Phylogeny of the Marine isopod genus *Idotea* in the Northeast Atlantic Ocean and Mediterranean Sea

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

O Presidente do Júri,

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Summary

Individuals of the family Idoteidae are still poorly studied and their phylogenetic relationships lack complete understanding, leading to biased studies and an overall confusion regarding the taxonomy and distribution of its species.

In this work a phylogenetic approach was used with the purpose of understanding the evolution of the isopod genus *Idotea* Fabricius, 1798, which currently has 27 species accepted, including 8 Northeast Atlantic and Mediterranean species. Resolving the phylogeny of *Idotea* species from the Northeast Atlantic Ocean and Mediterranean Sea allowed us to assess levels of diversity and understand the possible historical drivers (e.g. past climate, geological and ecological events) that promoted species diversity.

In the present work, the phylogeny of the genus *Idotea* was investigated on the basis of DNA sequencing data from one nuclear (28SrRNA) and two mitochondrial (COI, ND4) gene fragments obtained for six out of eight Atlantic and Mediterranean species. Furthermore, the status of *Idotea hectica*, which was transferred to the genus *Synischia* (which only comprises another species from Australia), was also assessed.

Interesting results point to a high genetic diversity regarding not only species from *Idotea* but also the outgroups as well. In fact, the phylogenetic analysis of this genus raised questions regarding the taxonomy of two outgroup species – *Pentidotea panousei* and *Synischia hectica* – which appear to belong to a different taxonomic group. Furthermore, the diversification of *Idotea balthica* and *Idotea chelipes* individuals collected in Turkey and Tunisia point, as was the case in other studies, to a high diversification of Idoteids in the Mediterranean Sea.

Although the present work approaches subjects that have not been truly discussed yet and represents a step forward in the comprehension of the relationships of idoteids, it is clear that more complete and vast studies are necessary for a thorough understanding of their phylogenetic relations.

Key words: Idoteidae; *Idotea; Pentidotea; Synischia;* marine invertebrates; phylogeny; Atlantic Ocean; Mediterranean Sea; taxonomy.

Resumo

Os indivíduos da família *Idoteidae* estão ainda pouco estudados e as suas relações filogenéticas ainda não estão bem compreendidas, o que leva a estudos tendenciosos e confusão geral no que toca à taxonomia e distribuição das suas espécies.

Neste trabalho, uma abordagem filogenética foi usada com o propósito de compreender a evolução do género de isópodes *Idotea* Fabricius, 1798, que, actualmente tem 27 espécies aceites, incluindo 8 no Nordeste do Atlântico e no Mediterrâneo. Resolver a filogenia destas espécies de *Idotea* permitiu-nos avaliar os níveis de diversidade e compreender os possíveis eventos históricos (p.e. eventos climáticos, geológicos e ecológicos do passado) que promoveram a diversidade deste género.

No presente trabalho, a filogenia do género *Idotea* foi investigada com base em dados de sequenciação de ADN. Foram utilizados fragmentos de um gene nuclear (28SrRNA) e dois fragmentos de genes mitocondriais (COI, ND4). Estes fragmentos foram obtidos para 6 das 8 espécies Atlânticas e Mediterrâneas. Para além disso, o estatuto da espécie *Idotea hectica,* que foi transferida para o género *Synischia* (que contém apenas uma outra espécie Australiana) foi também avaliado.

Resultados interessantes apontam para uma elevada diversidade genética, não só nas espécies de *Idotea,* mas também nos outgroups. De facto, as análises filogenéticas a este género levantaram questões relacionadas com a taxonomia de duas espécies incluídas como outgroups – *Pentidotea panousei* e *Synischia hectica* – que parecem pertencer a um grupo taxonómico diferente do actual. Mais ainda, a diversificação dos indivíduos de *Idotea balthica* e *Idotea chelipes* recolhidos na Turquia e na Tunisia apontam, como já foi o caso em outros estudos, para uma grande diversificação de Idoteídos no Mar Mediterrâneo.

Apesar do presente trabalho abordar temas que ainda não foram verdadeiramente discutidos e de representar um passo em frente na compreensão das relações entre idoteídos, é bastante claro que estudos mais vastos são necessários para um compreensão mais minusiosa da suas relações filogenéticas.

Palavras-chave:Idoteidae; *Idotea; Pentidotea; Synischia;* invertebrados marinhos; filogenia; Oceano Atlântico; Mar Mediterrâneo; Taxonomia;.

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List of abbreviations

28S rRNA : 28s Ribosomal RNA (28S)

COI : Cytochrome C Oxidase Subunit I

ND4: NADH Dehydrogenase 4

mtDNA: Mitochondrial DNA

MSC: Messinian Salinity Crisis

16S rRNA: 16S Ribosoma RNA (16S)

TMRCA: Time to Most Recent Common Ancestor

BI: Bayesian Inference

ML: Maximum Likelihood

1. General Introduction

The order Isopoda is one of the most diversified groups of the Superorder Peracarida (Crustacea), with 10,207 species described (Schotte et al., 2011). Isopods inhabit a wide range of marine, freshwater and terrestrial habitats. Among strictly marine isopods, which comprise roughly half of the known isopod species, the family Idoteidae (Valvifera) is one of the largest. However, it is clear that this family and the phylogenetic relationships between its genera/species are still poorly studied. Up to 2001, family-level arrangements were still not well established, no modern keys to the genera/species existed, and some species placed in the Idoteidae and its sister family Arcturidae were thought to belong to other groups (Poore, 2001). The latest comprehensive revision of the Idoteidae family was made by Miers (1881). Genus-level knowledge is also very incomplete. For example, in the second most diversified genus of this family – *Idotea* (Fabricius, 1798) - the diagnosis of some species is yet poorly defined or highly simplified, pre-dating the 20th century. Some species are likely junior synonyms, while others await reassignment to different genera. The current awareness that there are numerous cryptic species, not only among idoteids (Xavier et al., 2012), but also in a vast number of other species (Bickford et al., 2007), further complicates the already intricate and problematic taxonomy of the Idoteidae (Wares et al., 2007).

Using a phylogenetic approach, the aim of this work is to understand how the distribution, genetic diversity and evolution of the genus *Idotea* have been influenced by past climate and geological events. The expectation is that past climatic, oceanographic and geological processes, such as the formation of barriers (e.g. the emergence of the Siculo-Tunisian strait during Pleistocene or the closure of the strait of Gibraltar in the Messinian) or changes in water circulation patterns have shaped the diversification of this group. This type of approach is exciting not just for improving knowledge about the evolutionary history of the genus *Idotea* but it will also contribute to clarify many aspects of its complex taxonomy.

1.1. Genus *Idotea*: characteristics and distribution

The family Idoteidae Samouelle, 1819 includes 22 genera and more than 130 species, reaching its greater diversity in the southern hemisphere (Poore, 2001). They are mainly herbivores and/or scavengers (Brusca, 1984) and have, as all peracarids, direct development within the female brooding pouch, from which juveniles (mancas)

emerge (Naylor, 1972). Among idoteids, the genus *Idotea* comprises 27 species (Poore, 2001) and is currently considered cosmopolitan, being absent only in the tropical zones (Brusca, 1984). This cosmopolitan status is misleading, since only one species – *Idotea metallica* (Bosc, 1802) – is known to be truly cosmopolitan, being an obligatory rafter (Thiel and Gutow, 2005). All other species have fairly restricted distributions and, with a few dubious exceptions, do not extend across oceans nor hemispheres. Hence, the present work was restricted to a smaller geographical coverage, the Northeast Atlantic and Mediterranean, from where the type species of the genus – *Idotea emarginata* (Fabricius, 1793) – was described.

This genus has eight known species in the NE Atlantic: *Idotea neglecta* (Sars, 1897)*, I. metallica, Idotea linearis* (Linnaeus, 1766), *Idotea granulosa* (Rathke, 1843)*, Idotea chelipes* (Pallas, 1766), *Idotea pelagica* (Leach, 1815)*, I. emarginata* and *Idotea balthica* (Pallas, 1772). Only four occur in the Mediterranean (*I. chelipes, I. balthica, I. metallica* and *I. linearis*). An additional species, *Idotea ostroumovi* (Sowinsky, 1895), is restricted to the Black Sea, but may be a synonym of *I. metallica*. Furthermore, an Atlantic and Mediterranean species previously belonging to the genus *Idotea* (*Idotea hectica* Pallas, 1772) was recently transferred to *Synischia* (Hale, 1924) by Poore and Ton (1993), a genus which only comprises another species from southern Australia.

