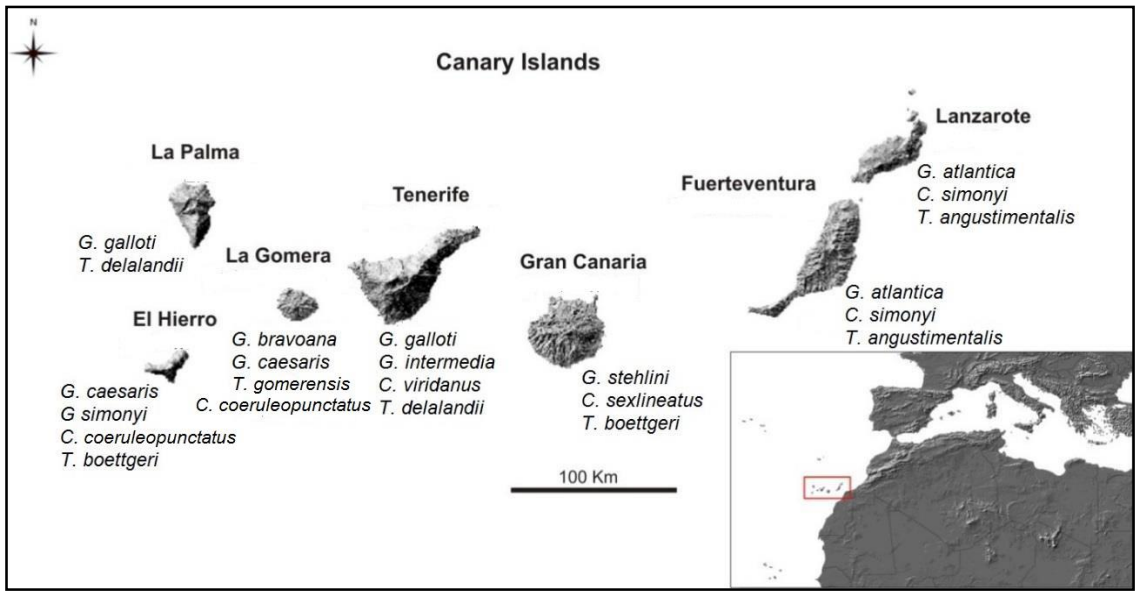

Figure 5 - Map of the Canary Islands archipelago with the indication of the recorded distribution of species of *Gallotia*, *Chalcides* and *Tarentola* (adapted from Jorge, 2009).

Gigantism in islands is very common, especially in rodents and marsupials (Lomolino, 1985). The Canary Islands are no exception, with several fossil records of extinct giants, for example the Gran Canaria and the Tenerife giant rats (genus *Canariomys*), the giant tortoise (genus *Geochelone*) or a giant and poorly known flightless bird (Francisco-Ortega et al., 2009). This trend of variation in body size in insular vertebrates is known as the *Island rule* (Foster, 1964; Van Valen, 1973). Several causes have been proposed to explain this phenomena including intraspecific competition, predation, limited resources and the challenge of dispersing to islands (Lomolino, 2005; Pafilis et al., 2009). However, comparing to the extinct forms Canary Island giant reptiles are smaller in size. This trend is particular important in *G. simonyi* group where its' ancestral form and *G. goliath* remains reveal that the living members of this group are smaller, probably because of anthropogenic pressure due to habitat degradation and predation by humans and introduced domestic animals (Maca-Meyer et al., 2003).

Gallotia species have an omnivorous diet, however these species show a higher trend to feed on plants than the rest of mainland Lacertidae family (Van Damme, 1999). Herbivory is less advantageous energetically because plants are more difficult to digest. Therefore herbivory is considered as a forced change often caused by low prey abundance and usually complementary to large body sizes (Van Damme, 1999). Although *Gallotia* species cannot be classified as herbivorous lizards the degree in which they consume plants is different depending on the species (in general *Gallotia* giant species ingest more plant forms than the other species; Van Damme, 1999) and several anatomic features have been associated with the degree of herbivory-change in this genus. The presence of monocuspid or bicuspid dentation is associated with a carnivorous diet, however only *G. atlantica* has a bicuspid dentition, while the other species are tricuspid which is indicative of a diet more based on plant forms (López-Jurado and Mateo, 1995; Valido and Nogales, 2003; Carretero, 2004). Moreover adaptations such as enlarged caecum, longer transit period, and intestinal flora capable of digest cellulose – characteristics related with herbivorous animals - have been found in giant *Gallotia* species (Carretero, 2004). Also, Gallotia species have bigger vagilities (when compared to other lizard genus such as *Tarentola* spp.), and do tongue flick (Arnold, 2002) which might make them more vulnerable to a given helminth infection, such as *Parapharyngodon* spp.

Chalcides spp.

The genus *Chalcides* represents the Scincidae family in the Canary Islands. There are around 24 species of *Chalcides* described, with four of them endemic to the Canary Islands: *C. sexlineatus*, *C. viridanus, C. coeruleopunctatus* and *C. simonyi* (Figure 5). *C. coeruleopunctatus* had been considered a subspecies of *C. viridanus*, but actually is genetically very different from *C. viridanus* and may be more closely related to *C. sexlineatus* (Carranza et al. 2008).

Colonization of the Canary Islands probably occurred via independent colonization events where the groups then differentiated within each island (Brown and Pestano, 1998). A double colonization is most likely to have occurred where the ancestral of *C. viridanus* reached the most western islands around 7 Mya while that of *C. simonyi* colonized Lanzarote and Fuerteventura around 5 Mya (Carranza et al, 2008). Moreover,

within-island differentiation has been recorded in *C. sexlineatus* on Gran Canaria where a northern and southern unit emerged around 2.2 Mya due to a possible barrier caused by volcanic activity around 2.8 Mya (Pestano and Brown, 1999; Carranza et al., 2008). Furthermore, genetic analyses suggest that there is differentiation between *C. viridanus* populations from Agana – Tenerife – and individuals from Teno and La Laguna regions (Brown et al. 2000).

With the exception of La Palma, all the other six main islands harbour representatives of the genus *Chalcides*. *C. viridadus*, is present in Tenerife and introduced in La Palma, while *C. coeruleopunctatus* can be found in La Gomera and El Hierro islands (Salvador, 2015b). Both species inhabit moist and arid coastal environments, with *C. viridanus* also occupying urban areas (Mateo, 2002c; Salvador, 2008; Sánchez-Hernández et al., 2013). *C. sexlineatus* is endemic to Gran Canaria where it is found in a wide variety of habitats (Mateo, 2002d; Roca et al. 2011). Finally, *C. simonyi* is present in field and rocky habitats from the most eastern islands of Lanzarote and Fuerteventura (Márquez and Acosta, 2002).

The genus *Chalcides* has a serpentine form body type caused by the elongation of the body and reduction of the limbs. This anatomical adaptation was previously described as evolutionary adaptive, since limbless taxa have the possibility to colonize many habitats that are not suitable for limb-developed animals, thus decreasing inter-specific competition and predation (Caputo et al., 1995). These form adaptations have been in fact described in numerous reptile and amphibian species (Caputo et al., 1995). Although not much information is known about ecological and behavioural traits in this genus, all the Canary Islands species seem to only bury themselves in the case of inclemental conditions at the surface, and prefer to look for refugee in bushes to escape predators (Greer et al. 1998). In terms of diet, *Chalcides* species in the Canary archipelago are insectivorous, feeding mainly on small insects and arachnids (Roca et al., 2012). Anatomical features of the tooth confirm the insect-based diet of this genus, with most of small species being equipment with bicuspid teeth while the larger ones – e.g. *C. oceelatus* - have blunt and flat crowns to allow them to crack other types of arthropods (Caputo, 2004). Moreover, an interesting behaviour was recorded in *C. viridanus* where in case of the presence of other individuals, this species use their tongue to explore not only the individual but also the adjacent environment (Sánchez-Hernández et al., 2012). Therefore this behaviour might expose *Chalcides* species to accidental parasite infections such as *Parapharyngodon* spp.

Tarentola spp.

The genus *Tarentola*, from the Phyllodactylidae family, comprises at least 21 species that range across the Mediterranean Basin, Macaronesian islands, Cuba, Jamaica and the Bahamas. The genus is represented in the Canary Islands by four endemic species: *T. angustimentalis*, *T. delalandii, T. gomerensis and T. boettgeri* (Figure 5) with the last one also being present in the Selvages Islands.

As with *Chalcides*, the colonization of the Canary Islands by these geckos seems to have occurred in three independent events (Carranza et al., 2000; Carranza et al., 2002). A first colonization where the ancestral of *T. boettgeri* colonized Gran Canaria and El Hierro islands – as well as the Selvages archipelago - (Carranza et al., 2002). A second colonization where *T. delalandii* and *T. gomerensis* ancestors colonized Tenerife, La Palma and La Gomera (Carranza et al., 2002). And a third independent colonization likely to have occurred with the dispersion of *T. mauritanica* from North Africa to the Lanzarote and Fuerteventura islands with *T. angustimentalis* being a lineage within a paraphyletic *T. mauritanica* species complex, and unrelated to the other species from the Canary Islands (Carranza, 2000; Rato et al. 2012).

Intraspecific differentiation within islands was recorded in several species from the Canary Islands. Based on average molecular distance results, *T. boettgeri* from Gran Canaria and *T. delalandii* from Tenerife reveal some degree of isolation when comparing both northern and southern populations probably due to population isolation resulting from north-south ecological differences in both islands (Nogales et al., 1998). However, in the case of Tenerife the union of the three independent edifices – that now constitute the main island – could also explain the observed levels of variation between populations in *T. delalandii* (Nogales et al., 1998; Ancochea et al., 2006). Moreover, significant values of genetic variation were also found between *T. angustimentalis* populations from Lanzarote and Fuerteventura (Nogales et al., 1998).

Tarentola geckos in the Canary Islands are generalist in terms of habitat and they can be found in a panoply of environments such as rocky areas, lava fields, scrublands and agricultural and urban areas. *T. angustimentalis* is endemic to the most eastern islands of Lanzarote and Fuerteventura (Mateo, 2002e) while *T. delalandii* is present in Tenerife and La Palma (Baéz, 2002b) and *T. gomerensis* can be found in the island of La Gomera (Nogales et al., 1998; Mateo, 2002f). *T. boettgeri* is represented by two subspecies – *T. boettgeri boettgeri* and *T. boettgeri hierrensis* – in the islands of Gran

Canaria and El Hierro, respectively - while a third subspecies *T. boettgeri bischoffi* is endemic to rocky and coastal areas of the Selvages Islands (Mateo, 2002g).

Members of this genus are mostly active at night, preferring open, rocky and dry environments. Species of the *Tarentola* genus are, in general, morphologically similar, although in the Canary Islands there is morphological variation between and within populations (Nogales et al., 1998). Traits such as the presence of osteoderms in the supraorbital region and claw reduction in digits 1, 2 and 5 can be used for morphological identification (Bauer and Russel, 1989; Carranza, 2002; Kahnnoon et al., 2015). *Tarentola* species are oviparous, however evidences suggest that the gender of the specimen is determined by the incubation temperature; while intermediate temperature produce females, higher temperatures result in males (Gamble, 2010). Tarentola species are crepuscular-nocturnal, have restrict vagilities, do not tongue flick and their diet is based in insect forms (Arnold, 2002). Although this specific behaviour makes this geckos unlikely to be infected by direct-life cycle helminths they still are infected by several nematodes (Roca et al., 1999) being therefore interesting host models to understand the forces that are shaping *Parapharyngodon spp.* evolution.

Gallotia, *Tarentola* and *Chalcides* helminthofauna

Helminths are worm-like parasites and in many cases – but not all - inhabit the intestine of the host. Although they can exhibit a wide variety of life cycles, in general, they have three life-cycle stages: eggs, larvae and adults. In general, adult worms infect the definitive host, whereas larvae might infect intermediate hosts, or be free-living.

Many studies have shown that the composition of the host diet may have influence in the helminthic community found in the intestine of the host (e.g. Martin et al., 2005; Carretero et al., 2006; Carretero et al., 2014). For instances, lizards with carnivorous diet are more likely to be infected by certain nematode genera from the family Pharyngodonidae than lizards that have a more herbivorous diet (Peter and Quentin, 1976; Roca et al., 2005; Carretero et al. 2014). Moreover, the helminth community found in herbivorous lizards is richer than the one found in carnivorous ones (Roca and Hornero, 1991) possibly due to the fact that herbivorous forms are more likely to ingest parasite eggs that were evacuated on plants by other infected animals (Carretero et al., 2006).

Diverse studies were conducted in order to infer the helminthofauna of the different Canary Island lizard species (e.g. Martin and Roca, 2004; Martin and Roca, 2005). All the Canary Island endemic lizards show a widely diverse helminthic community with high prevalence of the different parasite species (Table I).

Parapharyngodon and *Thelandros* species described in the Canary Islands seem to be host generalists, since they can be found in all the Canary endemic reptile genus except *Tarentola*, for which Thelandros species have not been reported (Roca et al., 1999). Moreover, variation in helmintho-fauna found in geckos and skinks suggest that differences in host environment, diet and immune system may influence the recruitment potential of the parasite – e.g. *Spauligodon sp*. was found in *Tarentola* but not in *Chalcides* (Roca et al., 2012).

Table I. Prevalences (%) of *P. echinatus* (P.e.), *P. bulbosus* (P.b.), *P. micipsae* (P.m.), *T. galloti* (T.g.), *T. tinerfensis* (T.t.) and *T. filiformis* (T.f.) helminths in *Gallotia* species (From Martin and Roca, 2004; Martin and Roca, 2005; Roca et al., 2005; Carretero 2006), *C. sexlineatus* (From Roca et al., 2012) and *Tarentola species* (From Roca et al., 1999). G.s. – *G. stehlini*; G.c.c; *G. c. caesaris*; G.c.g. – *G. c. gomerae*; G.a.a.- *G. a. atlantica*; G.a.m.- *G. a. mahoratae*; G.g.g.- *G. g. galloti*; G.g.p.- *G. g. palmae*; C.s.- *C. sexlineatus*; T.d.- *T. delalandii*; T.g.- *T. gomerae*; T.b.- *T. boettgeri*; T.a.- *T. angustimentalis*.

	Gs	G.c.c.	G.c.g.	G.a.a.	G.a.m.	G.g.g.	G.g.p	C.s.	T.d.	T.g.	T.b	T.a.
P.e.	9.1			26.2	44.0				42.9		2.2	
P.m.	15.2	15.4		23.8	11.4	14.8		53	64.3	81.3	39.1	63.2
P.b.	9.1											
T.g.		50.0	38.1			48.1	74.1					
T.t.		1.9	9.5				55.6					
T.f.	97	17.6	33.3				3.7					

Historical review of *Parapharyngodon* spp. and *Thelandros* spp.

The validity of the genus *Parapharyngodon* has been discussed various times since it was proposed in 1933 by Chatterji, and taxonomists do not agree if *Parapharyngodon* is by itself a genus or a subgenus of *Thelandros* – first described by Wedl in 1862 – or if there are no significant differences between *Thelandros* and *Parapharyngodon* that justify the separation between these two entities. Therefore an historical review on these two genera is needed to contextualize the reader.

Thelandros genus was first described by Wedl in 1862. This genus was later revised by Chatterji, who introduced *Parapharyngodon* as a separate genus based on

the presence of lateral alae (Chatterji, 1933; Pereira et al. 2011). This difference between the two groups was later corroborated by Yamaguti (1961) when he used the presence of the lateral alae to distinguish between specimens from both genera, but he proposed instead a different classification, by dividing the genus *Thelandros* into two subgenera, *Thelandros* (*Thelandros*) and *Thelandros* (*Parapharyngodon*). However, Petter and Quentin (1976) did not find the presence of lateral alae a consistent trait to separate both genera and therefore considered the species as all belonging to the genus *Thelandros*.

In 1981 Adamson insists on the separation of both genera, and argues that the presence or absence of lateral alae, as well as the differences in tail morphology in males and females are good evidences to distinguish the described species in two different genus. Moreover, this author considered, for the first time, differences in ecological and behavioural traits, reporting that *Parapharyngodon* spp. parasites are likely to be found in carnivorous reptiles and amphibians, while *Thelandros* spp. parasitize herbivorous or omnivorous reptiles. The separation of the two genera is also supported by several later studies including Roca (1985), Castano-Fernandez et al (1987) among others.

However, considering molecular and phylogenetic studies little attention has been paid to both genera. However the few studies that were published are of great value to a better comprehension of the diversity of some *Parapharyngodon* and *Thelandros* species. Phylogenetic studies on *P. cubensis* (endemic to the Caribbean) revealed well supported genetic variation of several “lineages”; however the authors were not able to morphological distinguish this different “lineages” suggesting that *“P. cubensis” is* possibly a complex of different cryptic species rather than a single species (Falk and Perkins, 2013) Moreover studies on *T. scleratus* phylogenetic position revealed that this species was grouped in the same clade as *P. echinatus* specimen (Chaudhary et al., 2014); although the results are in some extent preliminaries they do suggest that *T. scleratus* is closely related to P. echinatus, which possible suggest a synonymy between both genus or a taxonomical misclassification of this *Thelandros* species. Therefore the complex evolutionary patterns reported in Parapharyngodon and Thelandros nematode species make this genera a fascinating model to infer evolutionary patterns.

Thelandros spp.

The genus *Thelandros* Wendl, 1862 belong to the order Oxyurida, Superfamily Oxyuroidea, Family Pharyngodonidae. *Thelandros* species have direct life-cycles and have been described as parasites of omnivorous and herbivorous lizards (Adamson, 1981). There are more than 30 species described (Dung et al., 2009) and in the Canary Islands there are three recognized endemic species: *T. galloti*, *T. tinerfensis* and *T. filiformis.*

Thelandros galloti is a fusiform whitish nematode with striation at the level of the cuticle. *T. galloti* males are identified by the presence of two very long and wide lateral alae that start very close to the cephalic region and reach the level of the tail, being widest at the level of the cloaca; these males have an elliptical excretory pore situated below the oesophageal bulb and have three pairs of papillae being two of them cloacal and the third one caudal; the spicule is small and obtuse and the presence of caudal alae has not been recorded (Astasio-Arbiza et al., 1988; Figure 6). *T. galloti* females are bigger than males, have six lips in the mouth structure and the vulva is located at the level of the oesophagus; their tail is small and designed with conic shape and eggs are oval with one flatted side (Astasio-Arbiza et al., 1988; Figure 6).

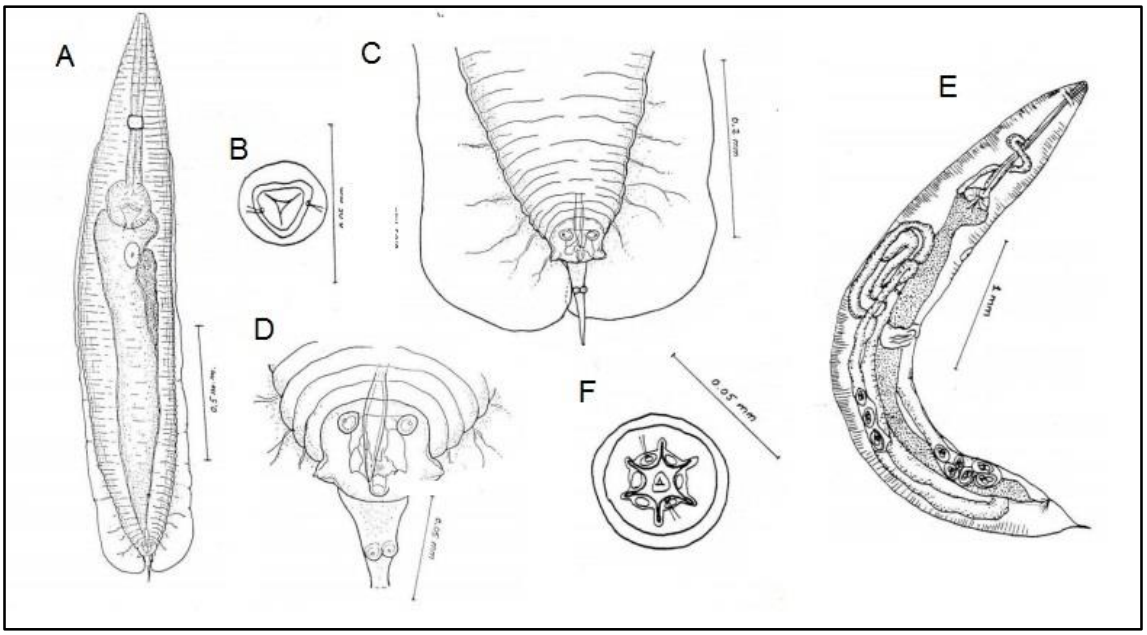

Figure 6 – Representation of *T. galloti* male (A) and female (E). Apical representation of *T. galloti* mouth structure in both male (B) and Female (F). Representation of *T.galloti* posterior region of the body in males with closer view of the alae (C) and the cloacal region (D) (Adapted from Astasio-Arbiza et al., 1988).

Thelandros filiformis is a small nematode with a white body that present striation slightly marked in the cuticle. Males of this species have a three-lip mouth, excretory pore that is located far from the end of the oesophageal bulb and small lateral alae that start in the posterior region of the body reaching the tail in an auricular form and being widest at the cloacal level; they have two pairs of cloacal papillae and one single caudal papillae. The spicule on these males is thin and a thin caudal alae has been recorded starting in the insertion of the tail with the body reaching the caudal papillae (Astasio-Arbiza et al., 1989; Figure 7). *T. filiformis* females have their vulva at the level of the middle body, a pointy and wide tail and the eggs have an elliptical form slightly flattened in both extremes (Astasio-Arbiza et al, 1989; Figure 7).

Thelandros tinerfensis males have a hexagonal mouth, excretory pore situated below the oesophageal bulb, 5 papillae (2 pairs in the cloaca and a single papillae in the tail); these males have small lateral alae that start in the final posterior region and reach the tail in an auricular form and a caudal alae that end at the level of the caudal papilla and the spicule is small and obtuse (Solera-Puertas et al., 1988; Figure 8). *T. tinerfensis* females have their vulva in the middle part of the body and their eggs have an elliptical shape (Solera-Puertas et al., 1988; Figure 8).

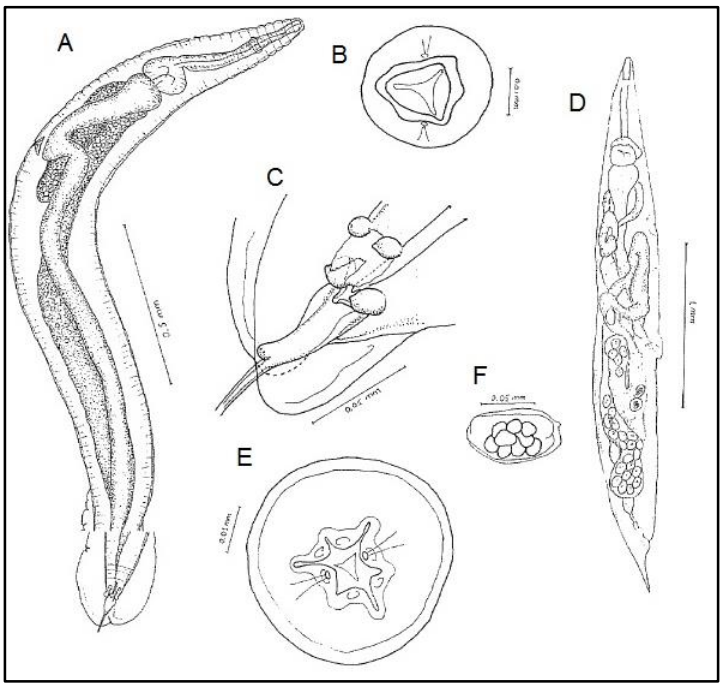

Figure 7 - Representation of *T. filiformis* male (A) and female (D). Apical representation of *T. filiformis* mouth structure in both male (B) and Female (E). Representation of *T.filiformis* posterior region of the body in males with closer view of the cloacal region (C). *T. filiformis* egg (F) (Adapted from Astasio-Arbiza et al., 1989)

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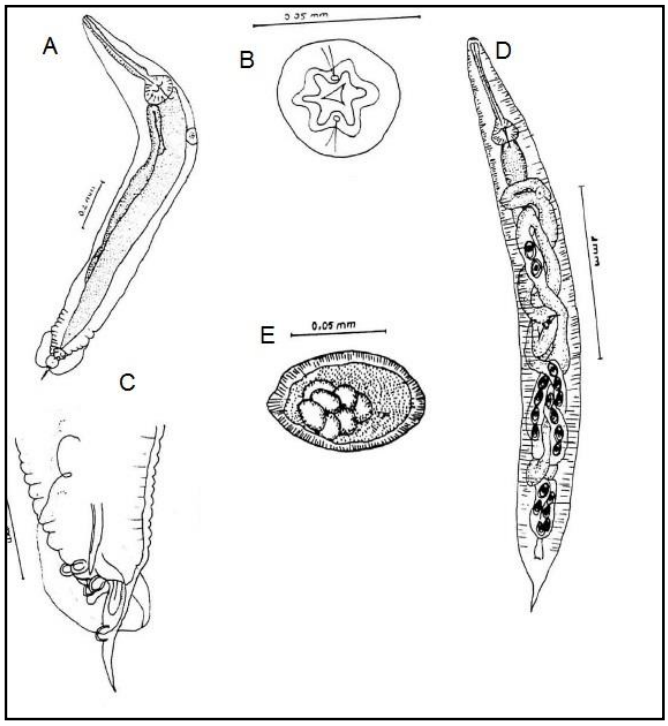

Figure 8 - Representation of *T. tinerfensis* male (A) and female (D). Apical representation of *T. tinerfensis* mouth structure (B). Representation of *T. tinerfensis* posterior region of the body in males with closer view of the cloacal region (C). *T. tinerfensis* egg (E) (Adapted from Solera-Puertas et al., 1988).

A resume with the main differences among the three Thelandros species described for the Canary Islands can be found in Table II

Table II. Distinctive morphological traits between T. galloti (From Astasio-Arbiza et al., 1988), T. tinerfensis (From Solera-Puertas et al., 1988) and T. filiformis (From Astasio-Arbiza et al., 1989) males.

	T. galloti	*T. tinerfensis*	*T. filiformis*
Body length	1270 µm	1680 µm	2310 µm
Body width	280 µm	210 µm	160 µm
Number of cloacal papillae	2 pairs	2 pairs	2 pairs
Number of caudal papillae	2	1	1
Presence of caudal alae	Absent	Present	Present
Length from the cephalic region to the alae	430 µm	1480 µm	2000 µm
Width of lateral alae	-	-	50 µm

Parapharyngodon spp.

There are currently 46 described species of *Parapharyngodon* (until 2011, see Pereira et al., 2011 for a partially updated table) distributed worldwide: 3 in the Australian region, 9 in the Ethiopian, 4 in the Nearctic, 13 in the Neotropical, 6 in the Oriental and 11 in the Palearctic region.

Parapharyngodon Chatterji 1933, is an intestinal nematode that belongs to the order Oxyurida, Superfamily Oxyuroidea, Family Pharyngodonidae. Like all the genera that belong to the order Oxyurida, *Parapharyngodon spp.* occurs in the intestines of the host, parasitizing mainly carnivorous forms (Adamson, 1981). *Parapharyngodon* species are haplodiploid, meaning that males are haploid and derived from unfertilized eggs and females are formed by fertilized eggs and are diploid (Adamson, 1990). They have direct life cycles and probably arose from lizards and then transferred to amphibian (Adamson, 1989). *Parapharyngodon* species are mainly identified based on the morphology of the anterior cloaca lip, form of the spicule and length and width of the lateral alae in males, and location of the ovary and egg size in females (Adamson and Nasher, 1984).

In the Canary Islands three species of *Parapharyngodon* have been described: *P. echinatus* Rudolphi, 1819, *P. bulbosus* Linstow, 1899 and *P. micipsae* Seraut, 1917 (Figure 10). Although *Parapharyngodon* has been described as part of the evolutionary lineage of Pharyngodonidae parasitizing carnivore lizards, in the Canary Islands they are found in all the endemic lizards, including *Gallotia* species that are known to have an omnivorous–herbivorous diet (Roca et al., 2005). Moreover, these three *Parapharyngodon* species are not endemic to the Canary Islands and have also been found infecting hosts across the Mediterranean basin and in Africa (e.g. Myers et al., 1962; Roca, 1985; Mašová et al., 2009).

Parapharyngodon echinatus (Figure 9) was first described by Rudolphi in 1819 from an unidentified gecko from Spain. These nematodes have a long fusiform body and exhibit a thick cuticle with transversal marks, a circular mouth with six platforms and 4 papillae and a post-bulb small excretory pore – both in males and in females (Roca, 1985). Males of this species exhibit maximum body width at the level of the excretory pore with long and wide lateral alae that start at the level of the oesophageal bulb and finish below the level of the cloaca where they reach the maximum width; *P. echinatus* males have an obtuse and long spicule alongside with three pairs of cloacal papillae and

one extra pair of caudal papillae present in a long tail inserted dorsally at the level of the upper lip of the cloaca opening (Roca, 1985; Mašová et al., 2008).

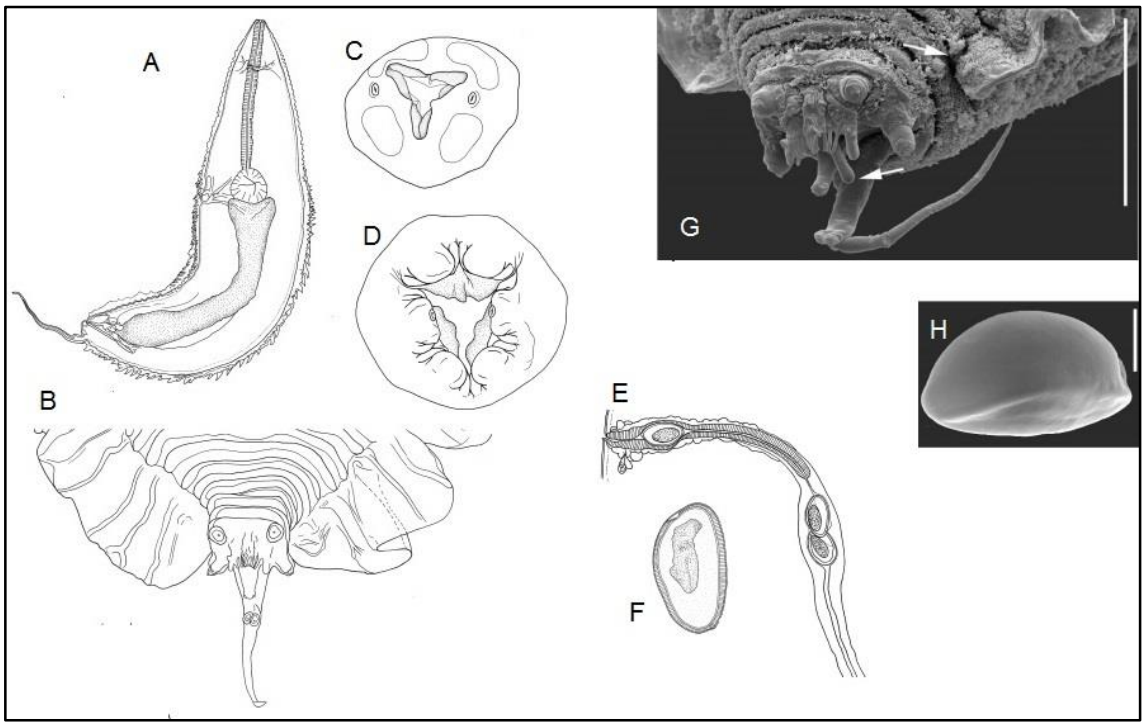

Figure 9 - Representation of *P. echinatus* male (A) with closer view on the posterior region of the body (B). Representation of *P. echinatus* mouth structure in both male (C) and female (D). Representation of *P. echinatus* proximal end of reproductive tract showing vulva (E) and egg (F). SEM of male *P. echinatus* posterior body (G), upper arrow indicates end of alae, lower arrow indicates end of spicule. SEM of *P. echinatus* egg (H) (Adapted from Mašová et al., 2008).

Females of this species are bigger than males and exhibit a mouth with six lips, a vulva that ends near the middle of the body and the ovaries reach the level of the oesophagus isthmus; females show a long pointy tail and the eggs have an ovoid form slightly flattened on one side with 2-8 blastomeres (Roca, 1985; Mašová et al., 2008). In terms of measurements, *P. echinatus* seems to have different length depending probably on the host or geographical region. A review on *P. echinatus* body measurements can be analysed in Table III.

Table III. *P. echinatus* females and males body measurements by Roca, 1985 and Mašová et al., 2008.

		Roca 1985	**Mašová et al., 2008**
Females	Body length	3431-6436 µm	1500-2930 µm
	Body width	450-994 µm	515-764 µm
	Oesophagus length	762-1158 µm	688-969 µm
	Nervous ring	180 µm	103-144 µm
	Excretory pore	1275-1834 µm	562-1118 µm
	Vulva from anterior end	2007-3312 µm	892-1452 µm
	Tail	97-191 µm	103-207 µm
	Eggs	81-110 x 43-66 µm	78-88 x 44-52 µm
Males	Body Length	1491-2839 µm	1341-1646 µm
	Body Width	125-540 µm	316-416 µm
	Oesophagus Length	240-435 µm	398-563 µm
	Nerve Ring	-	88-134 µm
	Excretory Pore	691-1035 µm	567-836 µm
	Spicule Length	61-112 µm	98-117 µm
	Spicule Shape	Obtuse	Obtuse
	Number of genital papillae	3+1 pairs	3+1 pairs
	Tail length	48-88 µm	80-126 µm
	Outgrowth	Present	Present (finger-like)

In 1917 Seraut described a nematode species as "*T." micipsae* from the gecko host *T. mauritanic*a and compared it with "*Thelandros" echinatus* (formerly *Parapharyngodon echinatus*). The author distinguished both species by the shape of the posterior extremity, the shape of the upper lip of the cloaca and the shape of the posterior part of the lateral alae (Mašová et al., 2009). However the author stated that, as happens in other genera of Oxyurids, females from both species were indistinguishable. This classification of "*T." micipsae* and "*T." echinatus* was reviewed later by Teixeira de Freitas (1957), who restored the previous classification of Chatterji as *P. micipsae* and *P. echinatus*. The same year, Chabaud and Golvan (1957) considered *P. micipsae* and *P. echinatus* as synonyms, based on the fact that the differences found at the level of the lateral alae and the superior lip of the cloaca vary with the fixation status of the

specimens. Roca (1985) also agreed with this synonymy between both species. However, Horner (1991) considered *P. echinatus* and *P. micipsae* as different species, despite females being indistinguishable, pointing to some anatomical traits that allow the differentiation between the two species (Table IV).

Tabela IV. Morphological differences that allow to distinguish between P. echinatus and P. micipsae males (Hornero, 1991) and *P. bulbosus* males (Moravec et al., 1987).

Parapharyngdon echinatus	***Parapharyngodon micipsae***	***Parapharyngodon bulbosus***
Lateral alae wide (50-80µm) and ending at the level of the cloaca	Lateral alae narrow and ending above the cloaca	Lateral alae wide and ending at the level of the cloaca
Large genital cone	Reduced genital cone	Long genital cone
Spicule obtuse	Spicule sharp	Spicule obtuse
Tail long and starting at the end of the body	Tail short and starting at the level of the tail papillae pair	Tail long

Parapharyngodon micipsae, Seraut 1917, (Figure 10) is found infecting all endemic lizard genera from the Canary archipelago. They are small white nematodes with a fusiform body with striations at the level of the cuticle. *P. micipsae* males have three lips with three papillae at the level of the mouth, and possess a narrow alae that is general smaller than the one from *P. echinatus (*Mašová et al., 2009*)*. *P. micipsae* specimens have 4 pairs of papillae – 3 pairs of cloacal papillae in a rosette-like form, and one extra pair in the tail structure - and the spicule is wide at the proximal end and sharp at the point (Mašová et al., 2009). Females from this species have cylindrical shape with the ovaries reaching the oesophagus isthmus; they have a small pointy tail and the eggs are asymmetrical flattened on one side (Mašová et al., 2009). In terms of body measurements, a review on different authors work can be analysed in Table V.

Parapharyngodon bulbosus, Linstow 1899, (Figure 11) is a small nematode with striations at the level of the cuticle and the presence of six lips in the mouth, with one papillae in the females (Roca, 1985). *P. bulbosus* males have long and wide lateral alae that start below the oesophageal bulb and end at the level of the tail with maximum width at the level of the cloaca; they have 4 papillae pairs with one present in the tail structure and the spicule is long and somewhat sharp (Roca, 1985; Moravec et al., 1987; Mašová, 2008). *P. bulbosus* females have the vulva in the middle of the body, small and wide tails

and the eggs have an oval form with 2-16 blastomeres (Roca, 1985; Moravec et al., 1987; Mašová, 2008). Measurements on this species are detailed in Table VI.

In all three described *Parapharyngodon* species, authors have slightly similar results in terms of body measurement. However the standard deviation of the different measurements is very high meaning that the size of the measured traits may fluctuate between specimens – possible due to different hosts or different geographical regions - and are likely dependent on the size of the individual (Mašová et al., 2009).

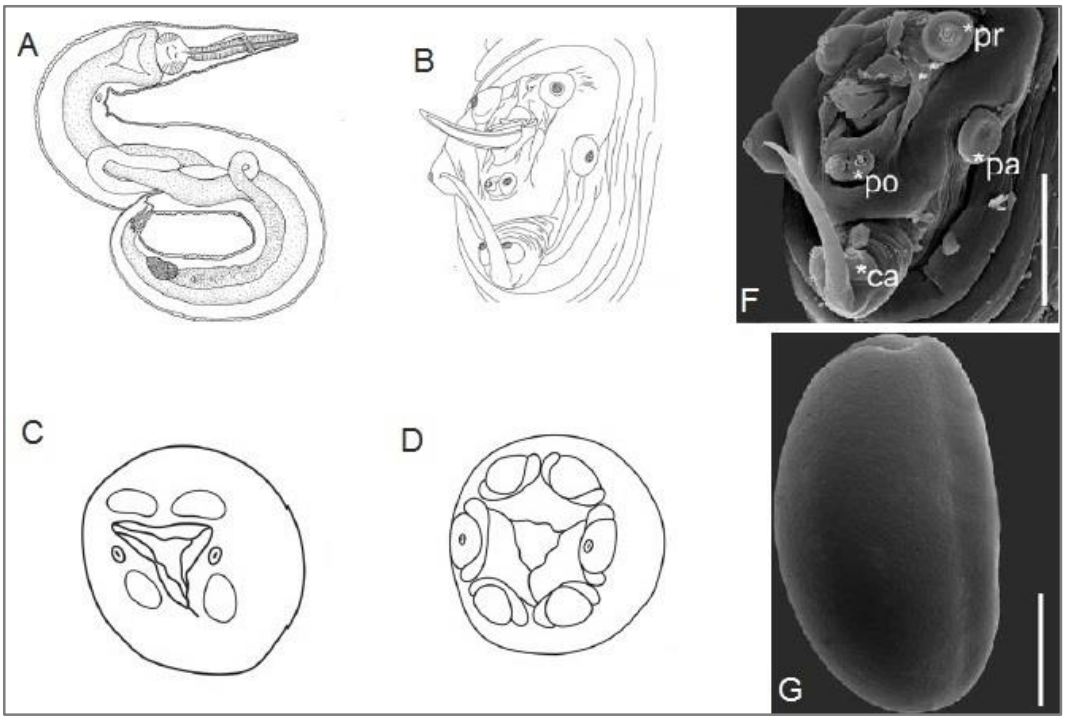

Figure 10 – Representation of *P. micipsae* (A) with view on cloacal region (B). *P. mcipsae* mouth structure in both male (C) and female (D). SEM of *P. micipsae* male posterior region (F) showing four pairs of papillae: precloacal (pr), paracloacal (pa), postcloacal (po) and caudal (ca). SEM of *P. micipsae* egg (G) (Adapted from Mašová et al., 2009b).

Table V. *P. micipsae* males and females body measurements by Seraut, 1917, Moravec et al., 1987, Ruiz Sanchez. 1996 and Mašová et al., 2009 (Adapted from Mašová et al., 2009).

		Seraut, 1917	**Moravec et al. 1987**	**Ruiz Sanchez 1996**	**Mašová et al., 2009**
Males	Body length	2350-3366 µm	1170 µm	1732 ± 97 µm	1164-2199 µm
	Body width	193 µm	95 µm	175 ± 15 µm	160 -310 µm
	Oesophagus length	462 µm	340 µm	460 ± 23 µm	309-435 µm
	Nervous ring	145 µm	102 µm	-	106-117 µm
	Excretory pore	1056 µm	625 µm	569-836 µm	409-644 µm
	Spicule length	88 µm	~40 µm	74 ± 9 µm	62-98 µm
	Spicule shape	Sharp	-	Sharp	Sharp
	Number of papillae	3+1 pairs	4 pairs	3+1 pairs	3+1 pairs
	Tail length	70 µm	-	57 ± 9 µm	77-106 µm
	Outgrowths	Simple	-	Trilobulated	Lobed
	Body length	8844 µm	4460-6770 µm	4283 ± 402 µm	1844-2982 µm
Females	Body width	924 µm	503-830 µm	489 ± 47 µm	720-977 µm
	Oesophagus length	1452 µm	1010-1060 µm	1117 ± 81 µm	809-1219 µm
	Nervous ring	130 µm	129-159 µm	-	116-158 µm
	Excretion pore	2442 µm	1580-1900 µm	1367 ± 190 µm	464-1129 µm
	Vulva from anterior end	4455 µm	2290-2920 µm	2243 ± 259 µm	828-1641 µm
	Tail length	120 µm	81-99 µm	107 ± 18 µm	81-137 µm

Table VI. *P. bulbosus* males and females body measurements by Roca, 1985 and Moravec et al., 1987.

		Roca, 1985	Moravec, 1987
Males	Body Length	1840-2839 µm	2140-2460 µm
	Body Width	312-425 µm	231-236 µm
	Oesophagus Length	384-521 µm	530-721 µm
	Nerve Ring	-	132-150 µm
	Excretory Pore	724-1081 µm	820-1090 µm
	Spicule Length	66-102 µm	51-63 µm
	Spicule Shape	-	Sharp
	Number of genital papillae	3 pairs	4 pairs
	Tail length	84-102 µm	51-63 µm
Females	Body length	3431-6459 µm	2860-4280 µm
	Body width	426-875 µm	340-449 µm
	Oesophagus length	820-1033 µm	790-1010 µm
	Nerve ring	-	159-183 µm
	Excretion pore	1275-1933 µm	1120-1540 µm
	Vulva from anterior end	1911-2958 µm	1360-2180 µm
	Tail length	106-145 µm	159-225 µm
	Eggs	84-101 x 43-60 µm	-

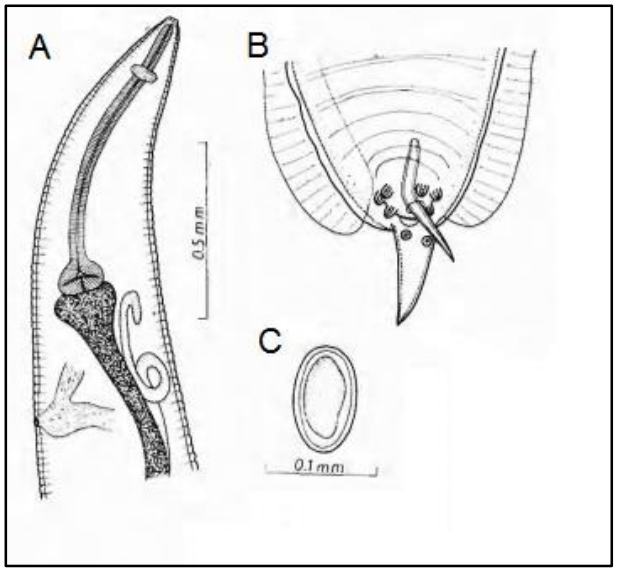

Figure 11 – Representation of *P. bulbosus* male apical region (A) and posterior region (B) and *P. bulbosus* egg (C) (Adapted from Moravec et al., 1987).

Phylogenetics

Linnaeus, known as the father of taxonomy, used common shared morphological characteristics in order to define a hierarchical structure between taxa (McKelvey, 1982). Since Linnaeus work, scientists have defined species based only on morphology (e.g. Costa et al., 1997; Weibo, 2000). However, convergent evolution or the presence of cryptic species may represent a problem in such taxonomic studies. In addition, in particular taxonomic groups, such as helminths, taxonomic studies based on morphological characters may fail due to factors such as the source of the host, the preservation method, how specimens are mounted and host-derived variation (Perkins et al., 2011). These factors plus small morphological characters of the parasites combined with specific life-history and similar selective pressures has led to erroneous classification of different species in the past (Banks and Patterson, 2005).

As we got close to the middle of the 20th century the use of molecular tools started to emerge. The principle concept that today observed biodiversity is related with changes at the level of specific genes that are consequence of accumulation of mutations during millions of years was the breaking point to the emersion of this tools. Polymerase Chain Reactions (PCR) and Sanger sequencing methods represent key tools not only in the identification and characterization of taxa from a taxonomical point of view, but also to study the evolutionary relationships between taxa and specifically between hosts and their parasite. However, today phylogenetic studies are typically carried out based on the use of a single gene (gene-tree). Of course that we are now entering a new era of Genomic approaches that promise to change our view in what is happening at the genomic level. However this still emerging field has its cons and, depending on the question, a genomic view may not be necessary. Still, the problem with using a single gene (or several) is that the time back to the common ancestor of two DNA sequences is different than the time back to the common ancestor of the two species (Nichols, 2001), where the different markers mutation rates will lead us into different results that may not be congruent between each other or may led us into erroneous conclusions concerning the evolution of the species concerned (Pamilo and Nei, 1988). Therefore the choice of the marker should be done in a way that allow us to answer specific questions, and results derived from a single gene should not be interpreted as the “true” phylogeny of a given taxa.

The 18S ribosomal RNA small subunit and 28S rRNA large subunit are two eukaryotic ribosomal RNA genes that are typically organized in arrays of tandem repeats on different chromosomes and are widely used in phylogenetic assessments in parasites groups (e.g. Jorge et al., 2011). This two markers are widely used in parasite phylogenetic assessment due to the (i) presence of multiple (but normally identical) copies in the genome, that means that laboratory techniques are relatively easy and (ii) because they contain both conserved and variable regions which allows the primer design to be relatively easy but still contained phylogenetic information (Perkins, 2011). However these markers have many insertion and deletions that may influence phylogenetic studies especially in more divergent taxa (Morrison and Ellis, 1997).

There are many algorithms that allow the reconstruction of phylogenetic relationships, of which the most widely used are neighbor-joining, NJ (Saitou and Nei, 1987), maximum parsimony, MP (Fitch, 1971), maximum likelihood, ML (Felsenstein, 1981) and Bayesian inference, BI (Huelsenbeck and Ronquist, 2001). Maximum likelihood (ML) approach use a stochastic model of evolution and branch length accounting for the fact that changes are more probable in long branches than in shorter ones incorporating uncertainty in ancestral state reconstruction (Sanmartín et al., 2008). ML algorithms search for the most probable tree where each tree likelihood calculation is done by summing over all possible nucleotide states in the internal nodes (Roots et al., 2009). However, this approach doesn't count for the phylogenetic uncertainty – the ancestral stage changes is reconstructed over a fixed tree – and to assess node support bootstrap analysis are typically employed (Sanmartín et al., 2008), although interpreting node support from bootstraps is not simple. Bayesian inferences (BI) have been proposed in the recent years and unlike ML they do not search only the best tree, instead they search for a set of plausible trees or hypotheses for the data that holds a confidence estimate of any evolutionary relationship within the input prior distribution model (Roots et al., 2009; Sanmartín et al., 2008). BI incorporate sources of uncertainty by sampling the posterior distribution of the phylogeny using Markov Chain Monte Carlo (MCMC) that simulate a random set of parameters and proposes a new set of parameters, by changing the parameters using random operators, calculating the likelihood and prior ratio and allowing the analysis to overcome local optima by running multiple times using a random starting point; if the likelihood ratio product is better the parameters are accepted and the analysis continues to the next step, if it is worse the probability that the state is rejected is inversely proportional to how much worse the new state is (Roots et al., 2009).

Following this methodology not only is a tree estimated, but a consensus tree can be calculated to give Bayesian Posterior Probabilities which in turn can be interpreted as levels of support for internal nodes.

Building phylogenies is the first step to reconstruct host-parasite interactions and therefore uncover their co-evolutionary history. Still the difficulty in isolating parasites from their hosts represent one of the biggest barriers in assessing phylogenies. However not much attention is paid to parasites, and when it is usually has the ultimate goal of eradicate them (Poulin and Morand, 2004). In consequence, we are dealing with limited availability concerning molecular markers and genetic information. A search in GenBank database revealed that for *Thelandros* spp. there are only two 18S sequences and five 28S sequences available. The same happens for *Parapharyngodon* spp. with only two 28S sequences and 173 18S sequences available (where 171 of them correspond to *P. cubensis* specimens; Falk and Perkins 2013). However, one of the few phylogenetic studies in a helminth species (*Spauligodon atlanticus*) revealed to be crucial not only in the taxonomic reassessment of this group but it also helped to have more insights in the evolutionary patterns of this parasite hosts (Jorge et al., 2011). Also studies in this group unveiled the presence of cryptic speciation that seem to be quite common in nematodes (Jorge et al., 2013). Unlike *S. atlanticus, Parapharyngodon* species in the Canary Islands are host generalists. Therefore there is the urgency in understanding which forces are shaping *Parapharyngodon* evolution and how they do relate to their own hosts evolution. Are *Thelandros* and *Parapharyngodon* different genus? Can we rely only on morphological data in a taxonomic assessment study? How *Parapharyngodon* species are evolving? Is there cryptic speciation in *Parapharyngodon* lineages? How the hosts evolutionary forces are shaping the evolution of *Parapharyngodon*, and vice-versa? Why do *Parapharyngodon* species host-switch between Gallotia, Chalcides and Tarentola species in the Canary Islands? How *Parapharyngodon* ancestors colonized the Canary Islands in first place? All this questions are of great interest for the scientific world, still they remain unknown. Combining both molecular and morphological tools this dissertation has the main purpose to uncover some crucial evolutionary traits in the Canary Islands *Parapharyngodon* species that hopefully will “open the door” to more future studies concerning not only this genus but also other nematode groups.

Objectives

The main aim of this dissertation was to investigate the co-phylogenetic patterns between the three different host genera (*Gallotia, Chalcides* and *Tarentola)* and the genus of parasite *Parapharyngodon spp.* in the Canary Islands. Four major goals were important to be achieved: (i) morphological and genetic characterization of the *Parapharyngodon* parasite species (ii) phylogenetic analysis of both host and parasite using 18s and 28s rRNA nuclear markers, (iii) inference of cospeciation patterns in the host-parasite relationship and (iv) inference of the main colonization events associated with the evolutionary history of *Parapharyngodon spp.* in the Canary Islands. All these goals were addressed in Manuscript II however, due to the ongoing discussion concerning the taxonomic status of *Parapharyngodon* as distinct from *Thelandros,* a first study that used an integrative taxonomic approach to infer how *Parapharyngodon* species relate to the ones of *Thelandros* was important to be accomplished. Therefore, in Manuscript I we combined both morphometric and genetic tools in order to (i) understand what are the major classification traits at the phenotypic level that allow a clear morphological distinction between both genera and, if in the case, (ii) reassess previous taxonomic classification.

Materials and Methods

Sampling procedures

A total of 110 samples were collected from 23 lizard host species (Appendix 1). Sampling was performed in the Canary Islands, Morocco, Spain, Portugal, Cape Verde and São Tomé. Specimens were mostly obtained from faecal pellets, or from intestines removed from individuals sacrificed or accidentally killed in the field. Sampling was approved by the authorities from the Canarian Government (Cabildos Insulares from Lanzarote, Fuerteventura, Gran Canaria, Tenerife, La Palma, La Gomera and El Hierro).All samples were stored in 96% ethanol and then separated, counted and identified using an Olympus SZX2-ILLT magnifying glass (Olympus®, Tokyo, Japan).