Idotea species are usually found in intertidal and shallow-water habitats or coastal lagoons, living among algae or sea-grasses on which they feed (Brusca, 1984; Leidenberger et al., 2012). Despite being active swimmers, their relatively small size (< 4cm) suggests that long-distance dispersal can only be achieved by rafting on seaweed (Clarkin et al., 2012; Thiel and Gutow, 2005). This may explain the restricted distribution of many *Idotea* species, although a few can have significantly larger ranges, as is the case of *I. balthica* that ranges from the Black Sea to Cape Hatteras in the USA.

1.2. Northeast Atlantic and Mediterranean Sea Species

1.2.1. *Idotea granulosa* (Rathke, 1843)

I. granulosa has an oval body and can exhibit several colors, varying from green, brown or red, depending on the algae it inhabits (Naylor, 1972). Their size can range from 5 to 20 mm on males and from 6 to 13 mm on females. Larger specimens seem to prefer algae like *Ascophyllum* and *Fucus,* while smaller specimens predominate on algae such as *Cladophora* and *Polysiphonia* (Naylor, 1972). It appears to be a northern

species, occurring on the Baltic Sea and NE coast of the Atlantic, ranging from Britain and Ireland to the coast of Portugal (Christie et al., 2003; Leidenberger et al., 2012; Miers, 1881; Müller, 2004; Naylor, 1972; Pereira et al., 2006). Recent data extends the range of this species to Morocco (Natal, 2013). Hence, *I. granulosa* is one of the most widespread idoteids in North Atlantic waters.

1.2.2.*Idotea chelipes* (Pallas, 1766)

I. chelipes has a slender body, colored uniformly green or brown, sometimes with white markings. Females are often darker than males. Males are recognizable from about 5-15 mm body length. Females range from 6 to 10 mm. They are very common among intertidal algae in sheltered estuaries or where streams flow over the shore, and also in sheltered brackish pools at or above high water mark. *I. chelipes* occurs along the European coasts of the Northeast Atlantic, from Britain and Norway into the Baltic Sea, including the gulfs of Bothnia and Finland (Leidenberger et al., 2012; Naylor, 1972), but extends further south into the Mediterranean Sea (Casagranda et al., 2006). *I. chelipes* has already been proposed as a polytypic species (Charfi-Cheikhrouha, 1996), including three subspecies: *bocqueti*, *mediterranea,* and *chelipes* that occupy, respectively, the eastern Mediterranean basin (eastern Tunisia coasts), the western Mediterranean basin (north Tunisia and south French lagoons) and the NE Atlantic coasts (Morocco, Spain, France), North Sea and the Baltic. Genetic evidence (Charfi-Cheikhrouha et al., 1998) based on gel electrophoresis, suggests a moderate to high level of differentiation between subspecies, although the taxonomic status of the three subspecies is still open to debate.

1.2.3.*Idotea neglecta* (Sars, 1897)

I. neglecta has an oblong oval body, often uniformly brownish, sometimes with white longitudinal lateral markings, and occasionally with white marbling over the whole dorsal surface. Males' size ranges from about 8 mm to nearly 30 mm; adult females range from 10 to 16 mm and are mostly darker than males. They are commonly found on accumulations of detached algae or fish waste, and often found between tidemarks, usually in company with *I. balthica* and *I. emarginata*. They occur only on fully marine European coasts from Norway to France (Naylor, 1972).

1.2.4. *Idotea metallica* (Bosc, 1802)

I. metallica has an oblong body, which is uniformly greyish or brown. Length in males varies from about 8 to 30 mm; female's size ranges from 9 to 18 mm. This species occasionally reaches British waters from the east coast of North America, among floating weed, timber and colonies *of Lepas* carried by the Gulf Stream and North Atlantic Drift (Naylor, 1972). It has been frequently recorded in the Atlantic Ocean, but there are few records in tropical and subtropical Atlantic areas (Riera, 2014). The Mediterranean and Black Seas are recognized as resident breeding localities for this species (Abelló and Frankland, 1997). Records of *I. metallica* have also occurred in the Atlantic-Mediterranean coasts of Spain, from Gibraltar to the Balearic Islands (Junoy and Castelló, 2011), and in the Irish Sea (Tully and McGrath, 1987). Since 1994 it has been frequently observed in the coasts of Germany (Franke et al., 1998) although being only a summer resident since populations go extinct in winter due to the low temperatures that are unsuitable for reproduction (Gutow and Franke, 2001). Moreover, this species has been recorded also in the Pacific Ocean, reaching Australia and New Zealand (Poore and Ton, 1993).

1.2.5. *Idotea linearis* (Linnaeus, 1776)

I. linearis has a very slender body with green or brown color, often with darker or lighter longitudinal stripes; adult females are normally darker than male, frequently with paler markings around the edges. Antennas are also very long and slender. Males often reach from 15 to over 40 mm in length; females are usually smaller. It is a sublittoral species that occasionally casts up on the shore and is often found swimming near the water's edge on sandy shores at low tide. This species is common in the Atlantic-Mediterranean region, ranging from Morocco and the Canaries as far as to Denmark and Britain (Leidenberger et al., 2012; Naylor, 1972).

1.2.6. *Idotea emarginata* (Fabricius, 1793)

I. emarginata has an oblong and oval body. Color in males is often uniformly brown though sometimes white markings are present; females are generally darker, often with longitudinal lateral white bands, or alternating white and darker transverse bands. Male size ranges from 7-9 to about 30 mm; females range from 9 to 18 mm. This species is

generally sublittoral and can be found on accumulations of detached algae, but can occasionally be spotted between tidemarks on attached algae and in large numbers among cast up drift weed. *I. emarginata* is present on marine European coasts from Norway to Northern Spain (Naylor, 1972).

1.2.7.*Idotea pelagica* (Leach, 1815)

I. pelagica has *a* short and stout body. Male size ranges from 4 to 11 mm; females' size ranges from 7 to 10 mm. Color merges well with typical background of barnacles, mostly dark purple or brown, with white diamond-shaped patches or elongated stripes and with white markings along the edges of the dorsal side; females are often darker than males. Resident on exposed shores among barnacles and stunted fucoid algae. This species is recorded from Norway to the French coast, northern Spain and ranging as far as southern Portugal, but it is not present in the low salinity waters of the inner Baltic (Naylor, 1972; Pereira et al., 2006).

1.2.8.*Idotea balthica* (Pallas, 1772)

I. balthica has an oblong oval body. Size in males ranges from 10 up to 30 mm; Females' size ranges from 10 to18 mm in length. Color normally green or brown but often with white spots or longitudinal lines; female are often darker than males. They can generally be found offshore, but it is not infrequent to find *I. balthica* among attached algae on the shore and often cast up in large numbers among drift weed. This species is widespread in Europe from northern Norway into the high salinity area of the Gulf of Bothnia and coasts of Finland, occurring in nearly the whole Baltic Sea. It extends further south down to Moroccan waters, and into the Mediterranean, reaching the Black Sea (Leidenberger et al., 2012; Naylor, 1972; Pereira et al., 2006). *I. balthica* is also abundant in the eastern coast of North America, where its southern limit reaches Cape Hatteras.

1.3. The phylogenetic approach and its useful tools in the studies of the marine environment

The extension of the marine habitat and the difficult access to this environment often complicate studies that otherwise would be simpler to accomplish. However, the development of molecular biology has granted new tools which are helpful in the study of marine biodiversity. Several new species have been distinguished due to the observation of the "typical" levels of genetic divergence between species which may be a sign that cryptic speciation is frequent in the marine habitat (Gomez et al., 2007; Leese and Held, 2008; Leese et al., 2008; Lefébure et al., 2006).