Morphological characterization

Semi-permanent slides were prepared using a glycerol – water solution (1:1) as described by Borges et al. 2012, and were observed under a light microscope (Olympus CX41, Olympus Australia Pty Ltd, Nothing Hill Victoria, Australia) in order to confirm identification of specimens from genera *Parapharyngodon* and *Thelandros*. Species identification was based on the actual classification of different morphological traits: body length (BL) and width (BW), tail length (TL), nervous ring distance (NR), oesophageal bulb length (OBL) and width (OBW) and oesophagus length (OL) and width (OW). In males the following traits were crucial for the parasitological characterization: alae length (AL) and width (AW), tail width anterior to the tail papillae pair (TW1) and tail width posterior to the tail papillae pair (TW2), spicule shape (SS), spicule length (SL), spicule width (SW) and number and position of genital papillae. In females vagina length (VL), vulva position (Vu), egg length (EL), egg length average (ELa), egg width (EW) and egg width average (EWa) were used to discriminate females from different genera. Photographs were taken using a digital camera Olympus DP25 (Olympus®, Tokyo, Japan) and pictures saved using Cell^{B} software version 3.4 (Olympus Soft Imaging Solutions GmbH). Linear measurements were taken using ImageJ software version 1.48 (Wayne Rasband, National Institute of Health, USA) and were recorded by the same person (AS). Body length was measured from the anterior edge of the lip down to the posterior edge of the body; body width was recorded right below the oesophageal bulb

– excluding lateral alae in males -; oesophageal bulb length and width were measured from the upper border that connects to the oesophagus down to the posterior border and at the broadest part, respectively; oesophagus length was measured from the anterior border to the border that connects to the oesophageal bulb and oesophagus width was measured at the third part of the organ. Position of the nervous ring was recorded from the anterior part of the nervous ring up to the anterior border of the oesophagus and tail length was measured from the border that connects to the body to the end of the tail. Excretory pore was not found in most of the specimens, therefore this measure was not considered in the morphological analysis. In males, alae length and width was measured from the anterior edge to the posterior border of the alae and at the widest point, respectively; spicule length was measured from the apical point to the border that connects with the body and spicule width was measured at the broadest point of the spicule; tail width anterior to the tail papillae pair was measured above the papillae pair present in the tail and tail width posterior to the tail papillae pair was recorded below it. In females, vagina was measured from the posterior border of the organ to the vulva; vulva position was measured at the anterior border, and egg length and width was measured at the longest and broadest points. Average egg length and width was calculated for a total of four eggs per female. Species and genus classification relied on actual classification purposed by several authors Roca, 1985; Moravec et al., 1987; Astasio-Arbiza et al., 1988; Mašová et al., 2008; Mašová et al., 2009) : *T. tinerfensis* and *T. filiformis* were classified according to the alae shape and size (alae in this species is smaller than the ones of Parapharyngodon species, and is slightly bigger in *T. filiformis*) and considering the number of posterior papillae (two pairs in the cloacal region and one single papilla in the tail). *T. galloti* classification relied on the size of the alae (bigger than the ones of *Parapharyngodon* and reaching the caudal papilla) and on the presence of a total of 6 papillae. *P. bulbosus* was classified according to the size of the lateral alae (bigger than the ones from *P. echinatus*). *P. micipsae* classification relied on the number of cloacal papillae (2 pairs plus one single post-cloacal papilla), in the size and width of the alae (smaller and narrow of the ones of the other *Parapharyngodon* species) and on the shape of the spicule (sharp at the point and with a sickle-like shape). *P. echinatus* classification considered the shape of the spicule (obtuse at the point and straight) and the size of the lateral alae (smaller than the ones of *P. bulbosus* and *T. galloti*).

Statistical analysis

Morphometric statistical analysis were performed in R software Version 3.2.3 (© 2015, The R Foundation for Statistical Computing). Analysis were performed in order to identify significate differences between individuals from different species and genus groups. Groups were defined according to phylogenetic tree results and also concerning biological traits such as the host and locality were they were collected (Appendix).

Measurements were log-transformed and checked for homoscedasticity (Bartlett test) and normality (Shapiro–Wilk test) using the functions bartlett.test and shapiro.test of the base package, respectively. Results revealed that many variables did not followed the normality and homoscedasticity assumptions. Therefore, a nonparametric approach was followed. To assess the presence of morphological clusters among individuals a Principal Component Analysis (PCA) was performed using the R function prcomp. Correlation inferences between BL and all the other variables were tested using Pearson moment-correlation test using the function rcorr included in the R package Hmisc (Harrell Jr. et al, 2015). Results shown that many variables were correlated with BL. Multivariate analysis of variance (MANOVA) and multivariate analysis of multiple covariance (MANCOVA) were performed to test differences among groups using function adonis from package Vegan (Oksanen et al., 2012). MANCOVA analysis were performed using BL as covariate and the least square means were calculated using R function lm. Tukey post-hoc tests were performed to assess which group were causing the differences observed (R function TukeyHSD). A Discriminant Function Analyses (DFA) was performed to investigate which combination of variables better discriminated among groups using function lda implemented in the R package MASS (Venables and Ripley, 2002). Posterior probabilities were calculated using the leave-one-out cross-validation option.

For the qualitative variables multiple correspondence analysis (MCA) tests were performed using function mca implemented in FactoMiner package (Lê et al., 2008) to detect and represent underlying structures in a data set.

Molecular analysis

DNA extraction was performed on individual specimens using DNeasy® Blool and Tissue Kit (QIAGEN) according to the manufacturer's protocol and using a total

volume of 50 µl of elution buffer in the final step; two elutions were obtained. DNA quantity was measured using NanoDrop 2000 spectrophotometer and NanoDrop 2000 software version 1.5 (Thermo Fisher Scientific Inc. 2009) and the elution with higher concentration of DNA was used. Two partial nuclear genes – 28s ribosomal RNA and 18s ribosomal RNA were amplified using PCR method. 18s fragment was amplified using the primers Nem_18s_F and Nem_18s_R as described by Floyd et al. (2005); 28s fragment was amplified using primers 28s rD1.2a and 28s B from Whiting (2002; see Table VIII).

Table VII. Primer sequence, estimated PCR product, annealing temperature of the primes to the DNA template and respective author and publication year.

Gene	Primer	Sequence (5' -3')	PCR product (bp)	Annealing temperature (ºC)	Reference
18s rRNA	NEM_18s_F	CGCGAATRGCTCATTACAACAGC	900 1200	54	Floyd et al. 2005
	NEM_18_R	GGGCGGTATCTGATCGCC		54	Floyd et al. 2005
28s rRNA	28s rd1a	CCCSSGTAATTTAAGCATATTA	1200 1200	54	Whiting, 2002

Polymerase chain reactions (PCR) were performed for a total volume of 20 µl under the following protocol: 4 µl of MyTaq™ Red reaction buffer (Bioline), 0.8 to 1 µl of each primers at the concentration of 0.5 mM, 0.1 µl of MyTaq™ Red DNA Polymerase (Bioline), 0.4 µl of BSA and 2-3 µl of DNA template; for samples that failed to amplify with this protocol, it was used a protocol with 0.1 µl of Platinum®Taq DNA Polymerase (Invitrogen), 2 µl of 10x PCR buffer (Invitrogen), 1 µl of $MgCl^2$ at the concentration of 50 mM, 1 µl of dNTPs at the concentration of 10 mM, 0.8 to 1 µl of each primer at the concentration of 0.5 mM, 0.4 µl of BSA and 2-3 µl of DNA template. Temperature cycles for 18s were set for 40 iterations of 30s at 95 ºC, 45s at 54 ºC and 45s at 72 ºC. For the 28s fragment, 40 iterations were set with the following cycle: 30s at 95 ºC, 30s at 54 ºC

and 1min at 72 ºC. For both genes amplified, PCR settings included an initial template denaturation step of 3 min at 95 ºC as well as a final extension of 10 min at 72 ºC.

Given the length of the fragment (1056 bp), amplified 28s fragments were sequenced for both strands, and 18s fragments (723 bp) were sequenced in a unidirectional way - except in cases where forward read was ambiguous and thus, reverse strand was also sequenced. PCR product purification and sequencing was performed by a commercial facility (Beckman Coulter Genomics, UK).

Phylogenetic analysis

Sequences obtained were blasted to discard contaminations and imported into Geneious Pro version 4.8.5 (Biomaters, 2009), where sequences obtained in both directions were assembled into a consensus sequence. Additional *Parapharyngodon cubensis* 18S sequences published in GenBank were included in the alignment (Genbank accession numbers KF028940, KF029083 and KF029107) in order to obtain a more congruent and complete dataset. *Spauligodon atlanticus* and *Spauligodon auziensis* sequences (Genbank accession numbers JF829225, *S. auziensis* 18s; JF829242, *S. auziensis* 28s; JF829230, *S. atlanticus* 18s; JF829251, *S. atlanticus* 28s) were used as outgroups for both 18s and 28s phylogenetic analysis in Manuscript I. For Manuscript II *Thelandros tinerfensis* (Tt19408) and *Thelandros filiformis* (Tf19344) sequences where used as outgroups for both gene phylogenetic analysis. Alignments were performed using Geneious alignment (Biomaters, 2009) using the default parameters, and then manual editing was performed if needed.

jModel Test software version 2.1.7 (Darriba et al. 2012) was used to choose the best-fit DNA substitution model and eighty-eight different models were tested according to the hierarchical likelihood ratio test by Akaike Information Criterion (AIC) (Akaike, 1974). The models selected for the first manuscript were: TIM2+I+G (18s) and TVM+G (28). For the second manuscript the selected models were: TPM2uf+I (18s) and TVM+I+G (28s). Phylogenetic analysis were done using maximum likelihood (ML) and Bayesian inference (BI) approaches. ML analyses were performed using PhyML 3.0 (Guindon et al., 2010). Node support was done by bootstrap method (Felsenstein, 1985) using 1000 replicates. BI analyses were performed using MrBayes software version 3.2.5 (Ronquist et al, 2012). The analysis was run for ten million generations, with random

starting trees, employing a Markov Chain Monte Carlo (MCMC) approach for sampling the joint posterior probability distribution saved every 100 generations. Two independent runs were performed to ensure consistent results. The twenty five thousand trees - 25% burn-in - were discarded in order to avoid subtoptimal trees and therefore bias results. Concatenated of genes phylogenetic analysis were performed using BI approach. BI and ML analysis were imported in FigTree v. 1.4.2 (Rambaut, 2014) to observe the resultant phylogenetic tree. p-values of genetic distances between and within group were accessed using the software Mega6 (Tamura et al., 2013) where the pre-establishment of genetic groups was done according to the phylogenetic tree results for both genes (Appendix 2 and 9).

---------- (Manuscripts) ----------

Manuscript I

Unveiling lizard parasites evolution
An integrative taxonomic approach on *Parapharyngpdon spp.* and *Thelandros spp.*

Abstract

The separation between the genera *Parapharyngodon* and *Thelandros* has been widely debated. Although some authors agree that *Parapharyngodon* should be recognized as a distinct genus, other disagree and argue that *Parapharyngodon* species should be classified within *Thelandros*. We use an integrative taxonomic approach to assess the status of *Parapharyngodon* spp., comparing phylogenetic analyses of 18S and 28S rRNA gene DNA sequences with statistical morphologic measurements. Our results suggest that *Parapharyngodon* sp. could be consider a genus different from the one of *Thelandros sp*. However, we found that "*Thelandros" galloti* is more closely related to other species of the genus *Parapharyngodon.* Based only on published 18S rRNA sequences, the same may be true for some other species typically assigned to Thelandros. Furthermore, *P. micipsae* appears in our estimates of relationships as a morphotype of both *P. echinatus* and "*T." galloti*. Based on our extensive analysis of morphological data, we suggest which are the most reliable morphological traits to accurately distinguish between the different species. However, the overall uncongruence between species from the different genera and the apparent misidentification of morphotypes of at least two species as a distinct species highlights the discordance between morphological and molecular data, and the need for species to be analyzed under an integrative approach in order to dissentangle its taxonomical status.

Introduction

The use of morphological traits to taxonomically assess a given taxa status, as it has been described by Linnaeus, led to enormous errors in past. This is especially true in nematodes with differences found at the microscopical level, where the morphological assessment and identification of distinctive characters is often translated into a "challenge". Moreover, a taxonomic study based only on morphological characters may fail due to factors such as the source of the host, the preservation method, how specimens are mounted and host-derived variation (Perkins et al., 2011) These factors plus the parasite usually simplistic morphology, combined with specific life-history and similar selective pressures may lead us to erroneous classification of different species (Banks and Patterson, 2005).

There are over 50 described species of *Parapharyngodon* distributed worldwide (Pereira et al., 2011), with several described in the last one or two years (e.g. de Araújo Filho et al., 2015; Velarde-Aguilar et al., 2015). Still, the validity of the genus *Parapharyngodon* has been discussed since it was proposed in 1933 by Chatterji. While many authors agree on *Parapharyngodon* as a distinct genus (e.g. Adamson, 1981; Roca, 1985; Castano-Fernandez et al., 1987), many consider it as subgenus of *Thelandros* Wendl, 1862 (e.g. Yamaguti, 1961) and some do not consider any taxonomic differentiation at all between *Thelandros* and *Parapharyngodon* (e.g. Petter and Quentin, 1976; Petter and Quentin, 2009). However, as well as some identified morphological traits, both genera also have distinctive host preferences with *Parapharyngodon* species being found in insectivorous reptiles and amphibians, while the *Thelandros* species are typically found in more herbivorous or omnivorous reptiles (Adamson, 1981). In various geographical regions species of both genera are found together, and this includes the species found in the endemic reptiles of the Canary Islands.

Thelandros galloti, *T. tinerfensis* and *T. filiformis* males have been described as small nematodes with a whitish body and striations at the level of the cuticle (more prominent in *T. galloti* than in the other species). While *T. galloti* exhibits a long lateral alae that reach from the cephalic region to the region of the caudal papillae, *T. tinerfensis* and *T. filiformis* are only equipped with a short lateral alae that starts near the third part of the body and ends in auricular shape reaching the tail structure. Moreover, *T. galloti* exhibits one pair of caudal papillae, while *T. tinerfensis* and *T. filliformis* have been characterized with a single caudal papilla likely the result of morphological convergence

of both papillae into a single one (Astasio-Arbiza et al., 1988; Astasio-Arbiza et al, 1989; Solera-Puertas et al., 1988). Although these characters allow to distinguish *T. galloti* specimens, *T. tinerfensis* and *T. filiformis* morphological assessment is conducted using subtitle differences at the level of the length of few characters (e.g. lateral alae in *T. filiformis* is bigger than the one from *T. tinerfensis*). However, differences in the females are not as simple to assess. In females, morphological classification depend on differences found at the level of the mouth, specific location of the vulva and shape of the eggs (Astasio-Arbiza et al., 1988; Astasio-Arbiza et al, 1989; Solera-Puertas et al., 1988).

Parapharyngodon species are mainly identified based on the morphology of the anterior cloaca lip, form of the spicule and length and width of the lateral alae in males, and location of the ovary and egg size in females (Adamson and Nasher, 1984). *P. echinatus*, *P. bulbosus* and *P. micipsae* males have been described as small white nematodes that reveal some degree of striation at the level of the cuticle and inhabit the intestine of more carnivorous reptiles. While *P. echinatus* and *P. bulbosus* are described by two long lateral alae (longer in *P. bulbosus*) and 4 pairs of papillae in total (3 at the cloacal region and 1 in the tail); *P. micipsae* individuals have a more small and narrow lateral alae and only exhibit 5 papillae in the cloacal region (two pre-cloacal and lateral pairs and one single post-cloacal papillae). Moreover, there are some morphological divergences at the level of the cloacal spicule of the three species: *P. echinatus* exhibits a long symmetrical spicule with an obtuse point, while *P. bulbosus* and *P. micipsae* are equipment with a spicule that end in a sharp point (Mašová et al., 2008; Roca, 1985; Mašová et al., 2009; Moravec et al., 1987; Roca, 1985). During the last decades, a “hot-scientific topic” concerning *P. micipsae* and *P. echinatus* taxonomical classification is dividing scientists in whether they argue, or not, with a taxonomic synonymy between *P. micipsae* and *P. echinatus* (e.g. Chabaud and Golvan, 1957; Hornero, 1991; Seraut, 1917; Roca, 1985). These possible synonymy gained posterior support when authors agreed that females of the two genera were not distinguisable using morphological traits (Mašová et al., 2009; Roca, 1985).

However, a taxonomic assessment using an integrative approach that combines morphological traits with molecular data it is now almost obligatory in a given taxa reassessment (Goldstein and DeSalle 2011). Molecular tools offer the unprecedented opportunity to include genetic diversity at the level of a specific molecular marker. The use of these tools have not only proven to be useful to describe biodiversity but also to

acknowledge specific phylogenetic relationships between taxa. Recent studies that aimed the use of molecular tools in the phylogenetic assessment of *P. cubensis* (endemic to the Caribbean) revealed that this "species" is possible to be under cryptic speciation phenomena, resulting in a complex of different cryptic species. Also, cryptic speciation has been previous described in *S. atlanticus* (a close related species to the ones of *Thelandros* and *Parapharyngodon*) in the Canary Islands (Jorge et al., 2013). Moreover studies on *T. scleratus* phylogenetic position revealed that this species was grouped in the same clade as *P. echinatus* revealing low levels of genetic variation with *P. echinatus* haplotypes (Chaudhary et al., 2014). Therefore the complex evolutionary patterns of *Parapharyngodon* and *Thelandros* nematodes make them a fascinating groups to study phylogenetic processes and to infer the level of interactions between both genera.

In the present study we combine morphologic statistical analysis with 18S rRNA and 28S rRNA nuclear markers to infer the evolutionary forces that trace the relationship of taxa from *Parapharyngodon* and *Thelandros* genera, and therefore help us to (i) understand if there is a clear genetic differentiation between this two genera, (ii) assess the power of the different morphological characters that allow us a clear discrimination between the different *Parapharyngodon* and *Thelandros* lineages and (iii) test for cryptic speciation

Materials and Methods

Sampling procedures

For this study a total of 56 samples were collected from 16 lizard host species (Appendix 1). Sampling was performed in different localities including the Canary Islands, Morocco, Spain, Portugal, Madeira, Cape Verde and São Tomé. Specimens were mostly obtained from faecal pellets, or from intestines removed from individuals sacrificed or dead accidentally in the field.

All samples were stored in 96% ethanol and then separated, counted and identified using an Olympus SZX2-ILLT magnifying glass (Olympus®, Tokyo, Japan).

Morphological traits

Semi-permanent slides were prepared and species identification was based on the recent morphological classification (see Roca, 1985; Moravec et al., 1987; Astasio-Arbiza et al., 1988; Solera-Puertas et al., 1988; Astasio-Arbiza et al, 1989; Mašová et al., 2008; Mašová et al., 2009) and performed under a light microscope (see General Methods – Morphological Traits).

Statistical analysis

Morphometric analysis were performed in R software Version 3.2.3 (© 2015, The R Foundation for Statistical Computing). Analysis were performed in order to identify significative differences between individuals from different species and genera. Groups were defined according to phylogenetic tree results and also concerning biological traits such as the host and locality were they were collected (Appendix1 and 3). Statistical morphometric analysis were performed using the methodology provided in this dissertation chapter General Materials and Methods – Statistical analysis.

Molecular analysis

DNA extraction from sample tissues, amplification of 18S and 28S ribosomal RNA genes and sequencing precedures were performed (see General Materials and Methods – Molecular Analysis).

Phylogenetic analysis

Sequences obtained were blasted and imported into Geneious Pro version 4.8.5 (Biomaters, 2009), where all reads were checked and sequences obtained in both directions were assembled into a consensus sequence. Additional *Parapharyngodon cubensis* 18S sequences published in GenBank were included in the alignment (Genbank accession numbers KF028940, KF029083 and KF029107) in order to obtain more congruent and complete phylogenies. *Spauligodon atlanticus* (Genbank accession number JF829230, 18s and JF829251, 28s) and *Spauligodon auziensis* (Genbank accession numbers JF829225, 18s and JF829242, 28s) sequences were used as outgroups for 18s and 28s. Alignments were performed using Geneious alignment

(Biomaters, 2009) using the default parameters, and then manual editing was performed if needed.

The best-fit DNA substitution model was choose using jModel Test software version 2.1.7 (Darriba et al. 2012) and phylogenetic analysis were done using maximum likelihood (ML) and Bayesian inference (BI) approaches, using PhyML 3.0 (Guindon et al., 2010) and MrBayes 3.2.5 (Ronquist et al, 2012) softwares, respectively (see General Materials and Methods – Phylogenetic Analysis). P-values of genetic distances between and within groups were assessed using the software Mega6 (Tamura et al., 2013) where the pre-establishment of genetic groups was done according to the phylogenetic tree results for both genes (Appendix 2).

Results

Morphological analysis

A total of 53 males were morphological characterized according to the different morphological traits. Overall, 20 individuals were identified as *P. micipsae*, 16 as *P. echinatus*, 1 as *P. bulbosus*, 1 as *Parapharyngodon* sp., 7 as *T. galloti*, 4 as *T. tinerfensis* and 3 as *T. filiformis* (Appendix 4 and 5). Morphological characterization of females indicated that all individuals belonged to the genus *Parapharyngodon*. However, due to the synonymy of traits in all the *Parapharyngodon* and *Thelandros* species, identification of females was not possible and therefore female individuals were excluded from the statistical analysis.

Statistical analysis

In order to perform statistical analysis concerning measurable variables, individuals were grouped according to genetic group, as determined by the phylogenetic analysis. Therefore *T. tinerfensis* and *T. filiformis* were grouped in the *Thelandros* clade, and all the other species (including *T. galloti* individuals) were grouped in the *Parapharyngodon* clade. This clustering of individuals in two main groups was performed to test how reliable were the morphological characters to discriminate between these two potential genera.

PCA analysis failed to reveal a clustering organization among the two groups of interest (Appendix 6). The first three axes explained 56% of the total variation within the dataset. PC1 explained 32% of the total variation - ε_1= 4.537 – that was highly related with body length (BL), body width (BW), tail length (TL), tail width at the level of the caudal papillae (TW2), oesophagus length (OL) and oesophageal bulb width (OBW) and length (OBL). PC2 explained only 13% of the total variation - ε_2= 1.865 – that revealed to be related with spicule width (SW), tail width at the level of the caudal papillae (TW2) and tail length (TL). PC3 explained a little less than 11% of total variation - ε_3= 1.522 – mainly related to nervous ring position (NR) and spicule width measured at the widest point (SW; Table II, left). Correlation analysis revealed that all variables were correlated to body length (BL) except for tail width measured at the insertion point (TW1), spicule width (SW) and position of nervous ring (NR). MANOVA analysis indicated significant differences for BL, with individuals from the *Thelandros* group being larger than the ones from *Parapharyngodon (*Table I). Concerning MANCOVA, significant differences were identified for body width (BW), lateral alae length (LAL), SW and TL (Table I). These results suggest that individuals from *Thelandros* group in comparison with *Parapharyngodon* can be characterized by a thinner body, a shorter lateral alae, a wider spicule and a longer tail (Figure 1). Nevertheless discriminant function analysis showed that 100% of *Parapharyngodon* species but only 71% of *Thelandros* species were assigned as correct, being mainly explained by body length (BL), lateral alae length (LAL) and spicula width (SW) variables.

Table I. p-values of the different measurements for both MANOVA and MANCOVA analysis when testing between *Thelandros* and *Parapharyngodon* groups. Significant values are marked with an (*).

	MANOVA	MANCOVA
Body length	0.029*	-
Body width	0.258	0.008*
Lateral alae width	0.06	0.059
Lateral ale length	0.025*	0.001*
Tail length	0.003*	0.013*
Tail width 1	0.066	0.069
Tail width 2	0.587	0.969
Spicule length	0.74	0.689
Spricule width	0.002*	0.005*
Nervous ring	0.102	0.14

Oesophagus length	0.443	0.793
Oesophagus width	0.857	0.445
Oesophagael bulb width	0.631	0.2
Oesophagael bulb length	0.335	0.909

To assess a more detailed morphological analysis concerning the *Parapharyngodon* group the individuals within the group were clustered according to genetic lineage (PH1, PH2, PH3, PH4 and PH5; Appendix 3) obtained in the phylogenetic analysis. In general, PH1 corresponded to individuals from Cape Verde, Madeira and São Tomé (plus two individuals from Gran Canaria monophyletic to the group), PH2 grouped individuals from Tenerife, La Palma and La Gomera (plus one individual from Cape Verde), PH3 corresponded to individuals assigned as *T. galloti* and *P. micipsae*, PH4 corresponded to individuals from the Iberian Peninsula and PH5 grouped individuals from Lanzarote, Fuerteventura, Gran Canaria and El Hierro (plus one *P. micipsae* that parasitized a *Q. moerens* from Morocco).

Regarding this analysis, PCA analysis did not reveal any kind of clustering organization among the different groups (Appendix 7). The first three axes explained 60% of the total variation within the dataset. PC1 explained 37% of the total variation - ε_1= 5.235 – that was highly related with body length, body width, tail length, tail width at the level of the caudal papillae, spicule length, oesophagus length and width (OW) and oesophageal bulb width and length. PC2 explained 13% of the total variation - ε_2= 1.804 – that correlated to spicule width and nervous ring position. PC3 explained only 11% of total variation - ε_3= 1.423 – related to spicule length and width. Correlation analysis indicated that all variables were correlated to body length tail width at the level of the caudal papillae, spicule width and nervous ring position (Table II, right). MANCOVA results indicated significant differences for lateral alae width (LAW) and oesophagus length (OL; Table III). Post-hoc Tuckey test corroborated MANCOVA results, highlighting that the differences found in both variables were caused by PH3 and PH4 groups, where individuals assigned as PH3 have the widest lateral alae and oesophagus biggest length and PH4 individuals have the narrowest lateral alae and smallest oesophagus length (Figure 2). Concerning discriminant analysis results, DF1 is mainly explained by LAL and none of the studied species were 100% correctly assigned (Table IV).

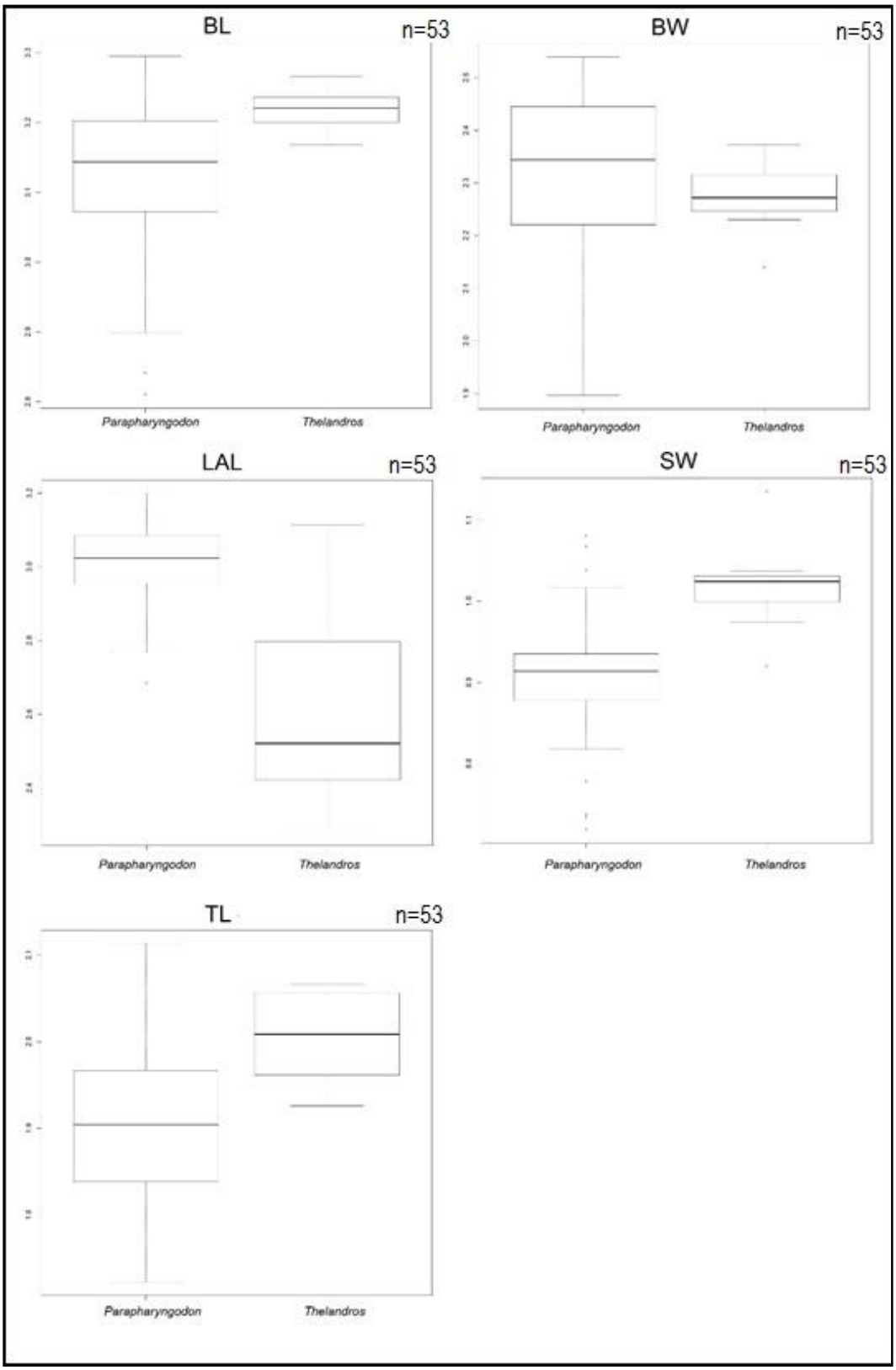

Figure 1 – Boxplot of the measurements with significant differences between *Thelandros* and *Parapharyngodon* groups for a total number of 53 individuals (nThelandros= 7; nParapharyngodon= 46).

Table II. Variable loadings (eigenvalues) extracted from the three-first principal components (PC) of the principal component analysis (PCA) on analysis between genus (GG; left) and species (SG; right). For each principal component, eigenvalues and % variance are shown.

	GG			SG		
	PC1	PC2	PC3	PC1	PC2	PC3
BL	0.644	-0.061	0.135	0.724	0.262	-0.348
BW	0.717	-0.416	-0.160	0.796	0.056	-0.109
LAW	0.401	0.237	0.073	0.457	-0.214	0.045
LAL	0.239	-0.644	-0.097	0.661	0.327	-0.402
TL	0.591	0.425	-0.004	0.592	-0.264	-0.065
TW1	0.343	0.538	-0.585	0.325	-0.767	0.054
TW2	0.625	0.411	-0.452	0.643	-0.558	0.201
Spi	0.550	0.235	0.177	0.640	0.048	0.448
SW	0.187	0.597	0.580	0.141	0.251	0.813
NR	0.078	0.024	0.711	0.045	0.650	0.343
OL	0.681	-0.062	0.104	0.582	-0.131	0.271
OW	0.659	-0.181	0.121	0.660	0.241	-0.163
OBW	0.785	-0.334	-0.020	0.837	0.085	0.007
OBL	0.822	-0.140	0.134	0.809	0.202	0.002
Eigenvalues	4.537	1.865	1.522	5.235	1.804	1.423
% variance	32	13	11	37	13	11

Table III. p-values of the different measurements for both MANOVA and MANCOVA analysis when testing between Ph1, Ph2, Ph3, Ph4 and Ph5 groups. Significant values are marked with an (*).

	MANOVA	MANCOVA
Body length	0.335	-
Body width	0.247	0.36
Lateral alae width	0.002*	0.006*
Lateral ale length	0.946	0.119
Tail length	0.96	0.86
Tail width 1	0.252	0.27
Tail width 2	0.325	0.507
Spicule length	0.936	0.974
Spricule width	0.062	0.061
Nervous ring	0.536	0.54

Oesophagus length	0.002*	0.001*
Oesophagus width	0.883	0.519
Oesophagael bulb width	0.394	0.634
Oesophagael bulb length	0.446	0.733

Table IV. Groups assignment results from Discriminant Function Analysis.

Groups / Species	PH1	PH2	PH3	PH4	PH5
P. echinatus	0	0	11	0	89
P. micipsae	20	0	10	35	35
T. galloti	0	0	63	0	37

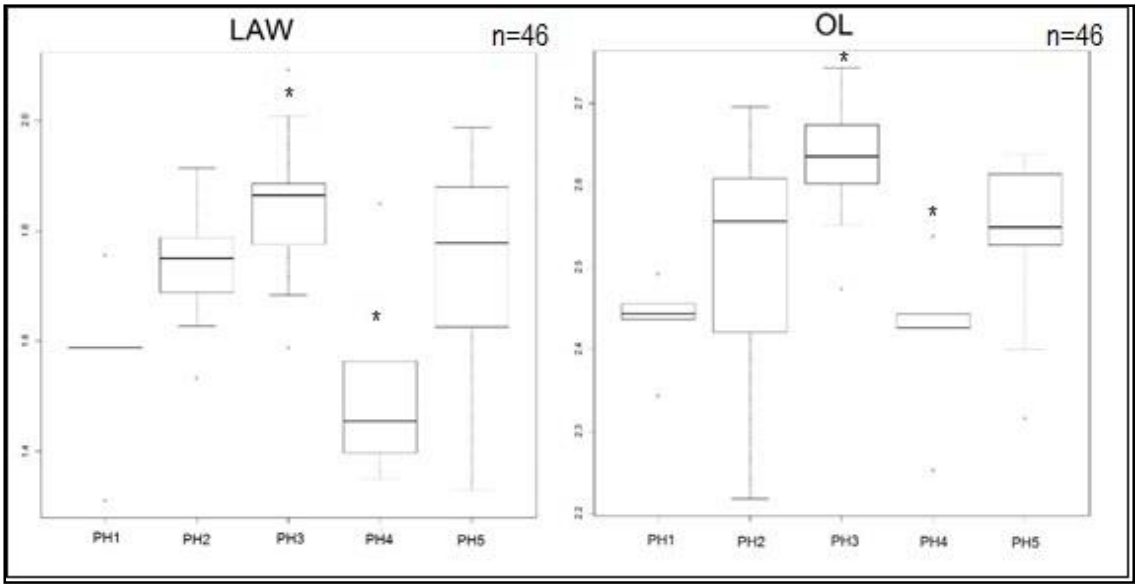

Figure 2 - Boxplot of the significant measurements found between the different *Parapharyngodon* groups in a total sample size of 46 individuals (nPH1= 5; nPH2= 7; nPH3= 11; nPH4= 5; nPH5= 18). Groups that are significant different concerning both variables are marked with an (*).

MCA results revealed three main groups according to our qualitative classification (Figure 3). An emerging group corresponding to *T. filiformis* and *T. tinerfensis* seems to be well defined by one caudal papillae, five total papillae, a small lateral alae and a short spicule. A second group corresponding to *P. micipsae* individuals emerges with the following characteristics: narrow and relatively small alae, a sharp spicule and a total of 7 papillae being 5 of them located in the cloacal structure. A third group is revealed concerning *P. echinatus* and *T. galloti* individuals where the alae size and number of

cloacal papillae traits seem to be crucial to distinguish between the two taxa, with *T. galloti* having a very long lateral alae and 4 cloacalpapillae while *P. echinatus* show to be classified due to its' long alae and 6 cloacal papillae

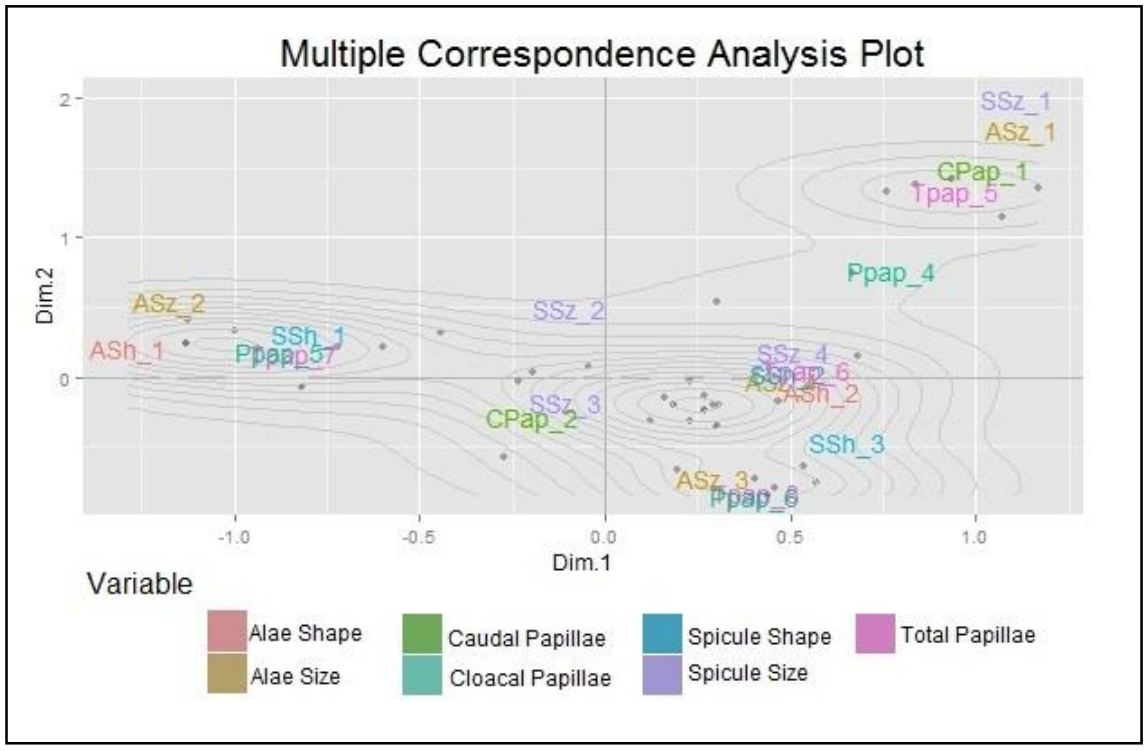

Figure 3 – Multiple correspondence analysis results concerning the qualitative morphological traits where the different variables are clustered in a two dimensional plot.

Phylogenetic analysis

For 18s rRNA molecular marker Bayesian Inference (Figure 4) and Maximum Likelihood (Appendix 8) analysis were calculated using the chosen model TIM2+I+G. However, both phylogenies were not fully concordant. In ML phylogenetic tree, for most of the emerging clades, bootstrap support was not strong enough to elucidate us in terms of phylogenetic variation. Still, the arranging of specimens within each clade is corroborated by both analysis which allow us some degree of comparison. In BI analysis four main groups emerge: (i) a group composed by *T. tinerfensis* and *T. filiformis* individuals, (ii) a group with some level of stratification that comprises individuals from the Iberian Peninsula, Lanzarote and Fuerteventura and Gran Canaria and El Hierro, (iii) an unresolved group derived from *Tarentola* species host from Tenerife, La Palma, La Gomera and Cape Verde and (iv) a group that comprise not only *T. galloti* individuals but

also *P. cubensis* from the Caribean, and *Parapharyngodon* individuals found in the intestine of *T. dugesii* (from Madeira and Gran Canaria), *Hemidactylus* (from São Tomé), *Chioninia* (from Cape Verde) and *G. stehlini* (from Gran Canaria). Although 18s phylogenetic tree does not elucidate us in terms of a fully resolved phylogenies, it allow us to answer some questions. First, there is a clear differentiation between *T. filiformis* and *T. tinerfensis* clade and all of the rest of the individuals (BI posterior probabilities are equal to 100%); although in ML results this group is in fact monophyletic to individuals from Cape Verde, São Tomé and the Caribbean bootstrap support is quite low (13.4 %) which does not allow us to interpret this result with confidence. Second, *P. micipsae* is found in every clade that respect not only *P. echinatus* individuals but also *T. galloti* individuals; however, no *P. micipsae* is found in *T. filiformis* and *T. tinerfensis* clade. Third, *T. galloti* appears to be more closely related to *P. echinatus* than to all the remain *Thelandros* species; furthermore a geographical clustering emerge with individuals from La Palma and Tenerife being monophyletic to a more basal lineage from La Gomera and El Hierro. Fourth, a clade comprising individuals from São Tomé, Cape Verde, Caribbean Islands and Gran Canaria reveals to be monophyletic to *T. galloti* lineage; although this clade is not very informative it reveal us that the individuals found in both *T. dugesii* from Madeira and Gran Canary are sister taxa to *P. cubesis* species. Fifth, a group comprising only Tarentolas from the Canary Islands (Tenerife, La Palma and La Gomera) and from Cape Verde seems to be basal to all the other *Parapharyngodon* lineages. Sixth, although all monophyletic to each other, there is some level of differentiation between the populations from the Iberian Penisula, populations from Lanzarote and Fuerteventura, individuals parasitizing T. boettgeri from Gran Canaria and El Hierro (and *Q. moernes* from Morroco) and individuals from Gran Canaria (*G. stehlini* and *C. sexlineatus*).

28s rRNA nuclear marker BI and ML analysis were calculated using the chosen model TVM+G. Both analysis revealed to be quite similar, therefore bootstrap values and posterior probabilities were combined in the same tree (Figure 5). Although 28s phylogeny corroborates in many aspects the one from 18s there are some results that should be discussed. In 28s analysis a clear differentiation between *T.filiformis* individuals and the ones from *T. tinerfensis* is observed resulting in two lineages that harbour each specie. Also, the lineage that comprise the individuals found in *T. dugesii* intestine appears as a basal lineage to all the other *Parapharyngodon* individuals. The *P. micipsae* individual found in *Q. moernes* (Marocco) is basal to all the

Parapharyngodon lineages except the ones from *T. delalandii* and *T. gomerensis* (La Palma and La Gomera). Moreover the nematode found in a *P. vaucheri* from Huelva appears as monophyletic to the *G. stehlini* and *C. sexlineatus* clade from Gran Canaria, but paraphyletic to the individuals from the Iberian Peninsula.

To estimate the phylogeny of both genus concatenated, BI analysis were performed. Despite the fact that this analysis take into account less individuals (comparing to 18s analysis) a much more resolved phylogeny emerge from this analysis that is powerful enough to be analysed giving us the opportunity to observe how the two genes together are influencing the phylogenetic relationships among taxa (Figure 6). In general the concatenated phylogenetic tree corroborates the ones from 28S single-gene analysis, still some results are not concordant and therefore of great interest. First, the Gran Canaria clade (*Parapharyngodon* individuals parasitizing *G. stehlini* and *C. sexlineatus*) appears to be basal to all the other *Parapharyngodon* clades except for the *T. delalandii* and *T. gomerensis* (from La Palma and La Gomera). Second, *P. vaucheri* individual is basal to the Iberian Peninsula clade. Finally, Lanzarote and Fuerteventura clade is monophyletic to the one of *T. boettgeri* (El Hierro and Gran Canaria).

Genetic Distances

The genetic diversity values, both from within and between groups were quite low in general. For 18s (Table V) no differentiation is found within almost every group where the ones that show some differentiation have a low p-value of genetic diversity (the highest is found for the clade of *P. cubensis* with a p-value of 0.006). Comparisons between groups also revealed low genetic diversity where the highest p-values found correspond to comparisons between the *Thelandros* clade and all the different groups (p-value ranging from 0.014 and 0.019) and between the *P. cubensis* clade and all the remain clades (p-values ranging from 0.004 to 0.016). Concerning *T. galloti* clade the highest genetic difference arise from the comparison between the *T. galloti* from Tenerife and La Palma and the Iberian Peninsula clade (p-value of 0.008).

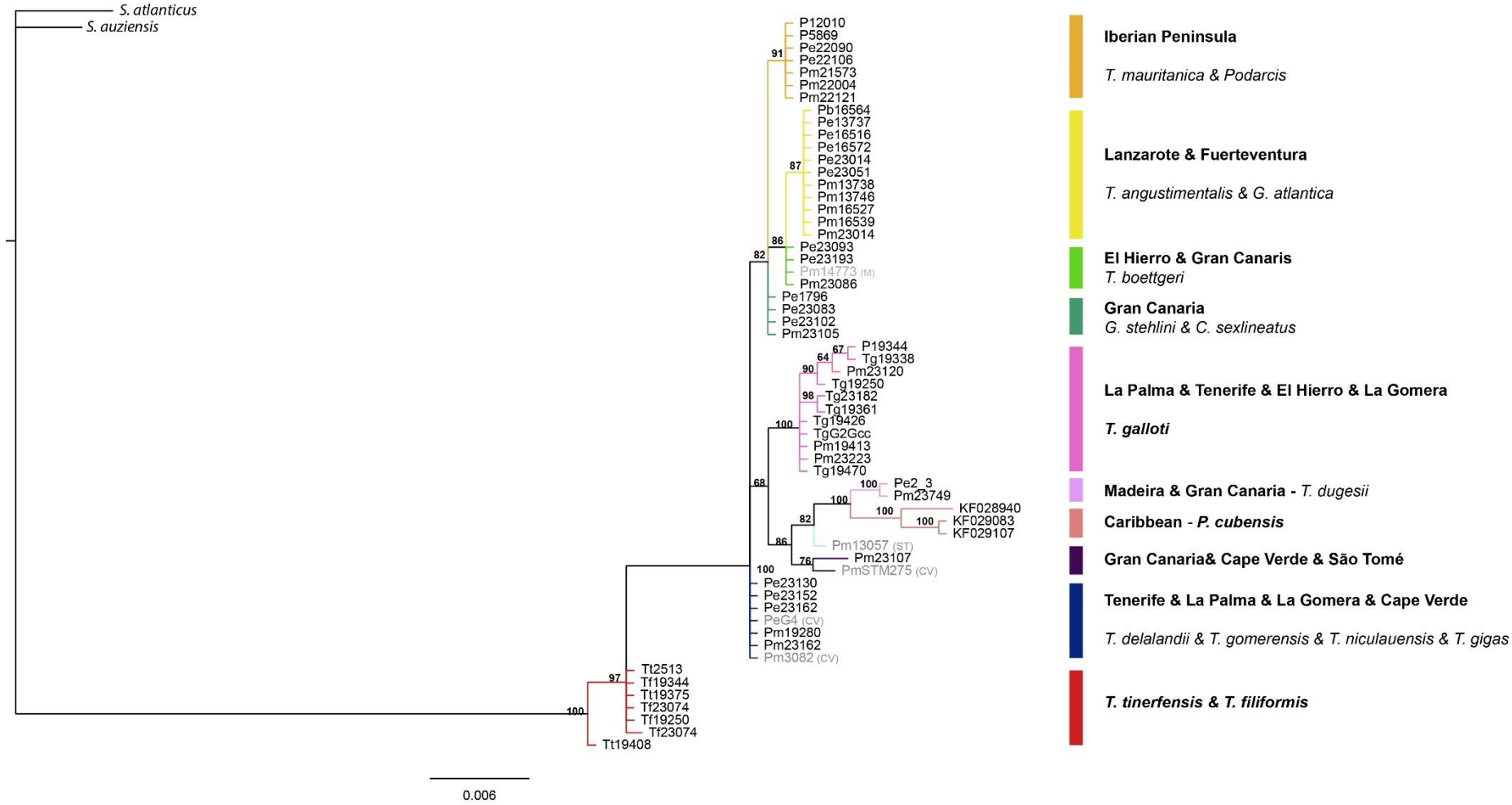

Figure 4 - Bayesian inference tree of the 18S RNA data. Values represent posterior probabilities. Each colour correspond to one clade represented in the right. Individuals that are grouped in a certain clade but have a different location are coloured in grey: M- Morocco, ST – São Tomé and CV – Cape Verde.

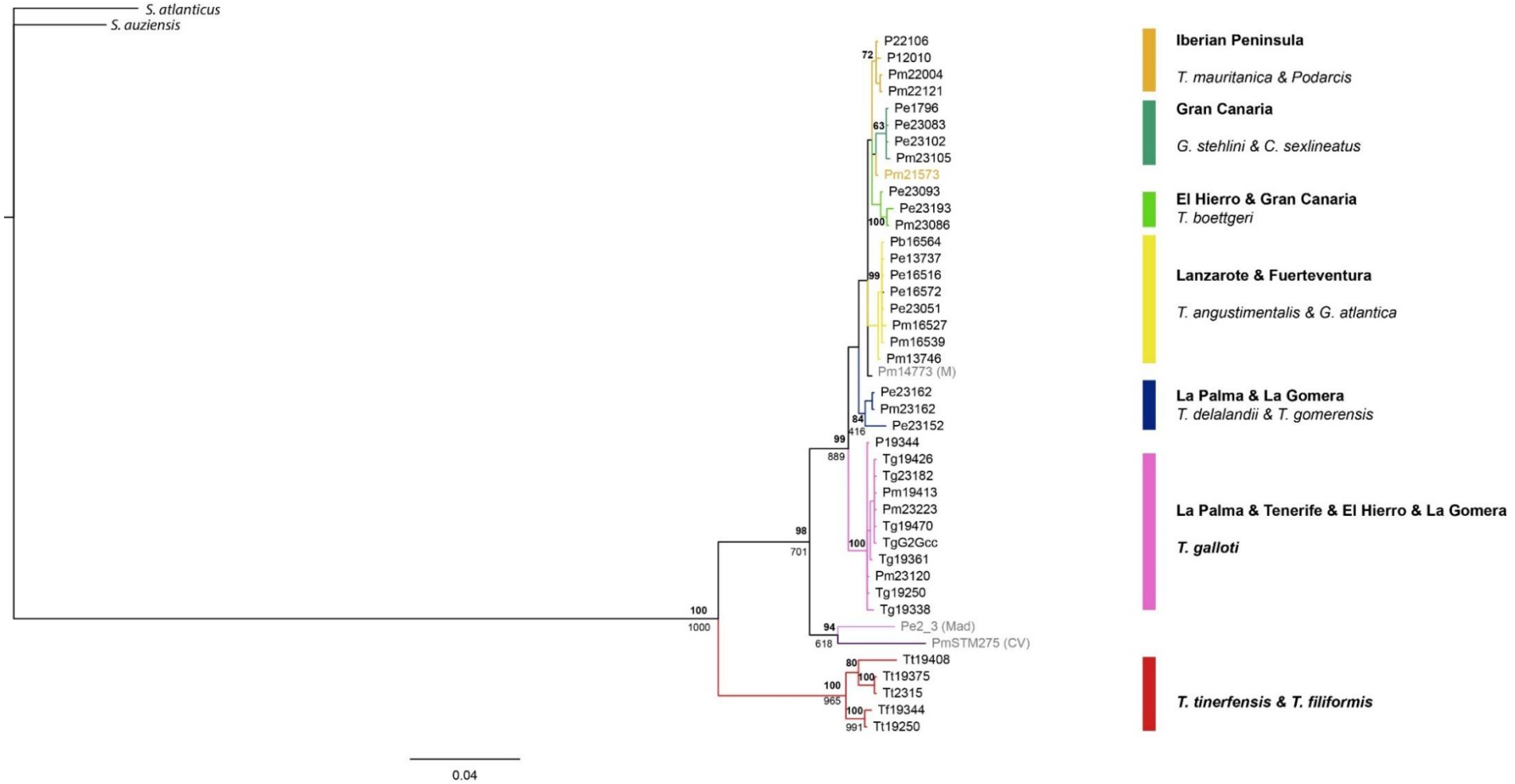

Figure 5 - Bayesian inference and Maximum likelihood phylogenetic tree of the 28S RNA data. Values in bold represent posterior probabilities, and values in plain represent bootstrap values. Each colour correspond to one clade represented in the right. Individuals that are grouped in a certain clade but have a different location are coloured in grey: M- Morocco, Mad - Madeira and CV – Cape Verde.

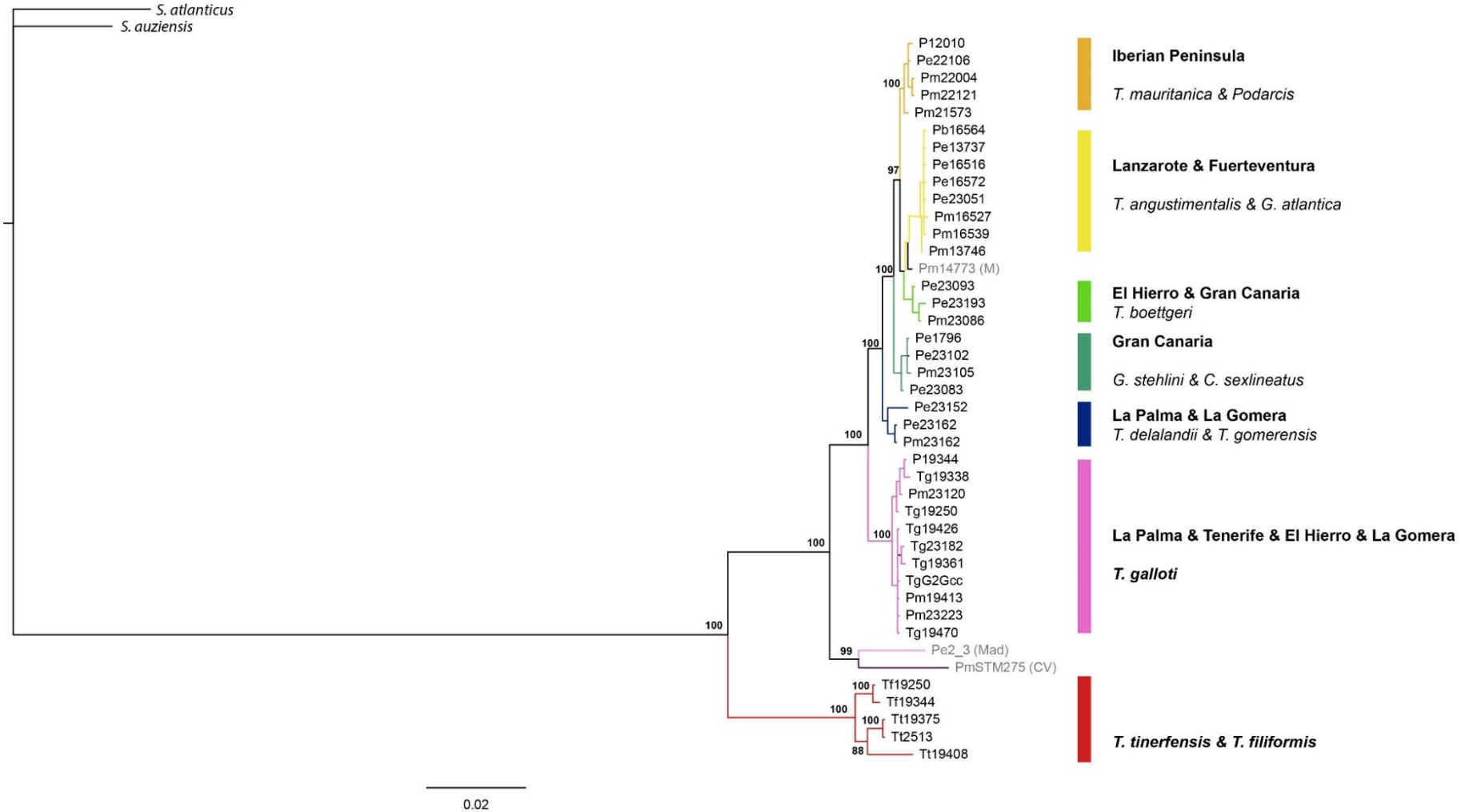

Figure 6 - Bayesian inference phylogenetic tree of both 18S and 28S concatenated genes data. Values represent posterior probabilities. Each colour correspond to one clade represented in the right. Individuals that are grouped in a certain clade but have a different location are coloured in grey: M- Morocco, Mad - Madeira and CV – Cape Verde.