Phylogenetic analyses have become essential and are currently used for many purposes. These molecular studies allow the ascertainment of the ancestral relations between species and have contributed to a more complete knowledge regarding the evolution and history of species, as well as to understand which historical mechanisms have influenced their evolution (Malaquias and Reid, 2009; Van Syoc et al., 2010). Improvements on taxonomy are also a consequence of phylogenetic reconstructions since these studies allow contextualizing of the evolution of morphological traits. One example is the ability to distinguish between characters that arise from a common ancestor (homologous) and those appearing due to convergent evolution (homoplasic), which may be useful to avoid misinterpretations and give a stable base to taxonomy (Collins and East, 1998; LaPolla et al., 2010; Levesque and De Cock, 2004).

Historical demographic events may also be detected by phylogenetic studies. Low levels of genetic variation and a shortage of rare alleles might be indicators of population bottlenecks. On the other hand, an excessive amount of rare alleles may be a sign of population expansion (Xavier, 2011). Another possible way of retrieving extra information from genetic data is to estimate the time of occurrence of certain events (such as historical climatic oscillations or geological processes) and correlate them with genetic signatures left on the genome, resorting, therefore, to the molecular clock hypothesis. Although useful, this premise is only effective if the calibration of the molecular clock is reliable which is often achieved by using fossil records (Donoghue and Benton, 2007; Warnock, 2013). While these conditions might be met for some organisms, it is easy to understand how this task is problematical in the case of small sized marine animals (Valentine et al., 2006). Nevertheless, successful studies have contributed to a better knowledge regarding the planet's biodiversity, and how it was affected by events such as the glacial periods of the Pleistocene, for example. It now seems clear that while species adapted to cold temperatures have expanded their

distribution during these stages (Stewart et al., 2010), warm-temperate species, on the other hand, were forced to inhabit much smaller geographic extensions, where temperatures and environmental conditions remained suitable (Graham, 1988; Haffer, 1969). Genetic drift and adaptation forced the divergence of these populations from their ancestors during the glacial refugia times. When suitable environment increased in geographical range, recolonization took place, leading to unique genetic patterns with high genetic diversity and differentiation and private haplotypes (Maggs et al., 2008).

1.4. Molecular markers: their usefulness and limitations

It is now clear that phylogenetic studies that rely on the analyses of a single molecular marker might not be demonstrating the evolutionary history of a species. Instead they are recovering information concerning the molecular marker itself. This occurs because a species tree is not inevitably identical to a gene tree (Nichols, 2001). There is, therefore, a necessity to merge the information collected from several molecular markers (mitochondrial and nuclear) in order to produce more consistent and trustworthy studies and results.

1.4.1.Mitochondrial DNA

When conducting phylogenetic studies, mitochondrial markers are often used, mainly because they are considered to be neutral markers and recombination is rare or absent. Moreover, they have a more rapid evolution when compared with nuclear genes. However, several studies have already shown that deviations to neutrality are not rare in mtDNA (Ballard and Kreitman, 1994; Ballard and Kreitman, 1995; Elson et al., 2004; Nachman et al., 1996; Rand, 2001). Because this molecule does not seem to suffer recombination, selection is likely to cause genetic hitchhiking, which can have important consequences in the inferences drawn from phylogenetic analyses. However, the belief that mtDNA is a molecule that does not suffer recombination has already been challenged and some authors claim that it might even be frequent in some animals (Piganeau et al., 2004). Furthermore, biparental inheritance has also been reported in several species which facilitates even more the events of recombination (Ballard and Whitlock, 2004; Galtier et al., 2009) and influences the analyses and interpretation on phylogenetic studies.

Despite all the drawbacks mentioned above, the usually lower effective population size of mtDNA (due to its maternally inheritance in most of the cases) when compared with nuclear DNA, carries a great advantage because it leads to a faster fixation of new alleles. However, more elevated mutation rates characteristic of mtDNA, also mean that this molecule may accumulate a great number of recurrent mutations, which may lead to a saturation of the molecule and a consequent decrease in its usefulness for phylogenetic inferences (Ballard and Whitlock, 2004).

In phylogenetic studies of crustacean species, the cytochrome c oxidase subunit I gene is one of the most used mitochondrial markers. This protein coding gene is very much used because it often has good resolution to distinguish between different families, species (Fransen and De Grave, 2009) and also populations.

1.4.2.Nuclear DNA

The recognition that studies based solely on the analysis of mtDNA might be biased has led to an increasing demand for more complete analyses. For this reason, nuclear molecular markers should also being included in genetic studies (Tollefsrud et al., 2009). The extra information provided by nuclear genes is used on phylogenetic studies to help overcoming the inaccuracies caused by deviations to neutrality and violations of recombination assumptions on mtDNA, especially when the effective population size is not gender balanced.

One of the main differences between mtDNA and nuclear DNA is the mutation rate. Nuclear DNA has a much slower mutation rate, which means the accumulation of recurrent mutations is lower. This characteristic might hold back some conclusions on phylogenetic studies but on the other hand makes nuclear molecules less affected by homoplasy (i.e. convergent evolution gives rise to equal genotypes instead of these being shared by a common ancestor).

Another difference between mitochondrial and nuclear markers is that recombination events are very common on nuclear DNA. This, however, induces homoplasy, which goes against the aforementioned advantage of nuclear DNA over mtDNA and undercuts and weakens phylogenetic studies. Many phylogenetic analyses involving crustacean species often rely on several nuclear genes. The 28S ribosomal gene, for example, is commonly used on phylogenetic reconstructions (Fransen and De Grave, 2009).

1.5. The dispersion ability of *Idotea*

When comparing the marine and terrestrial environments, it is easy to understand why the ocean is considered a much more challenging habitat, study wise. Its lack of visible and evident physical barriers united with large scale oceanographic currents facilitates the transport of individuals and the connection between populations, even at long distances (Carr et al., 2003), which increases the complexity of phylogenetic studies. This concept, associated with the idea that marine populations are open (recruitment of new individuals is not dependent on local production of offspring) suggests that only mild genetic differentiation and low levels of population structure exist in the ocean (Palumbi, 1994; Ribeiro, 2008). Although large panmitic populations might exist in highly mobile species (such as fishes), it is important to consider that the majority of marine species are benthonic, and adults are often sessile (Brunel, 2006). Although higher levels of genetic differentiation are expected in these cases, benthic organisms may have a larval life stage which can passively disperse in the water column. Eggs, spores or larvae dispersion may consequently increase population connectivity among geographically separated areas, thus interfering with population genetic structure and dynamics (Alexander and Roughgarden, 1996; Eckman, 1996; Levin, 2006; Pineda et al., 2007). The same logic is applied to species with external fertilization, where the transport of gametes stimulates crossing between distant individuals and populations (Young, 1994).

Idotea have, as all peracarids, direct and protected development within the female brooding pouch, from which juveniles (*mancas*) emerge, therefore, lacking early pelagic life-stages (Naylor, 1972). They are commonly found holding on to substrate (rocks and algae) and appear to be able to swim, but only in short distances, sinking if no other substrate is found meantime. These characteristics make individuals belonging to the genus *Idotea* poor dispersers and gene flow between populations is maintained mainly through rafting (Brusca, 1984; Clarkin et al., 2012; Thiel and Gutow, 2005). Due to these low levels of dispersal ability high population heterogeneity as well as high levels of divergence are expected within *Idotea* species, even between spatially close locations (Gibson et al., 2006). Peracarid species that disperse principally through rafting are also very dependent on oceanographic features such as currents, waves and local water circulation patterns, which will, most certainly, influence their population structure. Furthermore, and once again due to these organisms' restricted dispersal capacity, the current levels of gene flow are unlikely to delete the genetic signatures left by historical

events, such as allopatric isolations, range expansions or local extinctions. Hence the species from genus *Idotea*, like most peracarids, may be good models to study the impacts of historical, geological and climatic events in the evolutionary history of marine biota.