Table V. Results of the genetic distances found between and within groups for 18S rRNA nuclear marker. Genetic distance results are given in p-values.

	Genetic distance between groups											Genetic distance
Group	1	2	3	4	5	6	7	8	9	10	11	within groups
1	-											0
2	0.004	-										0
3	0.002	0.001	-									0
4	0.001	0.002	0.001	-								0
5	0.008	0.004	0.006	0.006	-							0.001
6	0.007	0.006	0.007	0.006	0.003	-						0
7	0.006	0.005	0.006	0.005	0.001	0.001	-					0
8	0.011	0.010	0.011	0.010	0.011	0.011	0.010	-				0
9	0.016	0.014	0.016	0.014	0.015	0.016	0.014	0.010	-			0.006
10	0.006	0.007	0.008	0.007	0.008	0.007	0.007	0.008	0.014	-		0.005
11	0.002	0.004	0.002	0.001	0.005	0.005	0.004	0.009	0.013	0.006	-	0
12	0.017	0.018	0.017	0.015	0.018	0.019	0.018	0.018	0.020	0.018	0.014	0.001

Table VI. Results of the genetic distances found between and within groups for 28s rRNA nuclear marker. Genetic distance results are given in p-values.

	Genetic distance between groups									Genetic distance within groups
Groups	1	2	3	4	5	6	7	8	9	
1										0.001
2	0.005									0.002
3	0.006	0.008								0.003
4	0.006	0.008	0.009							0.001
5	0.009	0.010	0.011	0.011						0.006
6	0.016	0.017	0.019	0.016	0.017					0.0002
7	0.015	0.016	0.017	0.015	0.016	0.002				0.001
8	0.050	0.051	0.053	0.052	0.053	0.052	0.052			0.049
9	0.090	0.091	0.096	0.093	0.090	0.092	0.092	0.092		0.013
10	0.090	0.092	0.095	0.093	0.090	0.089	0.091	0.092	0.019	0.002

28s results (Table VI) revealed a higher degree of differentiation between and within groups were the highest p-value of 0.006 is representative of the group from the host species *T. delalandii* and *T. gomerensis* from La Palma and La Gomera. Genetic distances between groups revealed in the case of 28s that the most distant genetic groups are the ones from *T. filiformis* and *T. tinerfensis* (p-value mean equals to 0.92 when comparing both groups with the ones from *Parapharyngodon spp*.). Moreover, comparisons between specimens from the host species *T. dugesii* from Madeira and *Chioninia* from the Cape Verde islands with the ones from *Parapharyngodon* and *T. galloti* also revealed some level of genetic distance (p-values ranging from 0.50 and 0.53).

Discussion

Studies with the purpose to assess a given taxa taxonomic classification that only rely on morphologic characters assessment might skew the scientific knowledge in a given group. That is not the exception in *Thelandros* and *Parapharyngodon* genera where the usual morphological traits might led scientists to erroneous or less clear conclusions. The ongoing discussion on *Parapharyngodon* and *Thelandros* taxonomy seems to be derived by (i) the high number of species described in these genera, (ii) the lack of information regarding these parasites and (iii) the lack of molecular markers and

sequences available. A recent study that tried to infer *T. scleratus* phylogenetic position grouped this species in the same clade of a *P. echinatus* specimen (Chaudhary et al., 2014). Moreover a study that was conducted using endemic Caribbean pinworms revealed that *P. cubesensis* is more likely to be a complex of different cryptic species than a single species per se (Falk and Perkins, 2013). Also cryptic speciation and complex phylogenetic patterns have been recorded in *S. atlanticus* species in the Canary Islands (Jorge et al., 2013). Our results seem to corroborate in many aspects the observed results of the previous mentioned studies.

Considering *Thelandros* genus, we classified the different *T. tinerfensis*, *T. filiformis* and *T. galloti* species as morphological belonging to this group. Still phylogenetic results revealed a clear differentiation between *T. tinerfensis* and *T. filiformis* specimens and all the other lineages. In fact, our phylogenetic analysis reconstructed the phylogenetic trees with a topology that grouped both *T. tinerfensis* and *T. filiformis* as a basal clade to all the other lineages. Moreover genetic distances on both genes revealed that the *Thelandros* clade is the one that exhibits higher genetic diversity when compared to all the other groups (mean p-value of 2% for 18S and 9% for 28S). These results suggest that *T. filiformis* and *T. tinerfensis* specific lineage should be taxonomically classified as a different taxa from the one of *Parapharyngodon spp.* as it has been proposed by several authors (e.g. Adamson, 1981; Roca, 1985; Castano-Fernandez et al., 1987). Statistical analysis on the morphological traits also revealed that the presence of 5 posterior papillae, the length of the lateral alae, the length and width of the body, the width of the spicule and the length of the tail are possible reliable traits to classify this species when comparing them to *Parapharyngodon sp*., where only 29% of the measured specimens were assigned as not belonging to *Thelandros* group. Therefore, *Thelandros* specimens, in comparison to the ones of *Parapharyngodon* revealed to have a thinner body, a shorter lateral alae, a widest spicule, a longer tail and the presence of 5 posterior papillae. However, considering Chaudhary and his co-workers study (2014), the taxonomic classification of the different species belonging to *Thelandros* group seems to be more complex than what expected, and therefore studies using type-species are needed to truly assess the taxonomical relationships within this group.

Moreover, our results allow us to go further in the complex taxonomic net that both *Thelandros* and *Parapharyngodon* species represent. "*T." galloti* is taxonomic clustered in *Thelandros* genus according to this parasite specific morphological characters (see Astasio-Arbiza et al., 1988). However, our results suggest that "*T." galloti* is genetically closer to *Parapharyngodon* spp. lineages than to the one of the other two

Thelandros species. In 18 and 28S concatenated gene analysis "*T." galloti* clade is basal to all the *Parapharyngodon* lineages except to the one with individuals from Cape Verde and Madeira. Also, a geographical stratification appears in this clade with individuals from La Palma and Tenerife being monophyletic to the ones from La Gomera and El Hierro. This results are corroborated with the genetic distance inference, where the different "*T." galloti* lineages are genetically closer to each other (but there is still some degree of differentiation) than to all the other lineages. Moreover genetic distance results reveal that for 18S and 28S "*T." galloti* is genetically more distant from *T. tinerfensis* and *T. filiformis* (mean p-value of 2% for 18S and 9% for 28S) clade than to the ones of *Parapharyngodon spp.* This results suggest that *T. galloti* might be considered a single species, but not belonging to the *Thelandros* group. Statistical results on the morphological traits were not strong enough to infer a morphologic structuration of *T. galloti*, with 37% of the individuals being misclassified and grouped in PH5 group (representative of *P. echinatus* lineage from Lanzarote, Fuerteventura, Gran Canaria and El Hierro). However, ANCOVA analysis revealed that there is some degree of morphological differentiation at the level of the lateral alae (where *T. galloti* exhibits a widest alae structure when compared to the other groups) and the oesophagus length (longer in *T. galloti*). Although the width of the alae was already predicted (Astasio-Arbiza et al., 1988), the differences found at the level of the oesophagus length have never been noticed; however, this result may arise with some degree of error associated with the microscopicaltraits of the nematodes and caution when analysing the results is advice. Furthermore, MCA analysis clustered the following traits together: wide and very long lateral alae, presence of 6 posterior papillae and semi-sharp shaped spicule. Although the first two traits are assigned as to belonging to *T. galloti*, the former one have also been observed in other *Parapharyngodon* species, therefore this last trait it may be possible to be used in a taxonomic assessment of "*T." galloti* if in the presence of the other distinctive characters.

P. micipsae specimens have haplotypes that fall into in every *P. echinatus* and "*T." galloti* lineage. Although morphological analysis allow a clear differentiation between *P. micipsae* specimens and the ones from *P. echinatus* and *T. galloti*, phylogenetic analysis of both genus revealed that *P. micipsae* may not be considered a species, as it was suggested by several authors (Chabaud and Golvan, 1957; Roca, 1985). The combination of both morphological and molecular traits suggest instead that *P. micipsae* may not only be a morphotype of *P. echinatus* but also one of *T. galloti*.

Considering *P. echinatus* phylogenies, results reveal that this group might represent a complex of several cryptic species as it was also recorded in *P. cubensis*

(Falk and Perkins, 2013). A clade with individuals from Cape Verde and Madeira appears as basal to all the other *Parapharyngodon* and "*T." galloti* species (5% of genetic variability in 28S when compared to the other *Parapharyngodon* and *T. galloti* lineages). A closer look on 18S phylogeny expose that individuals found in *T. dugesii* are closely related despite the fact that one was sampled in Madeira and the other one in Gran Canaria; moreover these individuals are not only sister lineages to the ones classified as *P. cubensis* but also to individuals from Cape Verde and Sao Tome; genetic distance results revealed that the genetic variation between this lineages is represented by a mean p-value of 1% for 18S. Additionally a study concerning the helmintofauna of *T. dugesii* individuals from Madeira suggest that this individuals have distinctive morphology than the *P. echinatus* paratype (differences were found at the level of the caudal papillae where some specimens only have one papilla, and at the level of the lower lip of the cloacal, where specimens exhibit a fringed or plain lip; Sánchez Gumiel et al., 1993). However these morphological differences were not noticed in our specimens. Therefore specific morphological and phylogenetic studies, using a more focused dataset, concerning this "lineage" are needed to confirm former assumptions, where a taxonomic reassessment is likely to "promote" this lineage to the species status.

Results on the remain *P. echinatus* phylogeny reveal the existence of 5 main lineages: (i) one from La Gomera and La Palma infecting *T. delalandii* e *T. gomerensis,* (ii) a second lineage from Gran Canaria infecting *G. stehlini* and *C. coeruleopunctatus*, (iii) a third lineage specific to *T. boettgeri* individuals from Gran Canaria and El Hierro, (iv) a fourth lineage parasitizing *Gallotia* and *Tarentola* species from Lanzarote and Fuerteventura islands and (v) a final lineage considering individuals from the Iberian Peninsula. Genetic distance results reveal that these lineages have a mean genetic variation of 0.7% (for 28S) when compared between each other. The divergence underlying this differentiation might be related (i) to the geographical location of the specimens but also, and more likely, (ii) to the colonization patterns associated. 18S phylogenetic results reveal that the lineage from La Palma and La Gomera clade also comprise individuals from *T. gigas* from Cape Verde suggesting that this *P. echinatus* lineage may be the result of a dependent colonization on the one of this *Tarentola* species common ancestral (see Carranza et al., 2002). Also, concatenated phylogenies results show that the two different lineage present in Gran Canaria (one from parasites of *Gallotia* and *Chalcides* and the other from nematodes of *T. boettgeri*) may be the result of independent colonization events associated with colonization of the ancestral of *T. boettgeri* (see Carranza et al., 2002), and with the colonization of a *Chalcides* or *Gallotia* ancestors. Also, the parasite from a gecko sampled in Morocco appear in a position basal

to the remain the individuals from Fuerteventura and Lanzarote possibly suggesting that the colonization of this islands by *P. echinatus* may be related to the species complex of *T. mauritanica* (see Rato et al. 2012). However, analysis that allow the estimation of this lineages ages were not performed, being all these hypothesis speculative.

Our results reveal to be quite congruent with a complex cryptic speciation pattern where each lineage (or species), according to our results, cannot be distinguished based only on a morphological taxonomic assessment as it has been previously recorded in *P. cubensis* (Falk and Perkins, 2013). However, this might not be the case for individuals belonging to the Iberian Peninsula lineage where a close morphological assessment reveals that these individuals exhibit a smaller lateral alae and a shorter oesophagus than individuals from other lineages. Therefore these distinctive traits might be crucial for the taxonomic assessment of this lineage as a species. However, more studies focusing in understand *P. echinatus* diversity complex patterns are needed to truly understand the forces that are shaping this group evolution.

As final notes, the present allow us to make some considerations considering the current taxonomic classification of *Parapharyngodon* and *Thelandros* species. One, our results suggest a taxonomic differentiation between *Parapharyngodon* species and *T. filiformis* and *T. tinerfensis* possible at the genus level. Two, we suggest a taxonomic reassessment of *"T." galloti* status as *P. galloti*. Three, *P. micipsae* should be treated as a morphotype of both *P. echinatus* and *P. galloti* instead of a single species. And finally *P. echinatus* is possible to be the case of a complex net of cryptic species rather than a single species. More studies are needed to fully understand the patterns of diversity found.

References

See General References.

Manuscript II

A phylogenetic assessment of Parapharyngodon spp. Chatterji, 1933 in the Canary Islands

Abstract

Host-parasite interactions are models of great interest to study coevolutionary patterns. However the inference of such relationships is not in most cases accessible to obtain due to events such as lineage sorting, duplication and pseudocospeciation that might lead to a dissociation between the host and the parasite phylogenies. In the Canary Islands *Parapharyngodon* species nematodes have been recorded to colonize each endemic lizard genus host endemic to this archipelago. Combined with the Canary Islands geological history, and the different colonization patterns of the endemic Canary lizards, *Parapharyngodon* host-generalist behaviour make this relationship an interesting model to observe host-parasite evolution. In this study we use 18s and 28s as molecular markers to infer the evolutionary forces that are tracing Parapharyngodon spp. phylogeny and to infer which is level of congruence between Parapharyngodon evolutionary history and the ones from their hosts. Accession of 18s and 28s phylogeny were not fully resolved. However they did evidence the differentiation of three different clades: one from *T. dugesii* nematodes, one from *P. gallotia* and the least basal from *P. echinatus*. Here, our results evidence the possibility of a cryptic species specific from *T. dugesii* individuals that appears as basal to the other clades. Also, our results reveal that *P. bulbosus* may not have a specie status. *P. galloti* and *P. echinatus* phylogenetic results reveal a differentiation between the most eastern and western islands lineages. Furthermore, when comparing this results with their host phylogenies is possible that both *P. echinatus* and *P. galloti* have colonized this islands via independent events that in the case of *P. echinatus* are possible to the related with the colonization of this islands by *Tarentolas* ancestrals.

Introduction

Coevolution, the process of reciprocal evolution between two or more species, is of great interest for evolutionary biologists and host-parasite interactions represent an exciting model to study these patterns (Page, 2003). Host-parasite interactions might arise by direct heritage from ancestral species (association by descent), or by host-switching events (association by colonization) (Banks and Paterson, 2005). Given the nature of parasites, and its tight dependence to the host, coevolutionary process might be expected to be the rule, however, far from that, .perfect phylogenetic matches are rarely found. This happens because events such as lineage sorting and duplication may lead to a dissociation between the host and the parasite evolutionary histories (Banks and Paterson, 2005). Moreover, shared biogeographic histories and parallel independent colonisations may cause pseudocospeciation confounding the analyses of interactions between parasites and hosts (Page, 2003).

Islands represent a useful scenario to study species evolution because of their isolated environment. The Canary Islands are located in the Macaronesia, in the Atlantic Ocean, and have a volcanic origin with well-known geological histories (see Sanmartín et al., 2008). This archipelago is positioned 100 km west from the African coast and is comprised by seven main islands: El Hierro, La Palma, La Gomera, Tenerife, Gran Canaria, Fuerteventura and Lanzarote. These islands have different origins with a gradient temporal pattern from East to West; the eastern islands are older than the western ones and emerged about 20 million years ago, while the youngest islands are only a little over 1 million years old (Ancoechea et al., 2006; Carracedo et al., 1998; Guillou et al., 2004). The Canary Islands patterns of biodiversity and geological history make this archipelago a unique system for studying evolutionary patterns and interactions (Nogales et al. 1998), including host-parasite interactions. Although the Canary Islands have a relatively poor vertebrate fauna, it shows high levels of endemicity, especially regarding reptiles and birds (Francisco-Ortega et al., 2009).

The genus *Gallotia* is endemic to the islands and currently there are seven recognized species: *G. galloti*, *G. caesaris*, *G simonyi*, *G. bravoana*, *G. intermedia*, *G. stehlini* and *G. atlantica*. The former ancestor of *Gallotia* colonized these islands in the Miocene where *G*. *stehlini* (from Gran Canaria) is basal to the other *Gallotia* species and *G. atlantica* (from the eastern islands) originates from the subsequent node*;* this suggests that *Gallotia* species seem to have colonized the Canary Islands in an eastern-western pattern (except for Gran Canaria that was the first island to be colonized) where

the western *Gallotia* lineages were originated from *G. atlantica*, rather than *G. stehlini* (Arnold et al. 2007; Cox et al. 2010). *Chalcides* genus, although it is not endemic to the Canary Islands, it has four endemic species: *C. sexlineatus*, *C. coeruleopunctatus*, *C. viridanus* and *C. simonyi*. This genus seems to have colonized the Canary Islands via independent colonization events followed by within-island differentiation (Brown and Pestano, 1998; Carranza et al, 2008; Pestano and Brown, 1999). The genus *Tarentola*, from the Phyllodactylidae family, is represented in the Canary Islands by four species: *T. angustimentalis*, *T. boettgeri*, *T. delalandii* and *T. gomerensis.* All species are endemic to the Canary archipelago except *T. boettgeri* that also inhabits the Selvagens islands. The colonization of the Canary Islands by these geckos seems to have occurred with multiple colonization events; a first colonization by the ancestral of *T. boettgeri* in Gran Canaria and El Hierro (Carranza et al., 2002), a second colonization where *T. delalandii* and *T. gomerensis* ancestral colonized Tenerife, La Palma and La Gomera (Carranza et al., 2002) and a third colonization likely to have occurred with the dispersion of *T. mauritanica* ancestral to the Lanzarote and Fuerteventura islands with *T. angustimentalis* being a lineage within a paraphyletic *T. mauritanica* (Carranza, 2000; Rato et al. 2012).

Regarding their parasites, all the reptile species in the Canary Islands are parasitized by the genus *Parapharyngodon* Chatterji 1933, family Parapharyngodonidae. These pinworms belong to a lineage that parasitize carnivorous lizards (Adamson,1981) although they have been recorded occurring in the gut of more omnivorous forms (e.g. Carretero et al., 2006; Martin and Roca, 2005). They are haplodiploids and show direct life cycles (Adamson, 1989, 1990). There are more than 46 species described across the world (Pereira et al., 2011), but in the Canary Islands there are three described *Parapharyngodon* species: *Parapharyngodon micipsae*, *P. echinatus* and *P. bulbosus* (Linstow, 1899; Rudolphi, 1819; Seraut et al. 1917). These species are distributed in the Macaronesia islands, Morocco and Mediterranean basin and in the Canary Islands they parasitize the genera *Gallotia*, *Tarentola* and *Chalcides*. However, preliminary results using genetic data (see Manuscript I) reveal several incongruences in its taxonomy; *T. galloti* probably belongs to the *Parapharyngodon* lineage and *P. micipsae* is probably a morphotype of *P. echinatus* and *T. galloti*.

While most of the current species were described based on morphological characters, the development of molecular tools provides a powerful tool not only to identify and characterize parasites from a taxonomical point of view, but also to study the evolutionary relationships of the parasite itself, and compare it to the ones of their hosts. In fact, recent molecular studies on another nematode genus, *Spauligodon*, also

infecting reptiles in the Canary Islands showed distinctive lineages in the more eastern and the more western islands (Jorge et al., 2011, 2013, 2014).

The present study aims to reveal the phylogenetic relationships of the genus *Parapharyngodon* infecting reptiles from the Canary Islands. For this, we use 18s and 28s rRNA nuclear markers. Furthermore, we compare the obtained phylogeny with the one of their host to assess to what extent the phylogeny of the parasite mirrors the one of the host, and to try to infer how *Parapharyngodon* colonized the Canary Islands.

Materials and Methods

In the present manuscript we will refer to *T. galloti* as *P. galloti* and *P. micipsae* individuals will be analysed as synonymous species of both *P. galloti* and *P. echinatus*, according to the results of Manuscript I.

Sampling procedures

Sampling was performed in 28 different localities across the seven Canary Islands between 2009-2014. Specimens were mostly obtained from faecal pellets, or from intestines removed from individuals sacrificed or dead accidentally in the field. From faeces, a total of 84 individuals of *Parapharyngodon* sp., including representatives of all lizard hosts and localities infected were selected for this study (Appendix 1). In addition, given the evidences from the first manuscript, a sample of *Parapharyngodon galloti* (Appendix 1) was also included. Sampling was approved by the authorities of the Canarian Government (Cabildos Insulares from Lanzarote, Fuerteventura, Gran Canaria, Tenerife, La Palma, La Gomera and El Hierro).

All samples were stored in 96% ethanol and specimens we separated counted and identified according to the general methodologies provided in this dissertation (See General Materials and Methods – Sampling Procedures).

Molecular analysis

DNA extraction from sample tissues, amplification of 18S and 28S ribosomal RNA genes and sequencing precedures were performed (see General Materials and Methods – Molecular Analysis).

Phylogenetic analysis

Sequences obtained were blasted and imported into Geneious Pro version 4.8.5 (Biomaters, 2009), where all reads were checked and sequences obtained in both directions were assembled into a consensus sequence. Additional *T. filiformis and T. tinerfensis* sequences were used as outgroups for both genes (see Manuscript I and Appendix 1). Alignments were performed using Geneious alignment (Biomaters, 2009) using the default parameters. The best-fit DNA substitution model was choose using jModel Test software version 2.1.7 (Darriba et al. 2012) and phylogenetic analysis were done using maximum likelihood (ML) and Bayesian inference (BI) approaches, using PhyML 3.0 (Guindon et al., 2010) and MrBayes 3.2.5 (Ronquist et al, 2012) softwares, respectively (see General Materials and Methods – Phylogenetic Analysis). P-values of genetic distances between and within groups were assessed using the software Mega6 (Tamura et al., 2013) where the pre-establishment of genetic groups was done according to the phylogenetic tree results for both genes (Appendix 9).

Results

A total of 79 nucleotide sequences of the 18S rRNA fragment with a length of 723 bp were included in the dataset (Appendix 9). The best model for this dataset was TPM2uf+I and it was the one implemented in our BI and ML analyses. The results of the BI and ML analyses (Figure 1), although not very informative, have in general a geographical structure, that allow to distinguish five main groups from the most basal to the more recent ones: (i) clade I, that includes *Parapharyngodon* infecting *Tarentola* and *Chalcides* from Tenerife, *Tarentola, Chalcides* and *Gallotia* from La Gomera, *Tarentola* from Gran Canaria and *Gallotia* from La Palma and El Hierro (ii) clade II, that includes individuals infecting *Tarentola* and *Chalcides* from Gran Canaria, as well as *Tarentola* from El Hierro (iii) clade III, a group with individuals parasitizing *Gallotia* and *Tarentola* from Lanzarote and Fuerteventura, (iv) clade IV a *P. galloti* group infecting *Tarentola* and *Gallotia* from La Gomera, Tenerife and El Hierro, and (v) clade V found in *Gallotia stehlini* from Gran Canaria and in a *T. dugesii* population recently introduced in the same island. Interestingly, *P. bulbosus* individuals have haplotypes that fall into two diffferent lineages, (Fuerteventura and Lanzarote- Lineage III, and in the group of the central and western islands - lineage I). As it was previous reported (see Manuscript I) *P. micipsae* haplotypes

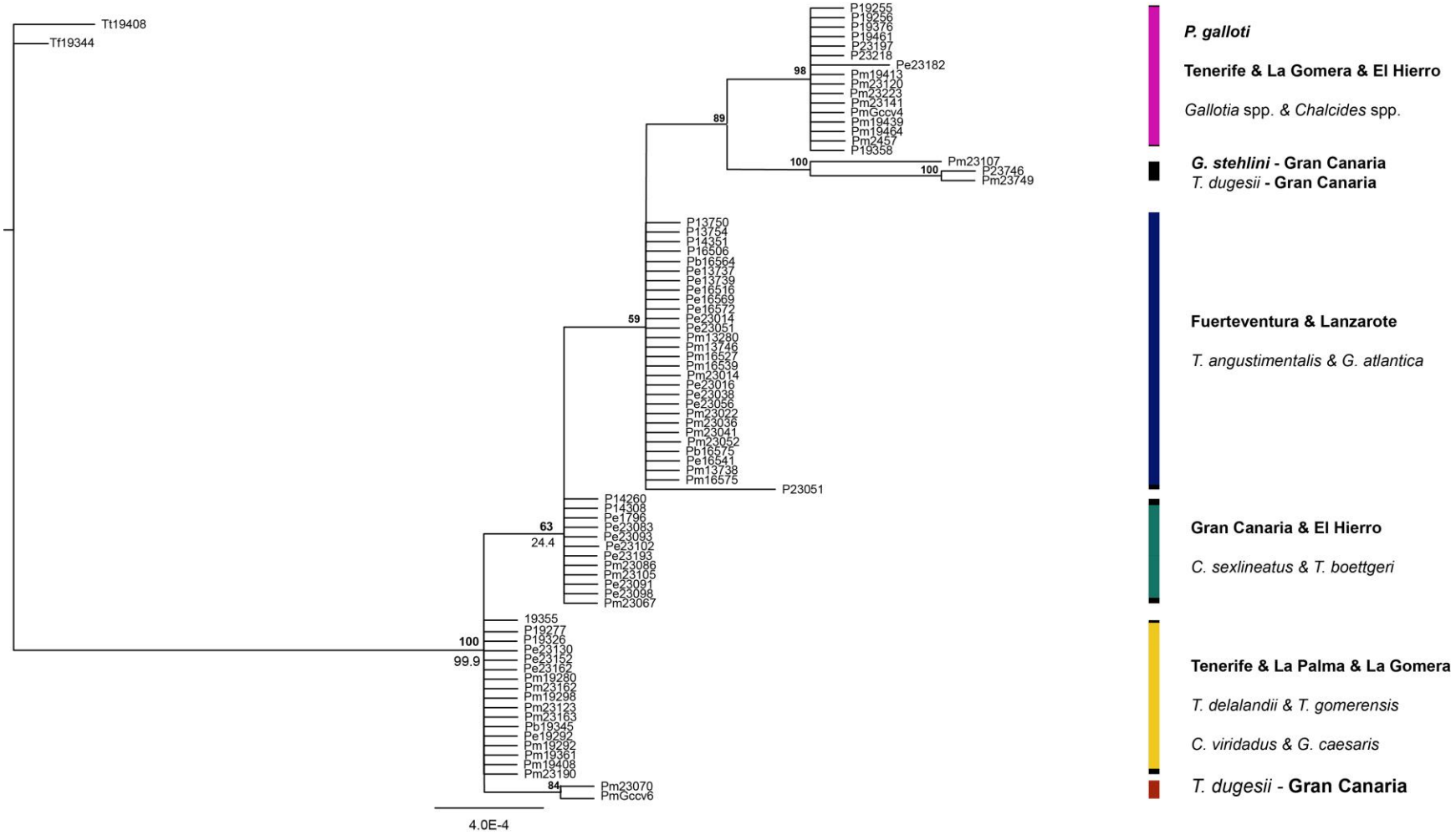

Figure 1 – 18S RNA Bayesian inference and Maximum likelihood phylogenetic tree. Values in bold represent posterior probabilities, and values in plain represent bootstrap values. Each colour correspond to one clade represented in the right.