1.6. Northeast Atlantic Ocean and its water movement patterns

The Northeast Atlantic region is associated with three main large-scale currents: the North Atlantic current, the Azores current and the Canary current (Mason et al., 2005). The North Atlantic current and the Azores current are both fed by the Gulf Stream. However, the first one splits into two branches, one flowing eastward towards northern Europe and whereas the other one flows in a northeastward direction, between Iceland and the British Isles (Mason et al., 2005). The Azores current also splits into two branches: one flowing North towards the Gulf of Cadiz (where upon its reaching turns south and flows along the northwest African coast) and one flowing Southeast towards the Canary Islands (Johnson and Stevens, 2000). When the Azores current reaches the western Iberian coasts, it feeds the Portugal Current, which flows southwards. The Canary current is also supplied by the Azores current and also receives a small input from the Portugal current, which interacts with the coastal upwelling waters (Barton, 2001; Relvas et al., 2007). Despite the fact that the Portuguese coast is influenced by a current pattern that predominantly flows in a southward direction, the coastal patterns of water circulation are very much influenced by seasonal winds, which can influence and change the established flowing direction (Relvas et al., 2007). In fact, seasonal changes in the currents pattern are registered in the coast of Portugal. Although water circulation is mainly southward during the summer months, due to the upwelling phenomenon, during winter the poleward direction is predominant along the West coast of Portugal and Northern Spain (Castro et al., 1997; Haynes and Barton, 1990).

The Portugal Current also enters the Mediterranean Sea, where the water circulation is very complex due to cyclonic formations and wind interactions (García-Lafuente et al., 2006). Finally, the Canary Current also suffers seasonal changes in its position: in the Summer it flows closer to North African Coasts, while in the Winter it has a more offshore position (Barton, 2001).

1.7. The Mediterranean Sea as center of diversification

The Mediterranean is a semi-enclosed sea, contacting to the west with the Atlantic Ocean and to the East with the Black Sea. The complex geological history of the formation of the Mediterranean was marked by two major historical events which are thought to be the main drivers of its fauna and flora diversification: the Messinian salinity crisis (MSC) and the Quaternary glaciations (Patarnello et al., 2007). The MSC occurred when the connection between the Atlantic and the Mediterranean was interrupted (Krijgsman et al., 1999) approximately at 5.96 – 5.33 Myr ago, which led to a long term evaporation of the Mediterranean basin, its consequent desiccation and to the extinction of a large proportion of the remnants of the Tethyan biota. Nevertheless, data suggests that refugial areas allowed the survival of shallow water species (Myers, 1996; Sotelo et al., 2009). It is now believed that the current Mediterranean diversity results from two different processes: The survival of some species in the Mediterranean during the MSC (paleoendemic species) and the recolonization of the Mediterranean after the MSC by individuals from the Atlantic Ocean (neoendemic species) (Xavier et al., 2012). Pleistocene glaciations have also influenced the current diversity of the Mediterranean Sea. Evidence suggests that during glacial periods marine species adapted to warm water were forced to retract their ranges to warmer habitats. For this reason it is assumed that locations in the south of the north-east Atlantic such as the North African coast were likely glacial refugia for many temperate species (Almada et al., 2005; Domingues et al., 2005; Xavier et al., 2011). Because the sea level drops during glacial periods, connection between the western and eastern Mediterranean basins may have been reduced, due to the emersion of land masses such as the Strait of Sicily (Giraudi, 2004; Lambeck and Purcell, 2005) which promoted allopatric speciation in many marine species (Maggs et al., 2008). Events such as the ones mentioned previously are expected to leave genetic signatures on many organisms (Maggs et al., 2008), especially in those with low dispersal abilities (Petit et al., 2003) such as idoteids.

1.8. Objectives of the thesis

The main objective of this Master's Thesis was to understand how the distribution, genetic diversity and evolution of the genus *Idotea* were influenced by past climate and

geological events, based on phylogenetic analysis. Although the genus is considered cosmopolitan (Brusca, 1984) only Northeast Atlantic and Mediterranean species were addressed.

With this approach it would be possible to correlate divergence events in *Idotea* to events such as the Messinian Salinity Crisis and to Pleistocene climatic shifts. The expectation for this work was that high levels of intra and inter-specific variation would be found, due not only to the poor level of detail in original descriptions and low dispersal capacity of these organisms but also because of the historical events that dominated the study area.

2. Materials and Methods

2.1 Taxon sampling, outgroup choice and PCR amplification

Samples included in this study were collected from several localities as detailed in Table and Image 1. Algae were gathered during low tide and subsequently washed with freshwater. All Isopoda were stored in 96% ethanol. Genomic DNA was extracted from 38 individuals belonging to *I. balthica* (n=14), *I. chelipes* (n=8), *I. pelagica* (n=2), *I. granulosa* (n=7), *I. metallica* (n=5) and *I. emarginata* (n=2) which were identified using the keys provided by Naylor (1972).

Outgroup choice has been reported to be of vital importance for reconstructing Crustacean phylogenies, especially when using mitochondrial data due to the fast rate of evolution of this molecule which can cause a number of artifacts such as long branch attraction. One way to overcome these problems is to include outgroups which are closely related to the ingroups (Brinkmann and Philippe, 2008; Caravas and Friedrich, 2010). Therefore, several specimens from the family Idoteidae were used as outgroups: *Stenosoma nadejda* (n=4) *Stenosoma cf. capito* (n=1) *Pentidotea stenops* (n=4), *Pentidotea panousei* (n=5), *Pentidotea wosnesenskii* (n=1) and *Synischia hectica* (n=3) (see Table 1 for details).

Portions of two mitochondrial, the cytochrome c oxidase subunit I (COI) and NADH dehydrogenase 4 (ND4) and one nuclear, the 28s ribosomal RNA (28S) genes were used for phylogenetic reconstructions. Two sets of primers were used for COI amplification: LCO1490 and HC02198 (Folmer et al., 1994) and their degenerate versions jgHCO2198 and jgLCO1490, (Geller et al., 2013). For PCR reaction, between 2 and 2.5 mM of $MgCl_2$ were used and annealing temperatures were set to range from

44–47ºC. For the ND4 gene, in addition to primers published in (Xavier et al., 2012) new sets of primers were designed using software Primer3 v.0.4.0 (Untergasser et al., 2012) based on previously published complete ND4 sequences from isopod species: *Idotea balthica* (Genbank accession number DQ442915) (Podsiadlowski and Bartolomaeus, 2006), *Ligia oceanica* (Linnaeus, 1767) (Genbank accession number: DQ442914) (Kilpert and Podsiadlowski, 2006) *Armadillidium vulgare* (Latreille, 1804) (Genbank accession number: EF643519) (Marcadé et al., 2007), *Limnoria quadripunctata* (Holthuis, 1949) (Genbank accession number: KF704000) (unpublished), *Eophreatoicus sp.* (Nicholls, 1926) (Genbank accession number: FJ790313) (Kilpert and Podsiadlowski, 2010), *Eurydice pulchra* (Leach, 1815), (Genbank accession number: GU130253) (Kilpert et al., 2012), *Sphaeroma serratum* (Fabricius, 1787), (Genbank accession number: GU130256) (unpublished), *Glyptonotusant arcticus* (Eights, 1852) (Genbank accession number: GU130254) (unpublished paper), *Janira maculosa* (Leach, 1814) (Genbank accession number: GU130255) (Kilpert et al., 2012), *Asellus aquatics* (Linnaeus, 1758) (Genbank accession number: GU130252) (Kilpert et al., 2012); and two unpublished complete ND4 sequences of *Stenosoma acuminatum* (Leach, 1814) and *Stenosoma nadejda* (Rezig, 1989) .

Sequences of new primers were ND4F2: 5’ TCTCCTARDARRTTHAGAG 3’, Nd4F5: 5’ ATTKGCYTCTCTTCCKCTTC 3’, ND4F6: 5’ CTACCTCCCTCCTCTCGACC 3’, ND4R3: 5’ RSADGRTTTACCVTGTTG 3’, ND4R4: 5’ TTKAGRGWRGGRGGRGCKGCT 3’, ND4R5: 5’ RGAAGSTTYSCCVTGTTG 3’. PCR amplification was achieved using 2-3 mM of $MgCl_2$ and annealing temperatures of 53-57ºC.

The 28S fragment was amplified using different combinations of the primers published by Whiting (2002). PCR amplification was achieved using 2.5-3 mM of $MgCl_2$ and 58–62ºC annealing temperatures.

For mitochondrial coding genes, ClustalW (Thompson et al., 1994) was used to align sequences as implemented in BioEdit (Hall, 1999). Moreover, sequences from these two genes were uploaded in DNASP (Librado and Rozas, 2009) and translated into aminoacids to search for premature stop codons that would be indicative of pseudogenes. Sequences of 28S were aligned using the MAFFT algorithm (Katoh and Standley, 2013) and highly variable regions were eliminated from the analysis using Gblocks (Castresana, 2000).

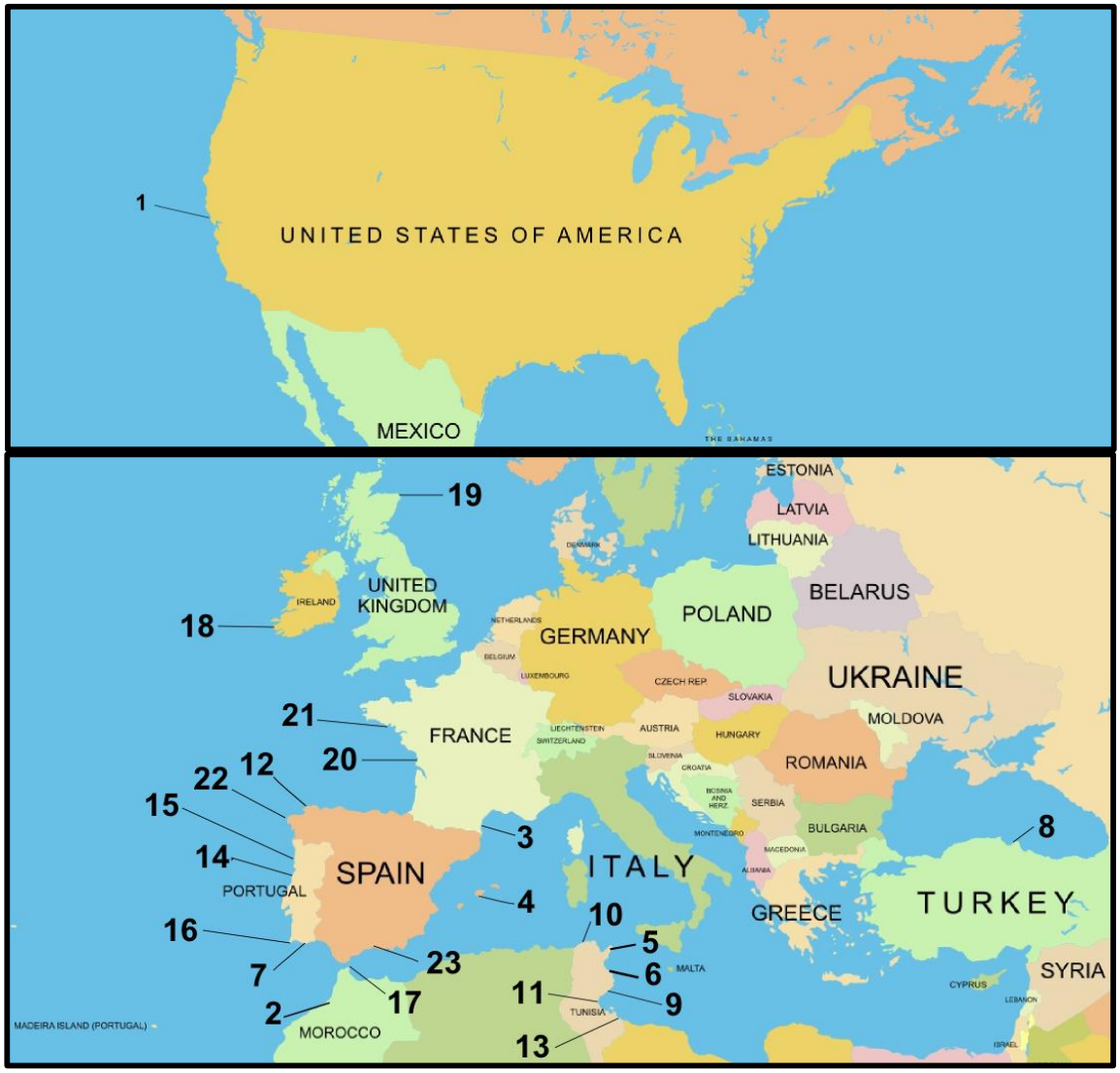

Fig. 1 - Locations sampled in this study: 1- Bodega Bay (USA); 2- Temara (Morocco); 3-Banyuls-sur-Mer (France); 4-Punta Negra, Spain; 5- Cap Bon (Tunisia); 6- Les Pyramides (Tunisia); 7-Ria Formosa (Portugal); 8-Sinop (Turquia); 9-Nabeul (Tunisia); 10-Cap Serrat (Tunisia); 11-Boughrara (Tunisia); 12- Espasante (Spain); 13-Djerba (Tunisia); 14-Ria de Aveiro (Portugal); 15- Vila Praia de Âncora (Portugal); 16-Castelejo (Portugal); 17- Cap Malabat (Morocco); 18-Minard Castle (Ireland); 19-Peterhead (England); 20-Royan (France); 21-Le Croisic (France); 22-Tóriñan (Spain); 23- El Morche (Spain)

2.2. Phylogenetic analysis

PartitionFinder v1.1.1 (Lanfear et al., 2012) was used to determine the best partition scheme as well as appropriate molecular evolution models using the Bayesian's Information Criterion for the two mitochondrial coding genes. This software compares all possible partition schemes in a timely manner and its outputs can be directly used in GARLI (Bazinet et al., 2014) and even MrBayes (Huelsenbeck and Ronquist, 2001;

Ronquist and Huelsenbeck, 2003). However PartitionFinder output might not always be implementable in BEAST (Drummond and Rambaut, 2007) as these analyses tend to contain more free parameters than the ones considered by PartitionFinder. For this reason JmodelTest (Posada, 2008) was also used to determine the best model of evolution for each gene as it provides detailed parameter estimates which improved phylogenetic inference in BEAST (based on ESS values).

Phylogenetic reconstructions were performed for the datasets separately, for the concatenated dataset of the two mitochondrial genes (Nd4+COI) and for the concatenated datasets of the three genes (ND4+COI+28S). Analyses were conducted using Bayesian inference (BI), implemented in MrBayes 3.2.4 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), Maximum Likelihood (ML), using a partitioned model implemented in GARLI 2.1 (Bazinet et al., 2014). Additionally, for the concatenated dataset, a coalescent approach for species tree reconstruction was implemented using BEAST v.2.2.1 (Drummond and Rambaut, 2007). This method uses a coalescent approach based on the Bayesian Markov chain Monte Carlo method to produce an estimate of the species trees, within which, single gene trees are embedded (Heled and Drummond, 2010). This method is considered to improve phylogenetic reconstruction when compared to cases where different genes were concatenated, since gene genealogies are not always concordant, therefore minimizing the potential discrepancy between the gene tree and the species tree. Priors and other details regarding this analysis are described in the following section of methods. For BI analysis, two separate runs were performed for $3x10^7$generations sampled every 1,000th generation (30,000 trees sampled). The approximate number of generations needed to obtain stationarity and good mixing was estimated graphically, i.e effective sample size (ESS)>200, using Tracer v1.6 (Drummond and Rambaut, 2007), and was set to 10% for all datasets. The default random tree option was used to begin the analysis. Analyses were made using four chains (one cold, three heated) with a temperature setting of 0.1. A majority-rule consensus tree was estimated after discarding the first 3,000 samples of each run. Robustness of the ML results was tested by bootstrap analyses with 1,000 replicates performed with GARLI (Bazinet et al., 2014). Only unique haplotypes were included in the phylogenetic analysis. To access typical interspecific levels of divergence uncorrected p-distances were calculated between *Idotea* species for each gene.

2.3. Dating divergence between major clades of *Idotea*

Different mutation rates for COI have been used to estimate time of divergence between isopod species, namely 1.4–2.6% estimated for decapod shrimps separated by the closure of the Isthmus of Panama (Spooner and Lessios, 2009), 2.3% for butterflies (Markow and Pfeiler, 2010) or 1.8% averaged over crustacean and non-crustacean intertidal species (Wares and Cunningham, 2001). Up until now no calibrated estimates of sequence divergence are available for idoteids, although a few exist for other isopods for COI (Ketmaier et al., 2003; Poulakakis and Sfenthourakis, 2008) and 16S rRNA genes (Held, 2002)

In this study, to date the divergence between the major clades within *Idotea*, two substitution rates for COI were used. The first was the rate of 1.25% estimated by Ketmaier et al. (2003) for *Stenasellus* dollfus, 1897 (Isopoda, Asellota). The second, was the upper bound of the range of 1.56–1.72% estimated for *Orthometopon* verhoeff, 1917 (Isopoda, Oniscidea) by Poulakakis and Sfenthourakis (2008).