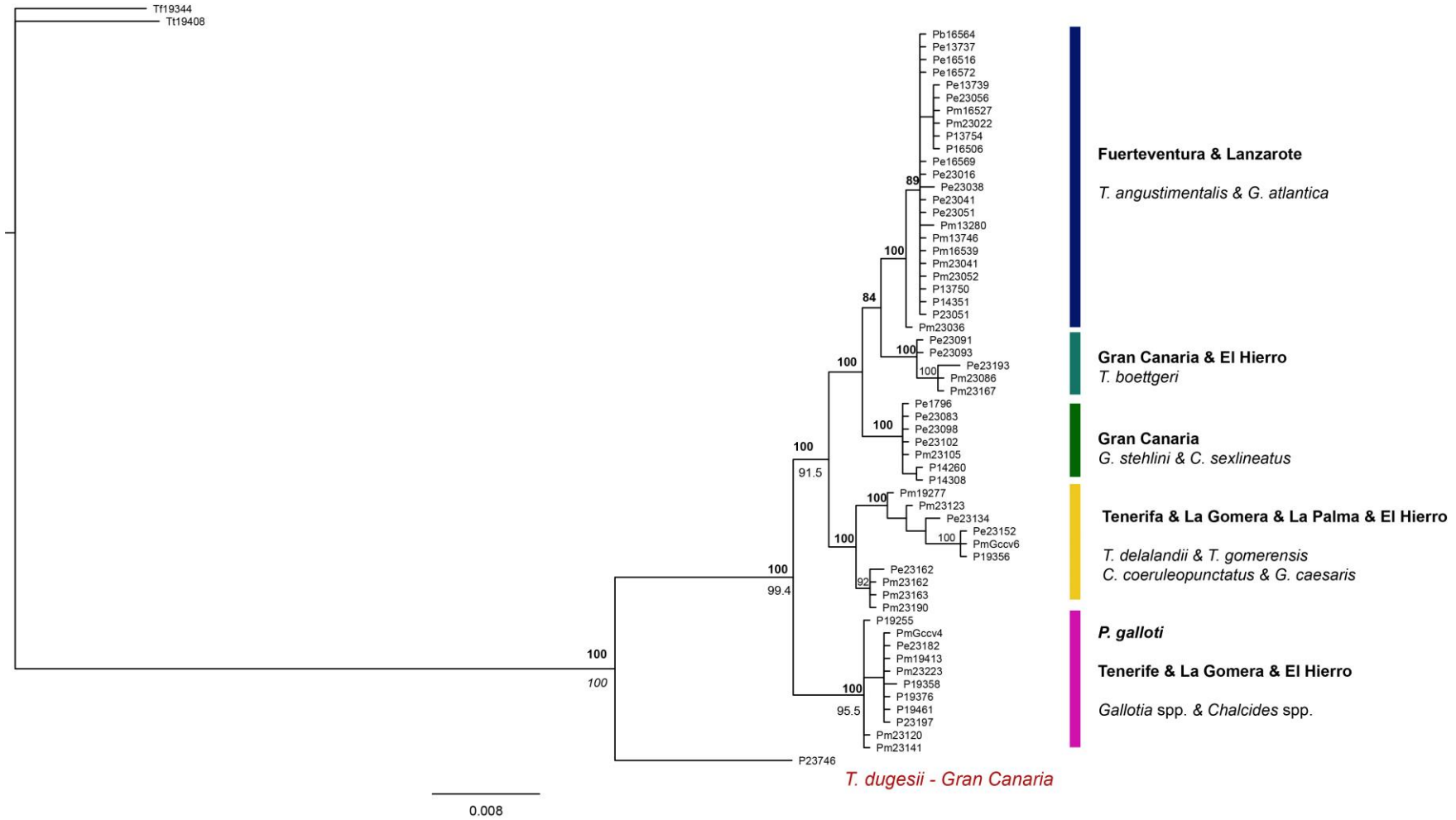

Figure 2 – 28S RNA Bayesian inference and Maximum likelihood phylogenetic tree. Values in bold represent posterior probabilities, and values in plain represent bootstrap values. Each colour correspond to one clade represented in the right

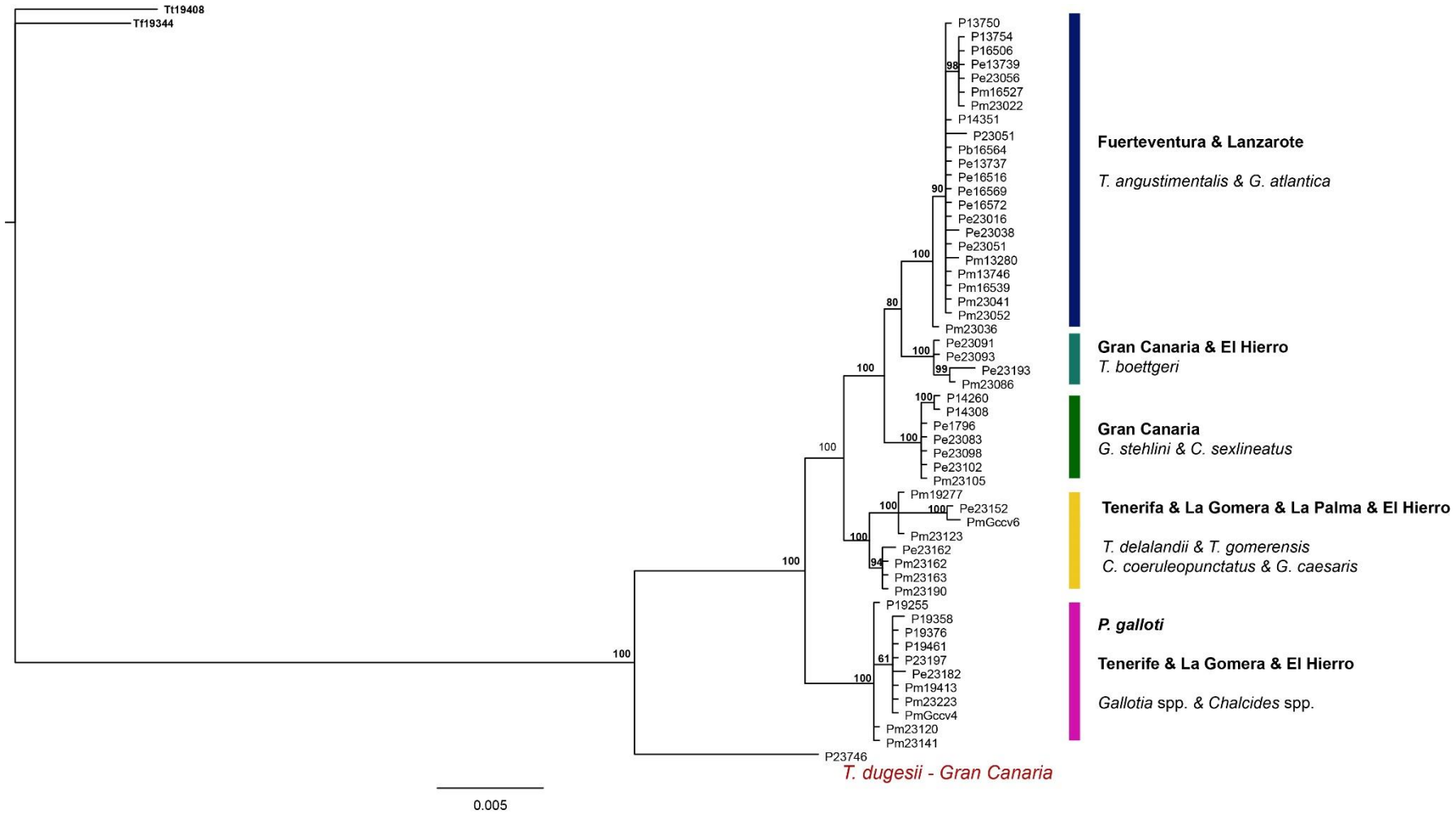

Figure 3 – 18S and 28S concateneted genes Bayesian inference pylogenetic tree. Node values represent posterior probabilities. Each colour correspond to one clade represented in the right

fall in every lineage from the phylogenetic tree. Moreover, there is a clear distinction between *P. galloti* and *P. echinatus*, where the first is constrained to clade IV and the last has representatives in every lineage, except for clades IV and V.

Nucleotide sequences of the 28S rRNA fragment were analysed from 58 specimens with a length of 1056 bp. The best model for this dataset, and was the one used in our BI and ML analysis, was TVM+I+G. 28s BI and ML phylogenetic analysis (Figure 2) is more informative than the 18s gene and interestingly shows different topology. (i) Clade I, is represented by one single individual of *Parapharyngodon* infecting *T. dugesii* appears as basal to all the other lineages, (ii) Clade II, includes *P. galloti* individuals and is monophyletic to Clade I, (iii) Clade III is representative of individuals found *in T. delalandii, T. gomerensis, C. coeruleopunctatus* and *C. viridanus* from Tenerife, La Gomera and La Palma, however results reveal the differentiation of two lineages, one from Tenerife and La Palma and the other from La Gomera,(iv) Clade IV includes individuals from Gran Canaria concerning *G. stehlini* and *C. sexlineatus* host species, (v) Clade V is represented by individuals specific to *T. boettgeri* from both Gran Canaria and El Hierro; however in this clade we can observe an individual found in *T. gomerensis* from La Gomera. (vii) Clade VI, a least basal clade emerges with individuals from *T. angustimentalis* and *G. atlantica*, both from Lanzarote and Fuerteventura; also, an individual from *T. angustimentalis* from Fuerteventura, appears as basal to this clade. Also comparisons between 18S and 28S phylogenies allow to infer that while some 18S lineages are retrieved in 28S tree topology (Clade 3, Clade 4 and Clade 5). Clades 1 and 2 from 18S phylogeny appear rearranged in the one of 28S. Moreover the posterior probabilities and bootstrap values from 28S phylogenetic tree strongly support 28S lineages. Concatenated gene phylogenetic tree results corroborate the results of 28s phylogeny (Figure 3).

Genetic distances

18S rRNA genetic distance analysis reveal low levels of genetic differentiation between and within lineages (Table I). Within lineage analysis revealed that the group that appears to be more genetic diverse is the one of the *T. dugesii* lineage (Clade V; p-value 0.04%) where the one more similar is the one of Gran Canaria (Clade II), with no genetic differentiation at all. When comparing genetic distances between groups the one with that shows less congruence with all the others appears to be the one of *T. dugesii* clade (Clade V; p-values equal to 0.06%).

Table I. Results of the genetic distances found between and within groups for 18S rRNA nuclear marker. Genetic distance results are given in p-values.

	Genetic distance between groups				Genetic distance
Groups	1	2	3	4	within groups
1	-				0.001
2	0.002	-			0
3	0.003	0.001	-		0.0002
4	0.006	0.007	0.006	-	0.004
5	0.003	0.004	0.003	0.006	0.0002

28S genetic distance results (Table II) revealed that the more distance group appears to be the one of *T. dugesii* (p-values are in most cases equal to 0.5%) but no differentiation is found within this group (due to the fact that only one individual constitute the group). Also there is a great level of differentiation between the *P. galloti* lineage and all the others (p-value between 0.15% and 0.18%). Within groups genetic distances have the highest values for the population of Tenerife, La Gomera, El Hierro and La Palma (p-value equal to 0.03%).

Table II. Results of the genetic distances found between and within groups for 28S rRNA nuclear marker. Genetic distance results are given in p-values.

	Genetic distance between groups						Genetic distance
Groups	1	2	3	4	5	6	within groups
1	-						-
2	0.044	-					0.001
3	0.044	0.015	-				0.0005
4	0.046	0.018	0.007	-			0.003
5	0.045	0.017	0.009	0.011	-		0.0005
6	0.048	0.018	0.010	0.011	0.009	-	0.002
7	0.045	0.016	0.010	0.013	0.010	0.009	0.001

Discussion

According to our concatenated genes results, *T. dugesii* lineage appears as basal to all the other ones. Furthermore, the genetic distances suggest that this lineage is very distinctive from the others. *T. dugesii* individuals were recently observed in a small population restricted to La Plaza de la Feria in Gran Canaria (López-Dos Santos et al., 2013). Moreover it appears that this population does not cohabit with any other lizard species. This lineage of *Parapharyngodon* is a direct result of the introduction of Madeira lizard individuals in Gran Canaria carrying this nematode. Interestingly, the sister taxa to the *Parapharyngodon* infecting *T. dugesii* is an individual of *P. micipsae* from *Gallotia stehlini* from the same island. Genetic distance analysis reveal that this lineage, for 28s, ranged between 4% and 5% of genetic variability when compared to the other groups. Studies concerning the helmintofauna of *T. dugesii* individuals from Madeira suggest that this individuals have distinctive morphology than the *P. echinatus* paratype (differences were found at the level of the caudal papillae where some specimens only have one papilla, and at the level of the lower lip of the cloacal, where specimens exhibit a fringed or plain lip; Sánchez Gumiel et al., 1993). However we did not record any of these morphological differences in our specimens. Still, studies sampling more individuals and using mitochondrial markers should be performed to assess the evolutionary history of this group.

In concatenate gene results *P. galloti* individuals appear as a basal clade to all the other *Parapharyngodon* ones (except the one from *T. dugesii*). We did not sampled many *P. galloti* but this nematodes are grouped in a fashion of two distinctive lineages: (i) one exclusive of Tenerife represented by parasites from *G. galloti* and *C. viridanus* and (ii) one from El Hierro and La Gomera from *G. caesaris* and *C. coeruleopunctatus* host individuals. This two different lineages are possible to be the result of two separated colonisations of *T. galloti* in the Canary archipelago that evolved separated due to the oceanic plates act as strong barrier to gene flow. However, we did not spot any *P. galloti* infecting *Tarentola* lizards. If in one hand is possible that by chance we did not sampled any *P. galloti* from a *Tarentola* in the other, we may not exclude the possibility that this nematode is not able to infect geckos. This is speciality likely to occur when reviewing Tarentolas ecological traits, being them lizards that have an insect base diet and that habit mainly in rocky environments, which makes an infection by P. *galloti* difficult to occur. *Gallotia* and *Chalcides* have very distinctive diets, with *Gallotia* species being

omnivorous (with some degree of herbivory) and *Chalcides* feeding on insects. *P. galloti* belongs to the lineage of nematodes that are believed to infect more carnivorous hosts, however in the Canary archipelago our results show that this nematode has the capacity to infect also *Gallotia* species (known to have and omnivorous-herbivorous diet). Therefore, taking into account their host diet, is more likely to infer that *P. galloti* colonized the Canary Islands by a *Chalcides* ancestral, and then had the capacity to infect *Gallotia* when they were feeding on plant forms. However, a colonization scenario by a *Gallotia* ancestral and posterior infection of *Chalcides* species should not be excluded.

In *P. echinatus* clade our results suggest a clear differentiation among the more western islands (La Gomera, La Palma, Tenerife and El Hierro) and the eastern (Lanzarote, Fuerteventura and Gran Canaria). In the more eastern islands lineage, Fuerteventura and Lanzarote clade appears as monophyletic to Gran Canaria and El Hierro clade (possible exclusive of *T. boettgeri*) and Gran Canaria clade is basal to the former ones. In the more western islands lineage an exclusive clade of La Gomera appears as monophyletic to a clade comprising individuals from Tenerife, La Palma and two individuals sampled in *G. caesaris* (one from El Hierro and the other from La Gomera). Genetic distances between this clades range around 1% for 28s. Although the value is quite low they are still indicative of differentiation between the different *P. echinatus* lineages. However, the almost inexistent genetic variability within each clade reveal that *P. echinatus*, as a host generalist, may not be evolving within one specific host and therefore, not speciating at all.

Comparisons between *Parapharyngodon* and their hosts phylogenies (Figure 4) allow us to try to explain how these parasites colonized the islands. Colonization of Tenerife, La Palma and La Gomera might have occurred via *T. gomerensis* and *T. delalandii* common ancestral. Direct comparisons between *Tarentola* species and *P. echinatus* phylogeny seem to corroborate this colonization where there is a clear distinction between *T. gomerensis* and *T. delalandii* lineages, both monophyletic and sister taxa. Moreover, results from Manuscript I showed nematodes from *T. gigas*, *T. delalandi* and *T. gomerensis* in the same clade corroborating our hypothesis that a common ancestral of these *Tarentola* species colonized the Canary Islands and Cape Verde in the same event (approximately 8-4.1 Mya; Carranza et al., 2002) bringing with them *P. echinatus* to these islands. *P. echinatus*, known to be host generalist (see Carretero et al., 2006; Martin and Roca, 2005; Roca et al., 1999), may had the chance to infect other lizard

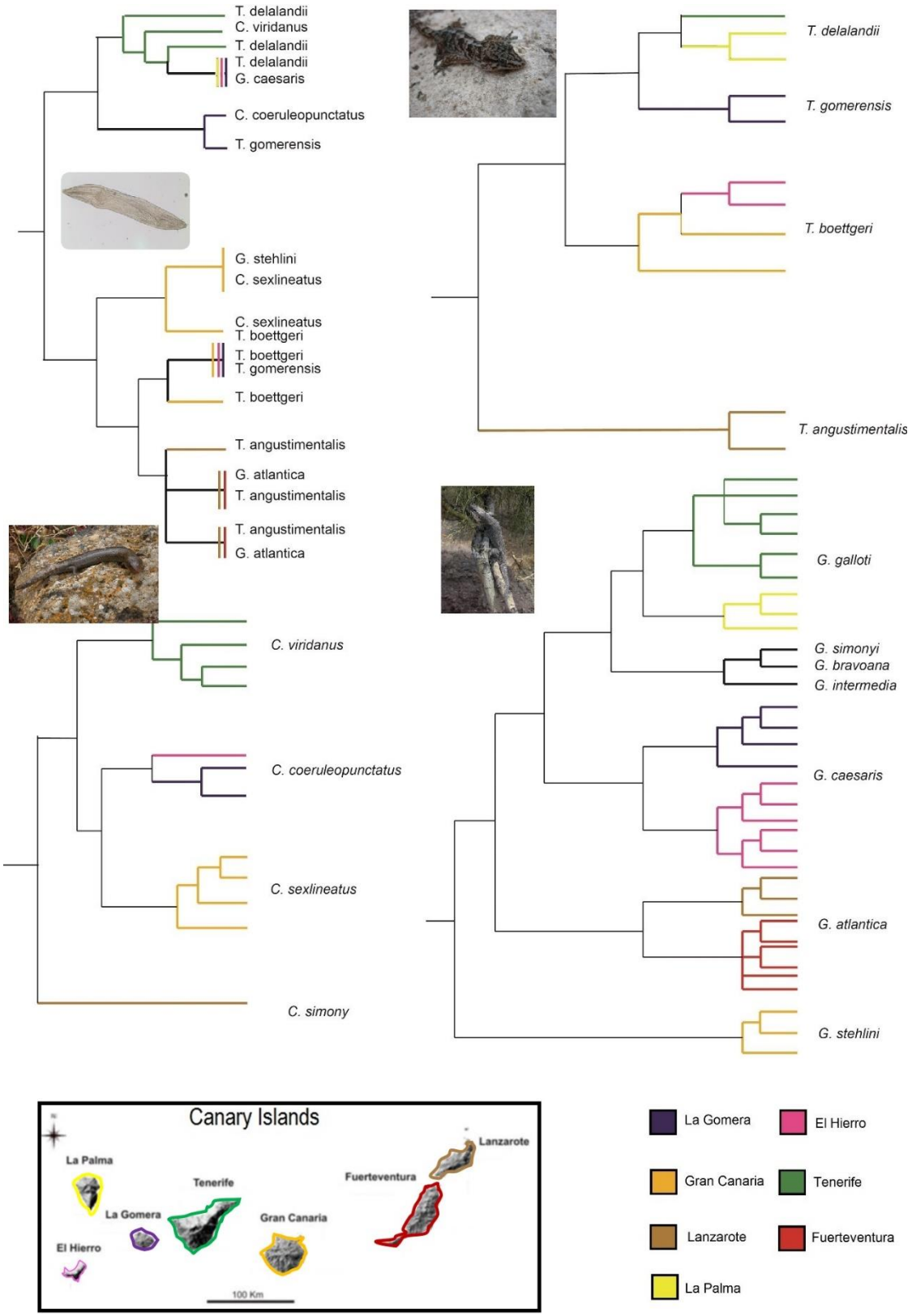

Figure 4 - Comparison of *P. echinatus* concatenate gene tree with the ones of their hosts, *Tarentola, Chalcides* and *Gallotia*. Colored branches represent the island where the specimens are from (Tarentola tree adapted from Carranza et al., 2002; Chalcides tree adapted from Carranza et al., 2008; Gallotia tree adapted from Cox et al., 2010).

species in La Gomera and then spread to El Hierro via a *Gallotia* or *Chalcides* ancestral at the time that they colonized these islands (approximately 0.68-0.89 Mya in the case of *G. caesaris* ancestral; Cox et al., 2010; and 1 Mya in the case of a colonization by *C. coeruleopunctatus* ancestral; Carranza et al., 2008).

Colonization of Gran Canaria, El Hierro, Fuerteventura and Lanzarote cannot be accessed by a straightforward comparison between *P. echinatus* phylogeny and the ones from its hosts. However, it is possible for us to infer some possible scenarios. Colonization by this nematode lineage may have happened via a *Gallotia* ancestral, where a lineage colonized Gran Canaria via *G. stehlini* ancestral, and then *G. atlantica* ancestral colonized Fuerteventura and Lanzarote followed by the other western islands. However, there are some aspects that fail to explain this colonization pattern. Our 18S and 28S concatenated phylogenetic tree results reveal the existence of a lineage (clade 6) that is possibly exclusive of *T. boettgeri* host (with the exception from one individual infecting *T. gomerensis* from La Gomera that may be an introduction). However we must not exclude any case scenario: if in one hand (i) exists the possibility that, by chance, we did not sample any *Gallotia* or *Chalcides* host that was parasitized by this lineage, in the other (ii) this lineage is in fact exclusive of *Tarentola* which is congruent with this geckos behaviour, that are characterized by an insectivorous diet, nocturnal activity and low dispersion rate. *T. boettgeri* ancestral is believed to have colonized the Canary Islands in an independent event related to the colonization of the Selvages Islands. Therefore, this lineage of *T. boettgeri* may be the result of a colonization by a *P. echinatus* ancestral brought to the island during host colonization and not related to all the others lineages. Authors believe that *T. angustimentalis* is not related to all the other Canary Islands *Tarentola* species and it is in fact the result of an independent colonization event of *T. mauritanica* to Lanzarote and Fuerteventura. In fact, recent studies confirm that *T. angustimentalis* is a lineage within a paraphyletic *T. mauritanica* species complex (Carranza, 2000; Rato et al. 2012). Therefore, the colonization of Lanzarote and Fuerteventura by *P. echinatus* is possible to have occurred via the ancestral of *T. mauritanica* and *T. angustimentalis*. Although all the previously discussed colonisations of *P. echinatus* lineages to the Canary archipelago are possibly related to *Tarentola (clades II, III, V, VI, 28S)*, the same might not have happened to the exclusive Gran Canaria clade (clade IV 28S). In fact, it is possible that this clade is in fact the result of the colonization by a *G. stehlini* or a *C. sexlineatus* ancestral. However, if that is the case, it does not make our previous inferences less likely to have happen. *G. stehlini* ancestral

colonized Gran Canaria approximately 13-6 Mya (according to Cox et al., 2010) however the western *Gallotia* lineages were originated from *G. atlantica*, rather than *G. stehlini* ancestral (Cox et al. 2010) and therefore this *P. echinatus* lineage may had been restrained to Gran Canaria island.

The colonization of the Canary archipelago by *P. echinatus* may have occurred in multiple independent events related to the *Tarentola* species colonization. *Tarentola* geckos are known to be insectivorous and do not disperse much from their populations. This ecological specific traits make *Tarentola* a much more difficult host to be infected when comparing to the other Canary Islands endemic hosts and therefore considering this information, is more likely that *Tarentola* has been the vector of colonization by *P. echinatus* than the result of a within-island infection. *Gallotia* lizards are known as omnivorous animals that may change for a more herbivorous diet in case of low prey abundance. *P. echinatus* infection occurs by the direct ingestion of the parasite. Therefore, *Gallotia* species are good candidates to be infected by *P. echinatus* after they had the chance to colonize these hosts habitats. *Chalcides* species are known to be insectivorous, however it has been recorded that some species from the Canary Islands are likely to hide in the vegetation in case of predatory stress. Although *Chalcides* species may not be the perfect candidate to be infected by this nematodes, it is possible that when feeding on prey in this vegetation areas, it may happen to accidentally intake *P. echinatus* individuals.

As final note, our results revealed that the sampled *P. bulbosus* individuals were grouped in the same clades of the ones of *P. echinatus*. This suggests that *P. bulbosus* possibly should not have the status of species, per se, but rather be described as a *P. echinatus* subspecies or morphotype (like *P. micipsae*; see Manuscript I). However, in our analysis we only had three *P. bulbosus* specimens which does not give us enough power to make this assumptions. However, we stress here the importance of conducting studies in this direction to infer *P. bulbosus* taxonomic status.

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See General References.