These substitution rates were implemented in BEAST to retrieve the time to the most recent common ancestor (TMRCA) for the major clades. For species tree reconstruction we followed the best partition scheme recommended by PartitionFinder. However, the same model of evolution was enforced to both partitions of each mitochondrial gene. As mentioned above, models recommended by PartitionFinder are not always adequate for implementation in BEAST, so for this analysis we specified model parameters for each gene based on Jmodeltest v. 2.1.3 (Posada, 2008). Clock models were set as independent between 28S, COI and ND4. However, mitochondrial genes were linked under the same tree.

3. Results

The two mitochondrial genes, COI and ND4, were amplified for 37 and 29 *Idotea* individuals, respectively (see Table 1). The COI alignment had a total of 416 - 609 base pairs (bp), and the ND4 alignment had 345 - 441 bp. The concatenated dataset of these two genes fragments (COI+ND4) had a maximum 1050 bp. Additionally COI and ND4 were amplified for six and five outgroups respectively (see Table 1 for details). When sequences were translated into proteins no premature stop codons were found in any of these protein coding genes and no gaps were postulated. An alignment, including 1515 bp, was obtained for the 28S ribosomal gene and included 23 *Idotea* individuals and six

outgroup species. Only 1155 bp were considered to be alignable using the software Gblocks (Castresana, 2000). Overall, for each dataset missing data was always below 10%, thus not compromising the accuracy of phylogenetic inference (Wiens and Moen, 2008).

Table 1 - List of individuals included in the phylogenetic analysis, their codes and sampling sites with respective geographical coordinates. The last three columns depict which genes were sequenced (x) for each individual and respective accession numbers. Dashes (-) represent genes that were not possible to amplify.

Species	Code	Locality	Coordenates	COI	28S	ND4
Pentidotea stenops	US_BBA_802	Bodega Bay, United States	38.319, -123.074	X	X	X
Pentidotea stenops	US_BBA_3238	Bodega Bay, United States	38.319, -123.074	X	X	-
Pentidotea stenops	US_BBA_3239	Bodega Bay, United States	38.319, -123.074	X	X	X
Pentidotea panousei	MA_FAL_151	Temara, Morocco	33.914, -6.980	X	X	-
Pentidotea panousei	MA_TEM_788	Temara, Morocco	33.914, -6.980	X	X	-
Pentidotea panousei	MA_TEM_789	Temara, Morocco	33.914, -6.980	X	-	-
Pentidotea panousei	MA_TEM_3237	Temara, Morocco	33.914, -6.980	X	-	-
Pentidotea wosnesenskii	US_BBA_803	Bodega Bay, United States	38.319, -123.074	X	X	X
Stenosoma nadejda	FR_BSM_434	Banyuls-sur-Mer, France	42.480, 3.144	JQ425504	JQ425566	JQ425555
Stenosoma cf. capito	IT_PNE_158	Punta Negra, Spain	40.593, 8.276	JQ425496	JQ425578	JQ425522
Stenosoma nadejda	MA_FAL_310	Hotel Le Falouque, Morocco	33.920, -6.969	JF915258	JF915302	JQ425539
Stenosoma nadejda	TN_CBO_435	Cap Bom, Tunisia	36.829, 11.084	JQ425503	-	JQ425538
Stenosoma nadejda	TN_PYR_439	Les Pyramides, Tunisia	36.441, 10.737	JQ425505	JQ425565	JQ425556
Synischia hectica	PT-RFO 2288	Ria Formosa, Portugal	36.974, -7.873	X	X	X
Synischia hectica	PT-RFO 2289	Ria Formosa, Portugal	36.974, -7.873	X	X	X
Synischia hectica	TN-CBO 415	Cap Bon, Tunisia	36.829,11.084	X	X	X
Idotea balthica	TR_SIN_2536	Sinop, Turkey	42.021, 35.153	X	X	X
Idotea balthica	TR-SIN 2535	Sinop, Turkey	42.021, 35.153	X	X	X
Idotea balthica	TN-NAB 801	Nabeul, Tunisia	36.439,10.730	X	-	X
Idotea balthica	TN-NAB 800	Nabeul, Tunisia	36.439,10.730	X	-	X
Idotea balthica	TN-CSE 810	CapSerrat, Tunisia	37.240, 9.218	X	-	X
Idotea balthica	TN-CSE 809	CapSerrat, Tunisia	37.240, 9.218	X	-	X
Idotea balthica	TN-BOU 2546	Boughrara, Tunisia	33.706,10.758	X	-	X
Idotea balthica	TN-BOU 2545	Boughrara, Tunisia	33.706,10.758	X	-	X
Idotea balthica	TN-BOU 2544	Boughrara, Tunisia	33.706,10.758	X	-	X
Idotea balthica	TN-BOU 2543	Boughrara, Tunisia	33.706,10.758	X	-	X
Idotea balthica	MA-FAL 58	Hotel Le Falouque, Morocco	33.920, -6.969	X	X	X
Idotea balthica	MA-FAL 1079	Hotel Le Falouque, Morocco	33.920, -6.969	X	X	X
Idotea balthica	ES-ESP 792	Espasante, Spain	43.733, -7.796	X	X	X
Idotea balthica	TN-DJE 412	Djerba, Tunisia	33.874, 10.924	-	X	-
Idotea chelipes	TN-DJE 413	Djerba, Tunisia	33.874, 10.924	X	X	X
Idotea chelipes	TN-DJE 2542	Djerba, Tunisia	33.874, 10.924	X	X	X
Idotea chelipes	TN-DJE 2541	Djerba, Tunisia	33.874, 10.924	X	-	X
Idotea chelipes	TN-DJE 2540	Djerba, Tunisia	33.874, 10.924	X	X	X
Idotea chelipes	TN-DJE 2539	Djerba, Tunisia	33.874, 10.924	X	-	X
Idotea chelipes	PT-RAV 38	Ria de Aveiro, Portugal	40.656, -8.657	X	X	-
Idotea chelipes	PT-RAV 37	Ria de Aveiro, Portugal	40.656, -8.657	X	X	-
Idotea chelipes	PT-RFO 2282	Ria Formosa, Portugal	36.974, -7.873	X	X	-
Idotea pelagica	PT-VPA 416	Vila Praia de Âncora, Portugal	41.816, -8.871	X	X	X

Idoteapelagica	PT-CAS 2657	Castelejo, Portugal	37.102, -8.945	X	X	X
Idotea granulosa	MA-CMT 1410	Cap Malabat, Morocco	35.817, -5.750	X	-	X
Idotea granulosa	MA-CMT 1409	Cap Malabat, Morocco	35.817, -5.750	X	X	X
Idotea granulosa	IE-MCA 1348	Minard Castle, Ireland	52.125, -10.11	X	X	X
Idotea granulosa	GB-PET 1331	Peterhead, Great Britain	57.512, -1.781	X	X	X
Idotea granulosa	FR-ROY 1375	Royan, France	45.619, -1.042	X	X	X
Idotea granulosa	FR-LCR 1384	Le Croisic, France	47.301, -2.533	X	-	-
Idotea granulosa	ES-TOR 414	Tóriñan, Spain	43.049, -9.286	X	X	X
Idotea metallica	ES-MOR 1995	El Morche, Spain	36.737, -3.993	X	X	X
Idotea metallica	ES-MOR 1997	El Morche, Spain	36.737, -3.993	X	X	-
Idotea metallica	ES-MOR-1996	El Morche, Spain	36.737, -3.993	X	-	-
Idotea metallica	ES-MOR-1998	El Morche, Spain	36.737, -3.993	X	-	-
Idotea metallica	ES-MOR-1999	El Morche, Spain	36.737, -3.993	X	-	-
Idotea emarginata	ES-ESP 791	Espasante, Spain	43.733, -7.796	X	X	X
Idotea emarginata	ES-ESP 790	Espasante, Spain	43.733, -7.796	X	X	X

For the two mitochondrial genes, PartitionFinder results indicated that two partitions should be built for each mitochondrial gene fragment, one encompassing first and second codon positions, and a second partition including third positions. This partition scheme allows minimizing potential effects of saturation on phylogenetic reconstructions. For both partitions of ND4, the best-fit model of nucleotide substitutions selected was HKY+G (lnL=-3193.50289). The recommended model for the first and second codon positions on the COI dataset was TrNef+I+G (lnL=-1131.85219) and the HKY+G (lnL=-2945.23402) for the third codon position. Results from JModelTest (Posada, 2008) indicated that GTR+I+G (lnL=-4747.13393), HKY+I+G (lnL=-4747.96600), and GTR+I+G (lnK=-3893.12837), were the best models for the 28S, COI, and ND4, respectively. In BI and ML analyses the models of evolution used were the ones found by PartitionFinder, accounting for partitions of mitochondrial data. Since neither MrBayes nor GARLI (see below) include the TrNEf+I+G model, the SYM+I+G (lnL=-1131.24598) and the GTR+I+G (lnL=1122.8859) models, which were the next best models that could be implemented, were used in MrBayes and GARLI, respectively. For the 28S we used the best model given by JModelTest as this also estimates several parameters that can be fixed, thus optimizing tree search. For the same reason, the species tree was reconstructed using the models determined by JModelTest.

The BI and ML analyses produced similar tree topologies (see Figures 1, 2, 3, 4 and 5). Based on mitochondrial genes, species of *Idotea* were always retrieved in two major clades: Clade I - encompassing strictly Atlantic species, *I. pelagica* and *I. granulosa*; Clade II - that included *I. balthica, I chelipes, I. emarginata,* and *I. metallica* (Figures 1 and 2). The best tree based on COI, shows that *Idotea* is not monophyletic as *Pentidotea panousei* is placed within the ingroup, sister to Clade I. However, this has

little or no support (<50%) and could be regarded as a trichotomy. As *P. panusei* could not be sequenced for ND4 it was not possible to ascertain its position based on this gene. Overall, there was a low support for the monophyly of *Idotea* (based on ND4) or *Idotea* + *P. panusei* (based on COI). Accordingly, the analyses based on the concatenated mitochondrial datasets, placed *Pentidotea* sister to Clade I, but its position was not well supported and was considered a trichotomy (Figure 3). The monophyly of *Idotea* + *P. panusei* was however reasonably supported with bootstrap values of 70% and posterior probability of 100%. The analyses of mitochondrial genes further revealed a deep divergence between two lineages within two named species: *I. chelipes* and *I. balthica.*

The best tree retrieved with the nuclear dataset (Figure 4) supports the monophyly of *Idotea* (bootstrap support of 86% and posterior probability of 100%), and places *P. panusei* as the sister taxon of this group (bootstrap support of 89% and posterior probability of 98%). Within *Idotea*, and similarly to the results of the mitochondrial dataset, two major clades were recovered. One including *I. pelagica* and *I. granulosa* (Clade I), and the other including the remaining species (Clade II). However, within Clade II there were differences between the nuclear and mitochondrial data sets. With nuclear data *I. balthica* was sister to *I. chelipes*, while for mitochondrial data, *I. chelipes* is sister to *I. metallica* and *I. emarginata*. However, in the analyses based on mitochondrial genes, these relations have little or no support. The deep phylogenetic divergence within *I. balthica* and *I. chelipes*, with two distinct lineages each, was also well evident in 28S gene.

According to Mason-Gamer and Kellogg (1996) the incongruence between mitochondrial and nuclear topologies was not significant, as bootstrap support for incongruent branches is lower than 70% (see Figs 3 and 4) and therefore datasets were concatenated. The topology retrieved with the mitochondrial and nuclear concatenated dataset (Figure 5) was very similar to the one based solely on the nuclear dataset. *P. panusei* was retrieved as sister taxa of *Idotea*, and consequently *Idotea* was monophyletic (96% bootstrap support and 100% posterior probability). Phylogenetic relations within Clade II were also identical to the ones recovered with 28S.

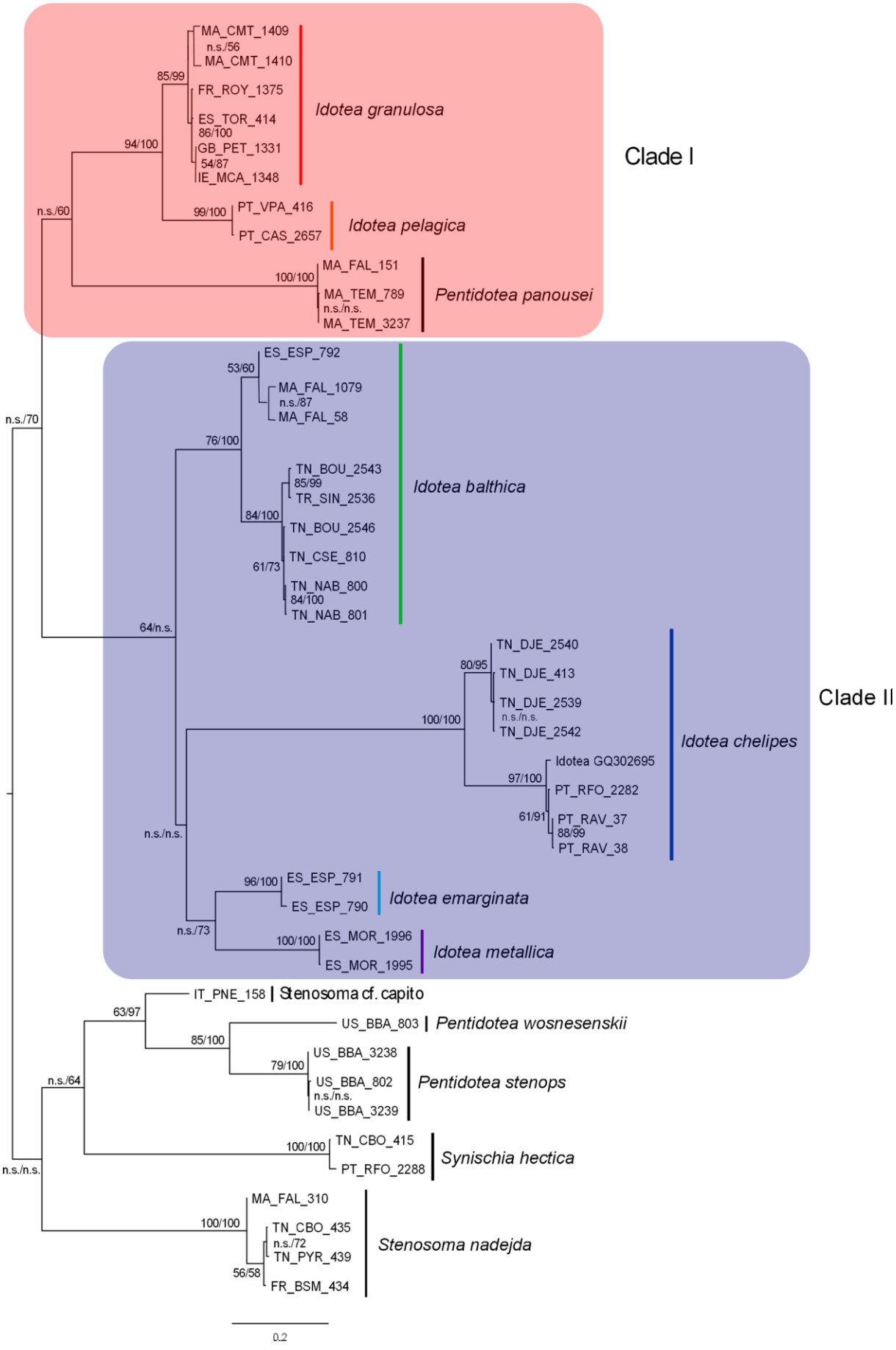

Fig. 2 - Maximum-Likelihood tree obtained for the COI gene. Values of nodes correspond to Bootstrap support and Bayesian posterior probability, respectively (n.s. indicates less than 50% support).The major clades of I*dotea* are depicted by different colours.