General Discussion

Although the use of molecular tools is now almost obligatory in any biodiversity study, less attention is being paid in this respect to many parasitic groups including the nematodes. Nematodes are in many cases small living forms with specific phenotype traits that are only possible to recognize at the microscopic level. If a classification of a given macroscopic taxa using only morphological traits is often a challenge, the one using microscopical individuals is even more difficult and in many cases may lead to erroneous conclusions. Indeed, many groups that were classically difficult to disentangle using only morphological characters are now being demystified. The present dissertation demonstrates the importance of combining both morphological and molecular tools to properly assess biodiversity in an integrative taxonomic approach. However, if molecular tools are evidently useful in this field, they are also critical for reconstructing the evolutionary history of groups. Moreover they demonstrate their utility not only in clarifying a given parasite phylogeny but also for comparing it to the ones of their host in a search for patterns that could explain the forces that are driving the evolution of both species together. This approach is also important to better understand the phylogeny of their hosts. Therefore the present dissertation also demonstrates the power of these tools for understanding host-parasite interactions that were obscured by limited knowledge, allowing to disentangle scenarios such as cryptic speciation, failure to speciate or how a certain locality was colonized by a given parasite. In this aspect it should be reinforced the need for additional studies that both allow us to better understand these phenomena but also to find new methodological approaches to analyze such complex datasets, accounting simultaneously for host and parasite related variables.

Parapharyngodon spp. are host generalists nematodes, and are found in amphibians and reptiles with an almost global distribution. Despite the morphological traits used to describe over 50 species, with more being described each year (e.g. de Araújo Filho et al., 2015; Velarde-Aguilar et al., 2015) little is known about how this genus evolved or how the different described species are related to each other. Although some authors believe that this genus should be synonymised with *Thelandros* (see Petter and Quentin, 1976; Petter and Quentin, 2009), *Parapharyngodon* is still a recognized genus by most authors, with particular morphological differences used to define it. In the Canary

Islands there are currently three accepted species: *P. echinatus*, *P. bulbosus* and *P. micipsae*. The choice of the Canary archipelago as the location to study the evolutionary histories of these nematodes was manly due to the geological and ecological distinctive traits that these islands harbour. Furthermore, the fact that other phylogenetic studies have been conducted using Oxyurida nematodes in these islands (Jorge et al. 2011, 2013, 2014) was important in the decision of choosing this system, in a way that allowed the comparison between the different nematode groups. Moreover the great input of helminthic fauna studies in endemic lizards of the Canary archipelago (see Carretero et al., 2006; Martin and Roca, 2005; Martin et al., 2005; Roca et al., 1999; Roca et al., 2005; Roca et al., 2012) represented a tremendous important starting point to choose *Parapharyngodon* as an ideal model.

The 18S rRNA nuclear marker was not very elucidative in estimating the phylogeny of *Parapharyngodon* species, primarily due to the slow evolving nature of this gene. However, it provided an initial molecular identification of the primary lineages identified. The 28S rRNA nuclear marker, a faster evolving gene than 18S, gave us some level of resolution in the phylogenetic inference being crucial to distinguish between related lineages. Moreover the level of congruence between both genes phylogenies allowed the concatenation of both analysis leading to a better estimation of the tree topology. Although we are aware that the information of these two rRNA genes is not independent, given that they both encode for the ribosomal structure, the use of additional markers such as COI, will be important to overcome misleading lineages.

The main aim of this dissertation was the assessment of the phylogenetic patterns found in *Parapharyngodon* species from the Canary Islands. However, due to the continuous discussion of the taxonomic status of this nematode, and because of the lack of evolutionary studies in this genus, a first work focused on understanding the relationship between *Parapharyngodon* species and those assigned to *Thelandros* was crucial to precede the work. At the same time the use of both morphological and molecular data made it possible to assess the potential occurrence of cryptic species.

In the first manuscript the results were of great interest in understanding the evolutionary relationships of both *Thelandros* and *Parapharyngodon*. Moreover this work revealed how morphological and molecular approaches can complement each other for a proper description of genera and species. Manuscript I analysis revealed that there is

a clear distinction between some *Parapharyngodon* spp. and *Thelandros* spp. as was previous suggested by several authors (Adamson, 1981; Castano-Fernandez et al., 1987; Chatterji, 1933; Roca, 1985). This differentiation was supported by a mean p-value of 9% (for 28s) indicative of the genetic variability found. Furthermore, these results suggest a differentiation of two lineages within the Thelandros group, one including *T. tinerfensis* individuals and the other comprising *T. filiformis* and therefore corroborating previous species status descriptions (see Astasio-Arbiza et al, 1989; Solera-Puertas et al., 1988). On the other hand "*Thelandros*" *galloti* was not assigned as a sister species to the other two of *Thelandros* spp. but instead appeared to be a member of *Parapharyngodon*. Interestingly, there was not a clear morphological differentiation between other *Parapharyngodon* species and *T. galloti*, where the length of the oesophagus and lateral alae width and the presence of six posterior papillae were described as the most reliable morphological differences. Although more studies are needed to fully understand *"T." galloti* evolutionary relationship with additional species, both *Thelandros* and *Parapharyngodon*, results on Manuscript I strongly suggest the need of a taxonomic reassessment of this species status as *Parapharyngodon galloti*. Moreover Manuscript I results were very elucidative concerning *P. micipsae* taxonomic status. In fact, *P. micipsae* does not appear to be a single species, but instead might be a morphotype of both *P. echinatus* and *P. galloti*. Although the potential synonymy of *P. micipsae* with *P. echinatus* was already discussed by several authors (Chabaud and Golvan, 1957; Roca, 1985) the same with *P. galloti* has never been suggested. The *P. echinatus* lineage is divided in five main clades, with geographical coherence. *Parapharyngodon* nematodes are characterized by a life-cycle with no free-living stages, and thus nematode dispersion strictly relies on the host dispersal abilities (Adamson, 1989). Indeed, this was observed in manuscript I where there seems to be no gene flow between populations from La Palma and La Gomera, populations of Gran Canaria, populations of Lanzarote and Fuerteventura and populations of the Iberian Peninsula. On the other hand, lineages of La Palma and La Gomera and Fuerteventura and Lanzarote include individuals from the Cape Verde islands and Morocco, respectively. One possible explanation could be that these lineages might have reached the islands during *Tarentola* colonization of the Canary Islands (see Carranza, 2000; Carranza et al., 2002; Rato et al. 2012) or by more recent sporadic introductions. Still, this are all possible hypothesis and more analysis regarding the divergence time of each *Parapharyngodon* lineage are needed to validate, if that is the case, one of these hypothesis.

The aim of Manuscript II was to understand the evolutionary history of *Parapharyngodon* spp. within the Canary Islands. The overall results corroborated much of the output from Manuscript I. In the Canary Islands an emerging pattern of a separation between the more western islands and the eastern ones appears. This pattern of a separation between the more western lineages and the ones from eastern islands has been previously recorded in another nematode, *S. atlanticus*, in the Canary archipelago (Jorge et al., 2011). However in the study in *S. atlanticus* published by Jorge et al (2011), the two lineages were not directly related to each other which might not be the case here. Still, comparing the host phylogenies and the ones from *Parapharyngodon*, the level of congruence between *P. echinatus* and *Tarentola* spp. is almost perfect. If this is the case then the possibility that *P. echinatus* colonized the Canary Islands following *Tarentola* is much likely. This hypothesis gain some power when considering how the distances between islands must act as a huge barrier to gene flow. Considering this, the different lineages of *P. echinatus* were shown to be very congruent with the widely-accepted colonization model of *Tarentola*. However, if a colonization via a *Tarentola* ancestral might explain much of the diversity of *P. echinatus* found in the Canary Islands, it does not explain all of the lineages found. Gran Canaria harbours two different evolutionary lineages, one specific to *T. boettgeri* (that also includes individuals from El Hierro) and the other that parasitizes *G. stehlini* and *C. sexlineatus* individuals. Although *P. echinatus* is believed to be a host generalist, something that is corroborated in the results from this thesis, the case of Gran Canaria seems to be the exception to the rule. However, we do not discard that a more detailed sampling might show the presence of this parasite species in other host genera. Still host-switching is not a linear phenomenon and relies on many aspects dependent on the host, such as behaviour, immune response, resources available to the parasite and habitat similarity with another infected host (Combes, 2001). If the failure to infect *T. boettgeri* could be explained by behaviours specific to gecko hosts (*Tarentola* species are crepuscular-nocturnal, have more restrict vagility, are strictly insectivorous, and do not tongue flick; Arnold, 2002), the same is not the case for the other two host genera, especially for *G. stehlini* (*Gallotia* lizards are diurnal, have higher vagility and an omnivorous diet; Van Damme, 1999; *Chalcides* are also diurnal but have low vagility and insectivorous diet; Salvador et al., 2015b). However, the lineage specific to *T. boettgeri* might not have host-switched to *G. stehlini* and *C. sexlineatus* due to the lower probability of physical encounter among these hosts, differences in hosts guts in terms of resources found or due to the differences in the

immunological system that prevent infection by this lineage, or due to competition with other lineages already infecting these species.

In both manuscripts, results revealed the differentiation of distinctive *Parapharyngodon* lineages potentialy evidencing cases of cryptic speciation. Cryptic species have already been recorded in *Spauligodon* nematodes (Oxyurida) from the Canary Islands (Jorge et al., 2013), as well as in *P. cubensis* from the Caribbean where the different lineages found are likely be explained by a complex of cryptic species rather than diversity at the interspecies level (Falk and Perkins, 2013). However, results concerning the Iberian Peninsula lineage revealed differences at the level of the lateral alae and oesophagus length. Therefore, more studies are needed to infer the validity of these morphological traits in this group for taxonomic purposes. Moreover *T. dugesii* specific lineage suggests a taxonomic reassessment of these *Parapharyngodon* individuals as belonging to a single species. This population was recorded in June 2011 in a city garden in Gran Canaria, where there are not other endemic lizards present with the exception of the introduced gecko *H. turcicus* (López-Dos Santos et al., 2013). On the other side, *T. dugesii* individuals from Madeira have a distinctive morphology than the ones from *P. echinatus* paratype (differences were found at the level of the caudal papillae where some specimens only have one papilla, and at the level of the lower lip of the cloacal, where specimens exhibit a fringed or plain lip; Sánchez Gumiel et al., 1993). However these morphological differences were not noticed in our specimens. Therefore specific morphological and phylogenetic studies, using a more detailed dataset focused on this “lineage” are needed to confirm former assumptions, where a taxonomic reassessment would likely promote this lineage to the species status. Despite the genetic variation that was found between the *P. echinatus* lineages, no significant variation was found at the interspecific level. However, 18S and 28S are slow evolving markers that in most cases only allow to infer what is happening at the species level. Therefore, an approach using mitochondrial genes is needed to infer patterns of diversification within-locality and within-host.

Another interesting result was that, using 28S and 18S markers, *P. bulbosus* was not differentiated from *P. echinatus*. These two species rely in morphological differences at the level of the lateral alae (*P. bulbosus* has larger alae than *P. echinatus* that in some specimens starts below the oesophageal bulb and overpass the cloacal region). Still, these morphological differences are to some extent subjective and in some cases do not

allow a clear differentiation between specimens from both species. They are possible related with specimen specific phenomena (such as preservation status of the specimen or host induced morphology) rather than divergent evolution. If that is the case, *P. bulbosus* and *P. echinatus* should be synonymised. However, more studies using a more robust dataset, mitochondrial genes and morphologic traits analyses are needed to understand what is happening in this case, prior to a taxonomic reassessment.

Overall, although this dissertation shed considerable light not only for understanding the evolutionary history of *Parapharyngodon* in the Canary Islands but also regarding the ongoing discussion of the taxonomic status of this genus, as a final balance several key issues remain unresolved. The challenge in amplifying COI as a mitochondrial marker proved to be one of them. COI is a much faster evolving marker than 18s and 28s. Although COI L/COI H universal primers (Folmer et al. 1994) and the nematode specific cocktail of primers Nem F/Nem R (Prosser et al. 2013) have been previously reported to successfully amplify COI fragments in other nematode species (e.g. Jorge et al., 2011) after intensive efforts, we only managed to amplify this mitochondrial marker for few specimens from our dataset. However COI is not only crucial to "barcode" species (Hebert and Gregory, 2005) but also to give more resolution at the intraspecific level. Therefore there is a clear need to redesign some of the currently used "nematode primers" so that they can also be used in these species. Regarding this, the use of Next Generation Sequencing tools will provide new primers with different levels of resolution to address some of the unresolved questions. Also, the difficulty of obtaining parasite samples (from more than 1000 host individuals collected in the field, we only detected 226 *Parapharyngodon* parasites), the fact that only males are used (females are undistinguishable among genera without molecular confirmation), the preservation status of the samples and the microscopical size of the specimens (that do not allow to extract large quantities of DNA) acted as stronger barriers to obtain a more complete dataset. Still the overcome of these practical barriers will allow to improve our dataset in order to understand (i) the taxonomic status of *P. bulbosus*, (ii) the possibility of the presence of cryptic species in the Canary Islands, (iii) how mitochondrial markers are evolving in *Parapharyngodon* nematodes (iv) how "*P. micipsae*" morphotype of *P. echinatus* relate to the one of *P. galloti*, and (v) whether an undescribed species occurs in Madeira. Regarding this, the analysis of the divergence time of each lineage (e.g. BEAST; Drummond et al., 2012) as well as the level of congruence between parasite and hosts phylogenies (e.g. PACo; Balbuena et al., 2013) will help not only to assess in

more detail the colonization patterns of the Canary archipelago but also to better understand host-parasite interactions that may be masked in the parasite phylogeny. Therefore such approaches will be implemented in the future for a better assessment of *Parapharyngodon* spp. evolution.

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Appendix 1

Appendix 1. General dataset regarding nematode species, sample database code (DB), sampling locality, host species and manuscripts specific dataset information. MI – Manuscript I, MII – Manucript II. x corresponde to samples used in Manuscript I and Manucript II; Samples used as outgroups are marked with an (*).

Nematode species	DB	Host Species	Locality	Island/Country	MI	MII
P. bulbosus	Pb16564	*G. a. atlantica*	Yaiza	Lanzarote	x	x
P. bulbosus	Pb16575	*G. a. atlantica*	Yaiza	Lanzarote		x
P. bulbosus	Pb19345	*G. g. palmae*	Playa Trigo	La Palma		x
P. echinatus	Pe2. 3	*T. dugesii*	H. Villa Galé	Madeira	x	
P. echinatus	Pe23130	*T. delalandii*	Buenavista Norte	Tenerife	x	x
P. echinatus	Pe23152	*T. delalandii*	La Lomada	La Palma	x	x
P. echinatus	Pe23162	*T. gomerensis*	El Jorado	La Gomera	x	x
P. echinatus	PeG4	*T. gigas*	Raso Island	Cape Verde	x	
P. echinatus	Pe22090	*T. mauritanica*	Huelva	Spain	x	
P. echinatus	Pe22106	*T. mauritanica*	Huelva	Spain	x	
P. echinatus	Pe16572	*G. a. atlantica*	Yaiza	Lanzarote	x	x
P. echinatus	Pe23093	*T. b. boettgeri*	Cercados Espino	Gran Canaria	x	x
P. echinatus	Pe13737	*G. a. laurae*	Mir. Rio	Lanzarote	x	x
P. echinatus	Pe23102	*C. s. sexlineatus*	Ccercados Espino	Gran Canaria	x	x
P. echinatus	Pe1796	*G. stehlini*	Ald Blanca	Gran Canaria	x	x
P. echinatus	Pe23083	*C. s. bistratus*	Ingenio	Gran Canaria	x	x
P. echinatus	Pe23051	*T. angustimentalis*	Jandia, Moro Jable	Fuerteventura	x	x
P. echinatus	Pe23193	*T. b. hierrensis*	E. Nue Señ Reyes	El Hierro	x	x
P. echinatus	Pe16516	*G. a. mahoratae*	La Oliva	Fuerteventura	x	x
P. echinatus	Pe23014	*T. angustimentalis*	Caleta de Famara	Lanzarote	x	x
P. echinatus	Pe16541	*G. a. mahoratae*	Butihondo	Fuerteventura		x
P. echinatus	Pe23038	*T. angustimentalis*	La Oliva	Fuerteventura		x
P. echinatus	Pe23041	*T. angustimentalis*	La Oliva	Fuerteventura		x
P. echinatus	Pe23056	*T. angustimentalis*	Jandia, Moro Jable	Fuerteventura		x
P. echinatus	Pe23098	*C. s. sexlineatus*	Cercados de Espino	Gran Canaria		x
P. echinatus	Pe23091	*T. b. boettgeri*	Cercados de Espino	Gran Canaria		x

P. echinatus	Pe23016	*T. angustimentalis*	Caleta de Famara	Lanzarote		x
P. echinatus	Pe16569	*G. a. atlantica*	Yaiza	Lanzarote		x
P. echinatus	Pm19292	*T. delalandii*	Guayonje	Tenerife		x
P. echinatus	Pe16572	*G. a. atlantica*	Yaiza	Lanzarote		x
P. echinatus	Pe19292	*T. delalandii*	Guayonje	Tenerife		x
P. echinatus	Pe23134	*T. delalandii*	Buenavista del Norte	Tenerife		x
P. micipsae	Pm23022	*T. angustimentalis*	Caleta de Famara	Lanzarote		x
P. micipsae	Pm23190	*C. coeruleopunctatus*	El Jorado	La Gomera		x
P. micipsae	Pm19298	*T. delalandii*	Playa Las Salimeras	La Palma		x
P. micipsae	Pm13057	*Hemidactylus*	Tinhosa Grande Isl.	São Tomé	x	
P. micipsae	Pm23107	*G. stehlini*	Cercados de Espino	Gran Canaria	x	x
P. micipsae	PmSTM275	*Chioninia delalandii*	Cape Verde	Cape Verde	x	
P. micipsae	Pm23749	*T. dugesii*	Plaza Feira Palmas	Gran Canaria	x	x
P. micipsae	Pm23162	*T. gomerensis*	El Jorado	La Gomera	x	x
P. micipsae	Pm19280	*T. delalandii*	Arm.-Las Cancelas	Tenerife	x	x
P. micipsae	Pm3082	*T. nicolauensis*	São Nicolau	Cape Verde	x	
P. micipsae	Pm23120	*C. viridanus*	San Miguel de Geneto	Tenerife	x	x
P. micipsae	Pm23223	*C. coeruleopunctatus*	Valverde	El Hierro	x	x
P. micipsae	Pm19413	*G. c. caesaris*	near Villa Valverde	El Hierro	x	x
P. micipsae	Pm22004	*T. mauritanica*	Huelva	Spain	x	
P. micipsae	Pm22121	*P. carbonelli*	Huelva	Spain	x	
P. micipsae	Pm21573	*P. vaucheri*	Huelva	Spain	x	
P. micipsae	Pm13746	*G. a. atlantica*	Nazaret-Teguise	Lanzarote	x	x
P. micipsae	Pm23014	*T. angustimentalis*	Caleta de Famara	Lanzarote	x	x
P. micipsae	Pm23105	*C. s. sexlineatus*	Cercados de Espino	Gran Canaria	x	x
P. micipsae	Pm23086	*T. b. boettgeri*	Cercados de Espino	Gran Canaria	x	x
P. micipsae	Pm14773	*Q. moerens*	Morocco	Morocco	x	
P. micipsae	Pm16527	*G. a. mahoratae*	La Oliva	Fuerteventura	x	x
P. micipsae	Pm16539	*G. a. mahoratae*	Butihondo	Fuerteventura	x	x
P. micipsae	Pm13738	*G. a. Laurae*	Mirador del Rio	Lanzarote	x	x
P. micipsae	Pm19464	*C. coeruleopunctatus*	Camiño de la Virgem	El Hierro		x
P. micipsae	Pm19439	*G. c. caesaris*	near Villa Valverde	El Hierro		x
P. micipsae	PmGccV4	*G. c. caesaris*	Valverde	El Hierro		x
P. micipsae	PmGccV6	*G. c. caesaris*	Valverde	El Hierro		x

P. micipsae	Pm2457	*G. c. caesaris*	Valverde	El Hierro		x
P. micipsae	Pm23036	*T. angustimentalis*	La Oliva	Fuerteventura		x
P. micipsae	Pm23041	*T. angustimentalis*	La Oliva	Fuerteventura		x
P. micipsae	Pm23070	*T. b. boettgeri*	Ingenio	Gran Canaria		x
P. micipsae	Pm23067	*T. b. boettgeri*	Ingenio	Gran Canaria		x
P. micipsae	Pm19361	*T. gomerensis*	El Atajo	La Gomera		x
P. micipsae	Pm23163	*T. gomerensis*	El Jorado	La Gomera		x
P. micipsae	Pm16575	*G. a. atlantica*	Yaiza	Lanzarote		x
P. micipsae	Pm13280	*T. angustimentalis*	Yaiza	Lanzarote		x
P. micipsae	Pm19277	*T. delalandii*	Arme-Las Cancelas	Tenerife		x
P. micipsae	Pm23123	*C. viridanus*	San Miguel de Geneto	Tenerife		x
P. micipsae	Pm23167	*T. gomerensis*	El Jorado	La Gomera		x
P. micipsae	Pm23052	*T. angustimentalis*	Jandia, Moro Jable	Fuerteventura		x
P. micipsae	Pm19408	*T. gomerensis*	Barranco S Sebastian	La Gomera		x
P. micipsae	Pm23141	*C. viridanus*	Buenavista del Norte	Tenerife		x
Parapharyngodon sp.	P14260	*C. sexlineatus*	Aldea Blanca	Gran Canaria		x
Parapharyngodon sp.	P19326	*T. delalandii*	Playa del Trigo	La Palma		x
Parapharyngodon sp.	P19255	*G. g. galloti*	Erjos	Tenerife		x
Parapharyngodon sp.	P19355	*G. c. gomerae*	El Atajo	La Gomera		x
Parapharyngodon sp.	P13754	*G. a. atlantica*	Nazaret-Teguise	Lanzarote		x
Parapharyngodon sp.	P13750	*G. a. atlantica*	Nazaret-Teguise	Lanzarote		x
Parapharyngodon sp.	P19376	*G. caesaris*	Barranco S Sebastian	La Gomera		x
Parapharyngodon sp.	P19358	*C. coeruleopunctatus*	El Atajo	La Gomera		x
Parapharyngodon sp.	P19356	*Gallotia caesaris*	El Atajo	La Gomera		x
Parapharyngodon sp.	P23051	*T. angustimentalis*	Jandia, Moro Jable	Fuerteventura		x
Parapharyngodon sp.	P19344	*G. g. palmae*	Playa del Trigo	La Palma	x	
Parapharyngodon sp.	P12010	*Podarcis lilfordi*	Maiorca	Spain	x	
Parapharyngodon sp.	P5869	*Podarcis sícula*	Lisboa	Portugal	x	
Parapharyngodon sp.	P19461	*C. coeruleopunctatus*	Camiño de la Virgem	El Hierro		x
Parapharyngodon sp.	P23197	*C. coeruleopunctatus*	E. Nue Señ Reyes	El Hierro		x
Parapharyngodon sp.	P23218	*C. coeruleopunctatus*	Valverde	El Hierro		x
Parapharyngodon sp.	P16506	*T. angustimentalis*	Butihondo	Fuerteventura		x
Parapharyngodon sp.	P14351	*T. angustimentalis*	Butihondo	Fuerteventura		x
Parapharyngodon sp.	P23746	*T. dugesii*	Plaza Feira, Palmas	Gran Canaria		x

Parapharyngodon sp.	P19256	*G. g. galloti*	Erjos	Tenerife		x
Parapharyngodon sp.	P13739	*G. a. atlantica*	Nazaret-Teguise	Lanzarote		x
Parapharyngodon sp.	P14308	*T. b. boettgeri*	Aldea Blanca	Gran Canaria		x
T. filiformis	Tf19250	*G. g. galloti*	Erjos	Tenerife	x	
T. filiformis	Tf19344	*G. g. palmae*	Playa del Trigo	La Palma	x	*
T. filiformis	Tf23074	*G. stehlini*	Ingenio	Gran Canaria	x	
T. galloti	Tg19338	*G. g. palmae*	Playa del Trigo	La Palma	x	
T. galloti	Tg19470	*G. c. caesaris*	E. Nue Señ Reyes	El Hierro	x	
T. galloti	TgG2Gcc	*G. c. caesaris*	E. Nue Señ Reyes	El Hierro	x	
T. galloti	Tg19426	*G. c. caesaris*	near Villa Valverde	El Hierro	x	
T. galloti	Tg19361	*T. gomerensis*	El Atajo	La Gomera	x	
T. galloti	Tg19250	*G. g. galloti*	Erjos	Tenerife	x	
T. galloti	Tg23182	*G. c. gomerae*	El Jorado	La Gomera	x	x
T. tinerfensis	Tt23074	*G. stehlini*	Ingenio	Gran Canaria	x	
T. tinerfensis	Tt2513	*G. c. gomerae*	Las Casetas	La Gomera	x	
T. tinerfensis	Tt19408	*T. gomerensis*	Barranco S Sebastian	La Gomera	x	*
T. tinerfensis	Tt19375	*G. caesaris*	Barranco S Sebastian	La Gomera	x	

Appendix 2

Apppendix 2. Markers amplified for each specimen and genetic distance groups information from Manuscript I. DB – Specimen Database code; Numbers correspond to different groups used in genetic distance analysis.

DB	18S	28S	DB	18S	28S
P12010	1	1	Tg19361	6	6
Pe22106	1	1	Tg23182	6	6
Pm22004	1	1	Pm19413	7	6
Pm22121	1	1	Pm23223	7	6
Pm21573	1	2	Tg19426	7	6
P5869	1		Tg19470	7	6
Pe22090	1		TgG2Gcc	7	6
Pb16564	2	4	Pe2. 3	8	8
Pe13737	2	4	Pm23749	8	
Pe16516	2	4	KF028940	9	
Pe16572	2	4	KF029083	9	
Pe23051	2	4	KF029107	9	
Pm13746	2	4	PmSTM275	10	8
Pm16527	2	4	Pm13057	10	
Pm16539	2	4	Pm23107	10	
Pe23014	2		Pe23152	11	5
Pm13738	2		Pe23162	11	5
Pm23014	2		Pm23162	11	5
Pe23093	3	3	Pe23130	11	
Pe23193	3	3	PeG4	11	
Pm23086	3	3	Pm19280	11	
Pm14773	3	4	Pm3082	11	
Pe1796	4	2	Tt19375	12	9
Pe23083	4	2	Tt19408	12	9
Pe23102	4	2	Tt2513	12	9
Pm23105	4	2	Tf19250	12	10
P19344	5	7	Tf19344	12	10
Pm23120	5	7	Tf23074	12	

Tg19250	5	7	Tt23074	12
Tg19338	5	7		

Appendix 3

Appendix 3. Statistical morphometric analysis groups from Manuscript I. DB – specimen database code; GM – Morphological genus; GG – Genetic genus; SM – morphological species; SG – Genetic species.

DB	GM	GG	SM	SG
Pm13057	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH1
Pm23107	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH1
Pe2_3	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH1
PmSTM275	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH1
Pm23749	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH1
Pm23162	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH2
Pm19280	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH2
Pe23130	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH2
Pe23152	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH2
Pe23162	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH2
PeG4	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH2
Pm3082	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH2
Tg19338	*Thelandros*	*Parapharyngodon*	*T. galloti*	PH3
Tg19470	*Thelandros*	*Parapharyngodon*	*T. galloti*	PH3
Pm23120	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH3
TgG2Gcc	*Thelandros*	*Parapharyngodon*	*T. galloti*	PH3
Tg19426	*Thelandros*	*Parapharyngodon*	*T. galloti*	PH3
Tg19361	*Thelandros*	*Parapharyngodon*	*T. galloti*	PH3
Tg19250	*Thelandros*	*Parapharyngodon*	*T. galloti*	PH3
Pm23223	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH3
19344	*Parapharyngodon*	*Parapharyngodon*	*P.*	PH3
Tg23182	*Thelandros*	*Thelandros*	*T. galloti*	PH3
Pm19413	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH3
Pm22004	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH4
Pe22090	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH4
Pm22121	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH4
Pe22106	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH4
Pm21573	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH4

Pm13746	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH5
Pm23014	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH5
Pe16572	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH5
Pe23093	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH5
Pm23105	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH5
Pm23086	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH5
Pe13737	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH5
Pe23102	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH5
Pe1796	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH5
Pe23083	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH5
Pb16564	*Parapharyngodon*	*Parapharyngodon*	*P. bulbosus*	PH5
Pe23051	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH5
Pm14773	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH5
Pm16527	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH5
Pe23193	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH5
Pm16539	*Parapharyngodon*	*Parapharyngodon*	*P. micipsae*	PH5
Pe16516	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH5
Pe23014	*Parapharyngodon*	*Parapharyngodon*	*P. echinatus*	PH5
Tt23074	*Thelandros*	*Thelandros*	*T. tinerfensis*	TH1
Tt2513	*Thelandros*	*Thelandros*	*T. tinerfensis*	TH1
Tt19408	*Thelandros*	*Thelandros*	*T. tinerfensis*	TH1
Tf19250	*Thelandros*	*Thelandros*	*T. filiformis*	TH1
Tf19344	*Thelandros*	*Thelandros*	*T. filiformis*	TH1
Tf23074	*Thelandros*	*Thelandros*	*T. filiformis*	TH1
Tt19375	*Thelandros*	*Thelandros*	*T. tinerfensis*	TH1

Appendix 4

Appendix 4. Specimens measurements from Manuscript I. Values are in µm. DB – Specimen database code; BL – Body length; BW – Body width; LAW – Lateral allae width; LAL – Lateral allae length; TL – Tail length; TW1 – Tail width at the insertion point; TW2 – Tail width at the narrowest point; Spi – Spicule length; SW – Spicule width; NR – Nervous ring position; OL – Oesophagus Length; OW – Oesophagus width; OBL – Oesophagael bulb length; OBW – Oesophagael bulb width.

DB	BL	BW	LAW	LAL	TL	TW1	TW2	Spi	SW	NR	OL	OW	OBW	OBL
Pm13057	1310.47	134.68		1074.73						38.26	220.27	28.85	65.84	62.9
Pm23107	646.02	78.79			71.91	14.28	6.98	71.9	7.54	133.64	310.1	29.9		
Pe2_3	1416.61	197.56	56.98	1023.48	129.71	15.15	8.3				285.16	32.24	107.13	81.64
PmSTM275	1404.18	293.35	20.47	1069.84	68.5	15.45	6.41	90.31	7.56		277.46	36.04	68.62	61.08
Pm23749	896.7				54.12	11.07	3.33	55.74	5.23					
Pm23162	1292.85	156.65						52.8		81.74	165.09	24.86	70.99	63.21
Pm19280	1210.08	166.19	34.06	1035.61	63.79	12.21	3.97			88.15	204.54	25.55	76.99	71.52
Pe23130	1527.42	216.1	42.36	1081.78	78.48	14.3	6.73	84.92	7.11		389.28	25.83	92.09	67.29
Pe23152	1623.27	265.87	58.72	1144.68	73.57			86.59	9.07	142.99	422.63	32	122.86	78.08
Pe23162	1359.97	246.16	81.89	1061.42	93.03	9.5	9.06	88.12		92.62	359.7	32.54	115.24	98.05
PeG4	1634.08	279.9	63.86	1138.3	93.62	13.5	7.65	110.5	9.78	59.33	495.55	32.84	117.38	101.02
Pm3082	693.89	151.07		483.57	59.91	12.13	5.25	43.36						
Tg19338	793.7	124.25	55.36	639.59	53.23	10.77	4.13	51.8		63.57		20	66.1	43.31

Tg19470	1175.15	161.97	123.33	948.98	52.82	10	3.01	55.94	9.54	87.17	297.25	23.28	59.23	54.28
Pm23120	1534.14	309.38		1083	60.94	21.9	10.95	84.71	7.31	45.14	355.33	24.89	116.94	81.99
TgG2Gcc	821.66	210.45	64.19	633.39	99.8	21.8	12.06			53.14	479.16	25.16	71.38	61.14
Tg19426	1199.53	304.74	80.63	901.62	94.63	16.52	9.82	75.86			433.59	31.33	116.52	93.98
Tg19361	1594.82	251.94	101.9	1286.47	100.03	25.62	13.43	97.51	7.84		552.75	32.33		
Tg19250	1869.66	210.57	48.23	1453.1	91.5	6.97	5.35	66.57	10.39	97.98	463.75	40.08	96.75	87.95
Pm23223	1058.26	195.05	38.7	744.75	68.96	17.07	8.13	96.99	8.92	73.68				
19344	936.4	211.63		724.4	100.33	17.76	7.53	80.37	9.23	65.86				
Tg23182	1827.96	333.57		1413.67	103.21					93.49	499.09	41.06	127.3	88.04
Pm19413	1971.99	282.78		1404.18	89.14	24.44	10.15	87.78	6.93		370.93	41.37	129.75	84.47
Pm22004	1697.88	246.92	28.5	1323.6				78.79	8.04		277.34	22.91	94.99	86.11
Pe22090	1500.88	298.34		1253.62	85.37	15.5	8.56			81.11	344.65	26.42	140.58	102.91
Pm22121	1099.93	198.78	25	640.93	68.95	16.77	6.36	84.08	9.7		178.61	29.24	80.5	63.65
Pe22106	1615.19	244.28	70.58	1314.27	69.49	10.34	8.69					41.82		
Pm21573	1189.91	241.17	22.35	1071.58										
Pm13746	1206.74	155.83	34.02	821.05				63.12	8.05	62.94	206.8	21.02	80.15	61.97
Pm23014	929.17	148.84	36.05	618.43	56.16			84.28	10.93	122.39	286.52	23.86	60.5	62.52
Pe16572	1386.48	218.37	69.48	893.36	68.13	13.33	7.91	65.16	5.42	50	386.81	24.03	108.17	85.84
Pe23093	1535.27	231.34	42.54	1211.19	92.58	12.72	5.73			121.83	417.45	26.26	87.85	77.74
Pm23105	1288.1	227.33		634.23	73.45	12.57		87.88	8.14	68.28	336.83	31.63	98.93	74.96
Pm23086	1576.13	150.64	59.15	1185.34				43.74	6.65	40.05	251.22	32.08	75.4	67.4

Pe13737	1853.5	304.11	90.78	1580.3				93.11	11.68	96.16	433.48	32.21	130.36	100.09
Pe23102	1322.35	210.48		1091.67	113.79	19.02	6.08	45.22	5.46		351.98	32.38	100.89	79.33
Pe1796	1940.09	305.54		1476.42				87.72	7.42	99.91	410.75	33.39	105.43	94.18
Pe23083	1500.95	274.34	96.96	1216.83	93.45	15.07	7.04	87.91	7.7	53.85	423.37	38.33	102.76	89.1
Pb16564	1743.11	345.99	84.27	1497.03	63	13.53	8.21	94.52	8.62	115.54	351.1	38.66	126.43	83.92
Pe23051	1938.55	303.45	75.64	1562.07	92.68	13.07	8.8	110.09	9.07	77.24	429.53	42.05	107.26	101.34
Pm14773	1398.56	133.93	21.42	585.3		7.8	5.87							
Pm16527	1480.59	190.9	39.68	962.18	70.42			67.32	6.58		314.23		70.73	47.9
Pe23193	1031.71	196.03			90.37	12.54	6.75	80.68						
Pm16539	1420	228	42.26	964	72			74	6					
Pe16516	1182.42	278.72	55.63	905.96	63.32					31.12				
Pe23014	1362.85	132.23	91.39	982.38	92.61	20.18	11.36	90.56	12.04	74.9				
Tt23074	1772	170		335.1	86.41	11.94	5.6	76.61		106.29	344.65	25.6	78.37	70.59
Tt2513	1580.57	187.18	72.9	1169.67	116.57	15.85	6.66	126.18	13.66	91.23	421.92	26.98	106.58	83.25
Tt19408	1662.27	236.05	71.27	1297.26	115.41	21.73	8.6	41.86		108.76	396.01	28.44	108.29	77.86
Tf19250	1679.71	137.97	73.42	331.97				85.41		126.97	261.82	29.26	52.26	66.3
Tf19344	1593.43	194.5	82.95	321	84.34	16.53		54.87	8.32	113	319.42	33.13	103.92	92.65
Tf23074	1845.14	182.81	82.03	190.65	112.02	18.77	7.74	94.7	9.44	52	442.62	35.83	86.66	88.67
Tt19375	1474.05	220.23	73.75	218.19	96.9	22.02	10.8	105.14	10.9	83.2	447.17	37.46	117.65	101.69

Appendix 5

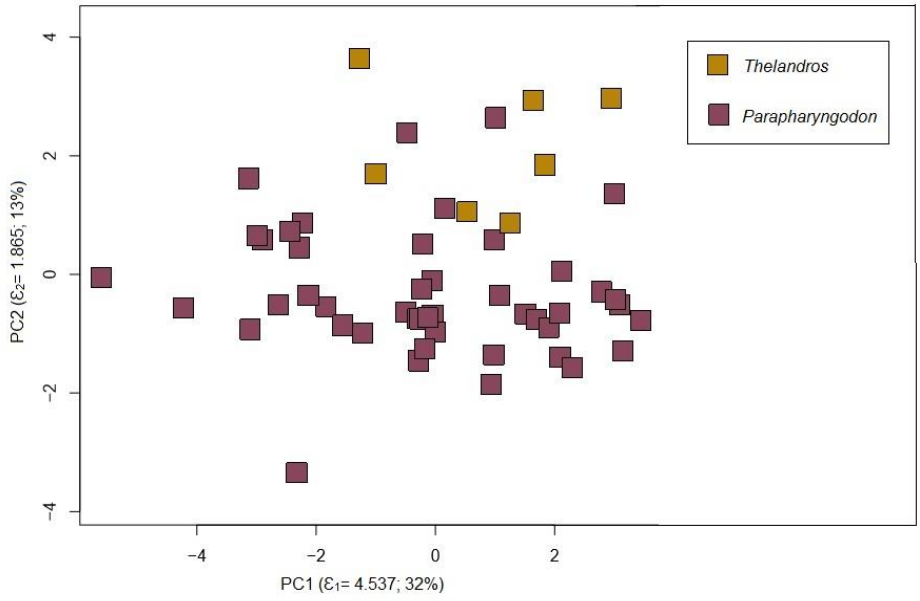
Appendix 5. Specimens qualitative morphological traits. DB – Specimen database code; Spi – Spicule; Tpap – Total number of posterior papillae; Ppap – Number of cloacal papillae; CPap – Number of caudal papillae

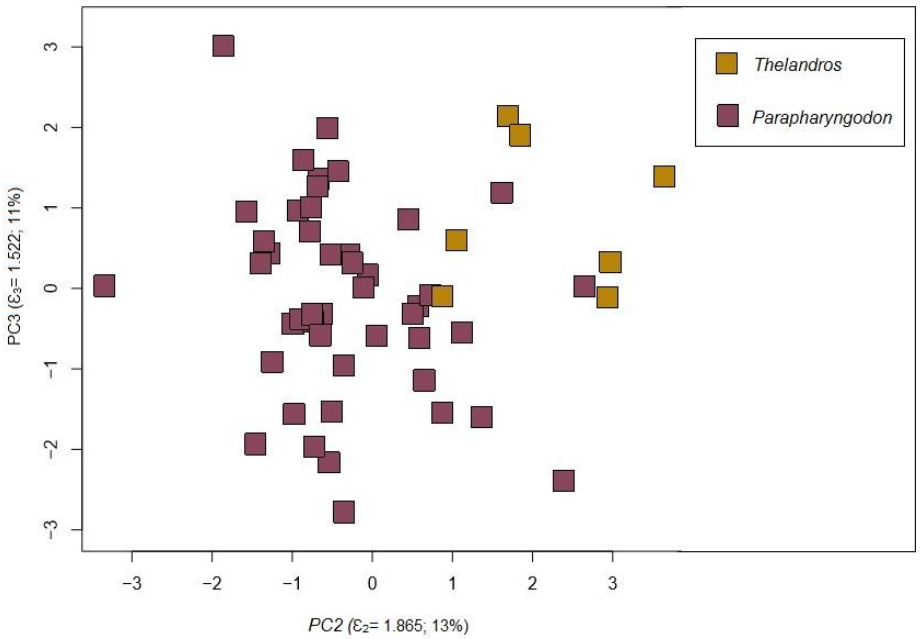
DB	Alae size	Alae shape	Spi shape	Spi size	Tpap	Ppap	CPap
Pm13057	Short	Narrow	Sharp	Long	7	5	2
Pm23107			Sharp	Long			
Pe2_3	Long	Wide	Obtuse	Long	8	6	2
PmSTM275	Short	Narrow	Sharp	Long			
Pm23749			Sharp	Long			2
Pm23162			Sharp	Short	7	5	2
Pm19280	Long	Narrow	Sharp	Long	7	5	2
Pe23130	Long	Wide	Obtuse	Long	8	6	2
Pe23152	Long		Obtuse	Long	8	6	2
Pe23162	Long	Wide	Obtuse	Long			
PeG4	Long	Wide	Obtuse	Very Long	8	6	2
Pm3082	Short	Narrow	Semisharp	Short			
Tg19338	Very Long	Wide		Long	6	4	2
Tg19470	Long	Wide		Short	6	4	2
Pm23120	Short	Narrow	Sharp	Long	7	5	2
TgG2Gcc	Long	Wide	Obtuse	Long		4	
Tg19426	Very Long	Wide	Semisharp	Long			2
Tg19361	Very Long	Wide	Semisharp	Very long	6	4	2
Tg19250	Very Long	Wide		Long			2
Pm23223	Short	Narrow	Sharp	Very Long	7	5	2
19344	Long	Wide	Semisharp	Long			2
Tg23182	Very long	Wide					
Pm19413	Long	Narrow	Sharp	Long	8	6	2
Pm22004	Long	Narrow	Sharp	Long	7	5	2
Pe22090	Long				8	6	2
Pm22121	Short	Narrow	Sharp	Long	7	5	2
Pe22106	Long	Wide	Obtuse		8	6	2
Pm21573	Long	Wide					

Pm13746	Short	Narrow	Sharp	Short	7	5	2
Pm23014	Short	Narrow	Sharp	Long	7	5	2
Pe16572	Long	Wide	Semisharp	Long	8	6	2
Pe23093	Long	Wide	Semisharp				
Pm23105	Short	Narrow	Sharp	Long	7	5	2
Pm23086	Short	Narrow	Sharp	Long	7	5	2
Pe13737	Very Long	Wide	Semisharp	Long			
Pe23102	Long	Wide	Obtuse	Short			
Pe1796	Long	Wide	Semisharp	Long			
Pe23083	Long	Wide	Semisharp	Long			2
Pb16564	Long	Wide	Semisharp	Very Long	8	6	2
Pe23051	Long	Wide	Obtuse	Long			
Pm14773	Short	Narrow			7	5	2
Pm16527	Short	Wide	Semisharp	Long			
Pe23193	Long	Wide	Obtuse	Long	8	6	2
Pm16539	Short	Narrow	Sharp	Long	7	5	2
Pe16516	Long	Wide					
Pe23014	Long	Wide	Obtuse	Long	8	6	2
Tt23074	Very Short	Wide	Obtuse	Long	5	4	1
Tt2513	Very Short	Wide	Sharp	Very long			
Tt19408	Short	Wide	Obtuse	Very short	5	4	1
Tf19250	Very Short	Wide	Sharp	Long	5	4	1
Tf19344	Very Short	Wide		Short	5	4	1
Tf23074	Very Short	Wide	Semisharp	Long			1
Tt19375	Very Short	Wide	Semisharp	Very long	5	4	1

Appendix 6

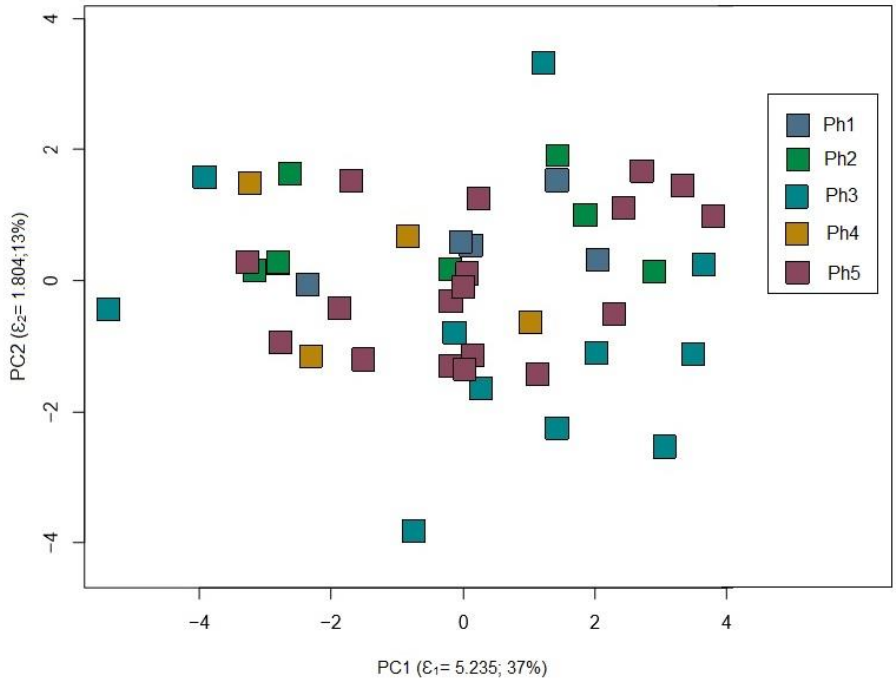
Appendix 6. Representation of the distribution of the individuals across the first three principal component axes. For each axis, eigenvalues (Ɛ) and% contribution of each axis to the total variance are detailed.

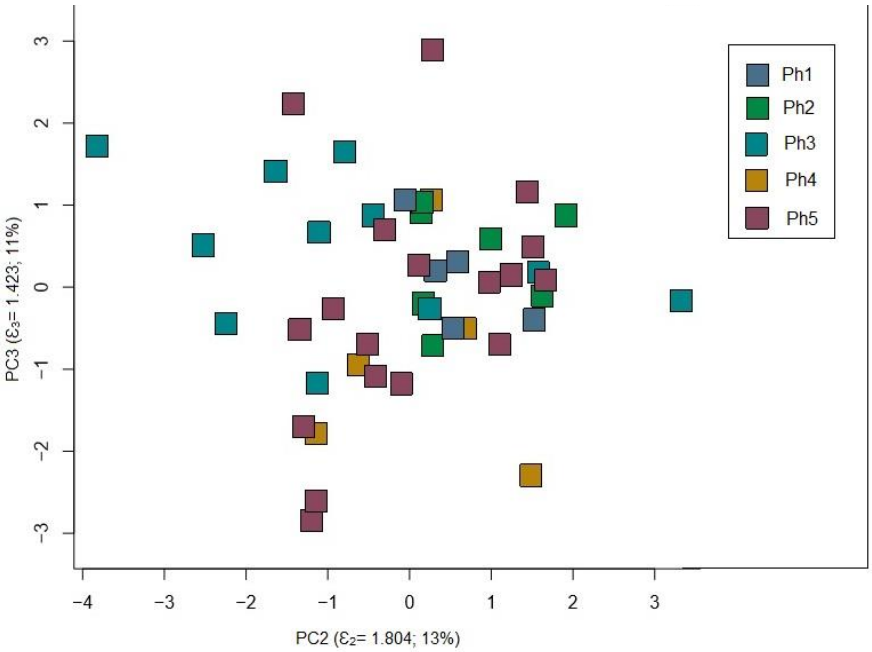




Appendix 7

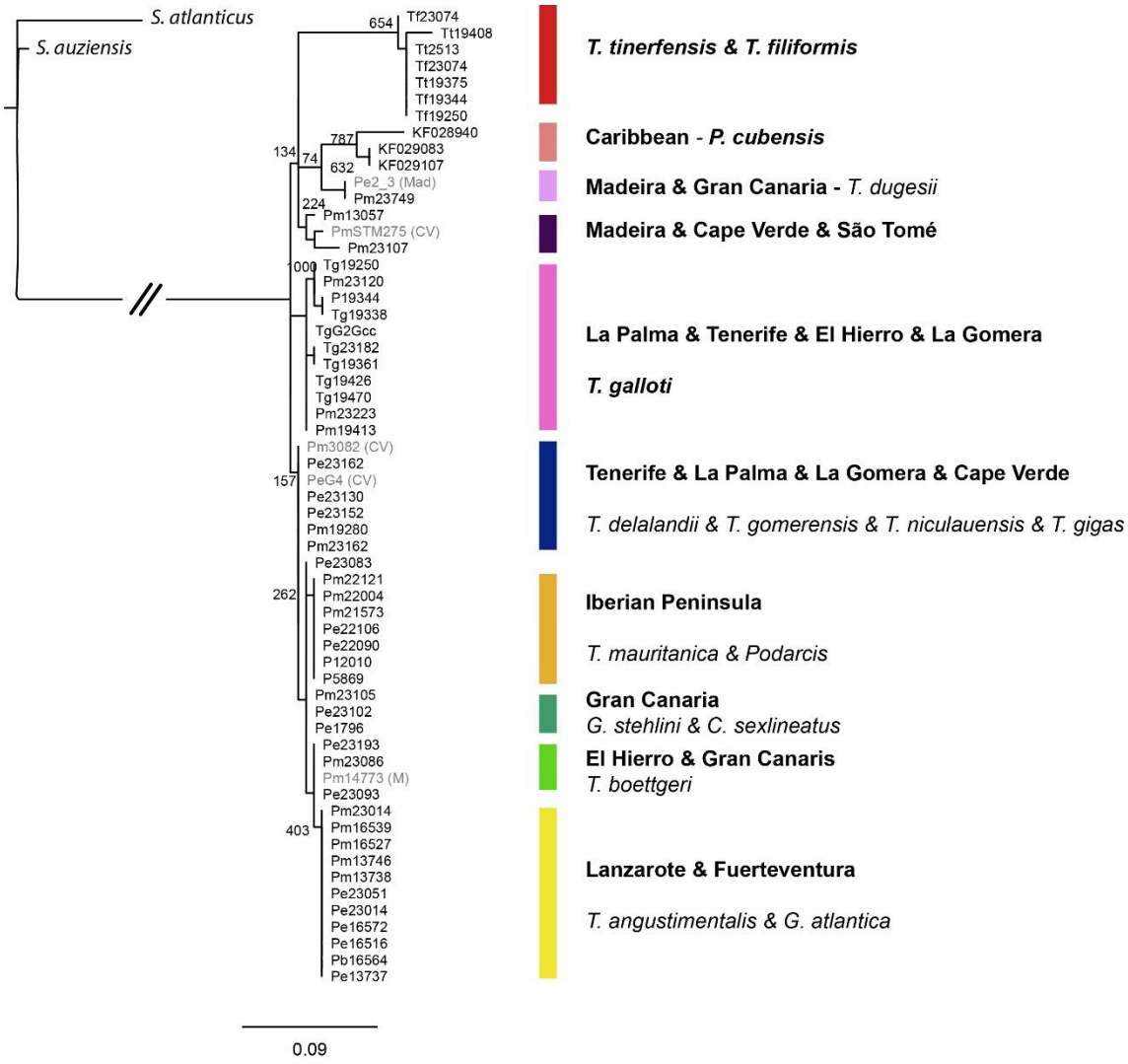
Appendix 7. Representation of the distribution of the individuals across the first three principal component axes. For each axis, eigenvalues (Ɛ) and% contribution of each axis to the total variance are detailed.





Appendix 8

Appendix 8. Maimum likelyhood phylogenetic tree of both 18S gene. Values represent bootstrap support. Each colour correspond to one clade represented in the right. Individuals that are grouped in a certain clade but have a different location are coloured in grey: M- Morocco, Mad - Madeira and CV – Cape Verde.



Appendix 9

Appendix 9. Markers amplified for each specimen and genetic distance groups information from Manuscript II. DB – Specimen Database code; Numbers correspond to different groups used in genetic distance analysis.

DB	18S	28S	DB	18S	28S
Pe23162	1	3	Pe23038	3	7
Pm23162	1	3	Pe23051	3	7
Pm23163	1	3	Pe23056	3	7
Pm23190	1	3	Pm13280	3	7
Pe23152	1	4	Pm13746	3	7
Pm19277	1	4	Pm16527	3	7
Pm23123	1	4	Pm16539	3	7
PmGccV6	1	4	Pm23022	3	7
P19326	1		Pm23036	3	7
Pb19345	1		Pm23041	3	7
Pe19292	1		Pm23052	3	7
Pe23130	1		Pb16575	3	
Pm19280	1		Pe16541	3	
Pm19298	1		Pe23014	3	
Pm19361	1		Pm13738	3	
Pm19408	1		Pm16575	3	
Pm23070	1		P23746	4	1
P14260	2	5	Pm23107	4	
P14308	2	5	Pm23749	4	
Pe1796	2	5	P19255	5	2
Pe23083	2	5	P19358	5	2
Pe23098	2	5	P19376	5	2
Pe23102	2	5	P19461	5	2
Pm23105	2	5	P23197	5	2
Pe23091	2	6	Pe23182	5	2
Pe23093	2	6	Pm19413	5	2
Pe23193	2	6	Pm23120	5	2
Pm23086	2	6	Pm23141	5	2

Pm23014	2		Pm23223	5	2
Pm23067	2		PmGccV4	5	2
P13739	3	7	P19256	5	
P13750	3	7	P19464	5	
P13754	3	7	P23218	5	
P14351	3	7	Pm19439	5	
P16506	3	7	Pm2457	5	
P23051	3	7	P19355	1	
Pb16564	3	7	Pm19292	1	
Pe13737	3	7	P19356		4
Pe16516	3	7	Pe23134		4
Pe16569	3	7	Pm23167		6
Pe16572	3	7	Pe23041		7
Pe23016	3	7			