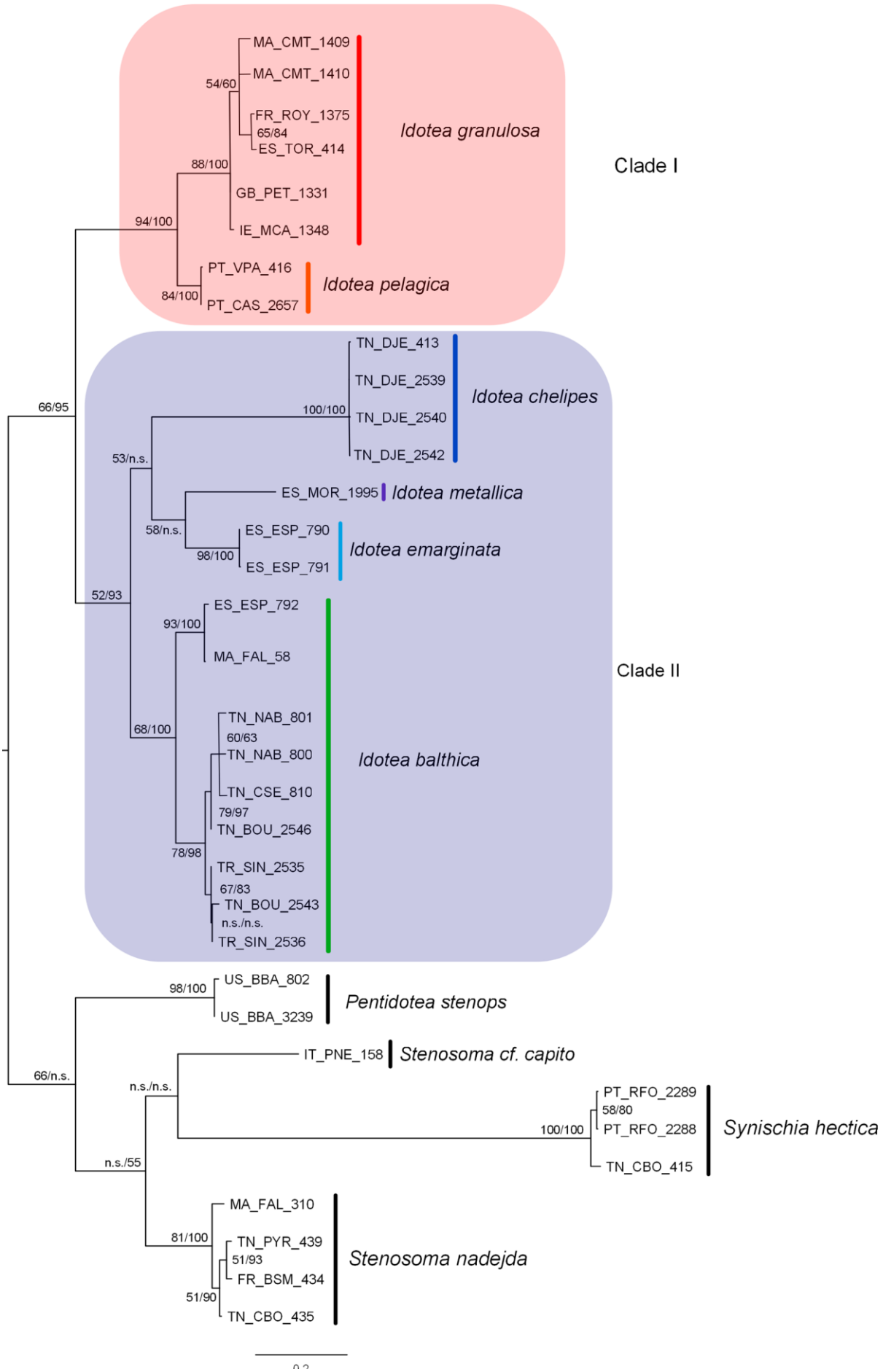

Fig. 3 - Maximum-Likelihood tree obtained for the ND4 gene. Values of nodes correspond to Bootstrap support and Bayesian posterior probability, respectively (n.s. indicates less than 50% support).The major clades of *Idotea* are depicted by different colours.

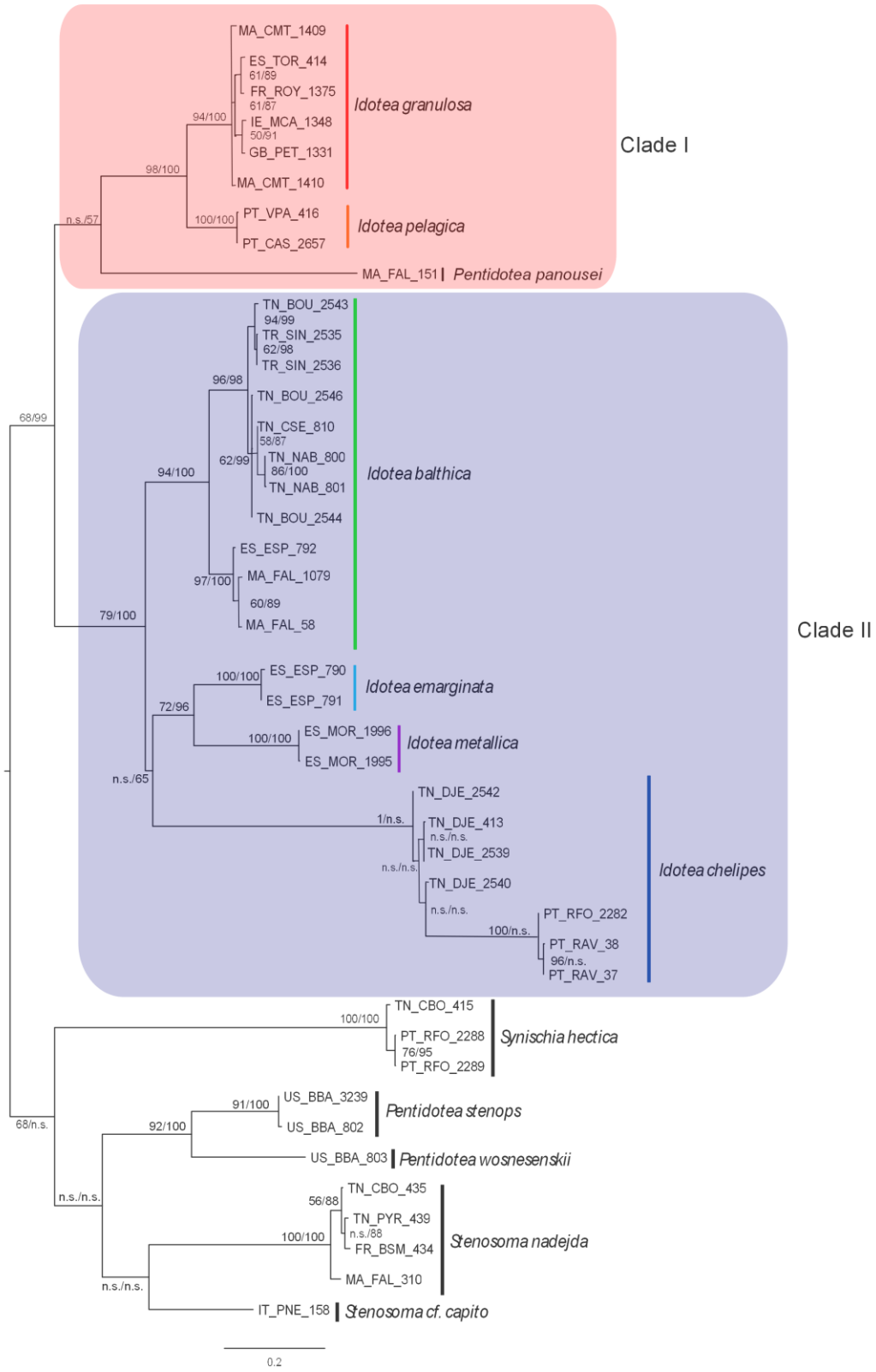

Fig. 4 - Maximum-Likelihood tree obtained for the concatenated dataset of mitochondrial genes (COI+ND4). Values at the nodes correspond to Bootstrap support and Bayesian posterior probability, respectively (n.s. indicates less than 50% support). The major clades of *Idotea* are depicted by different colours

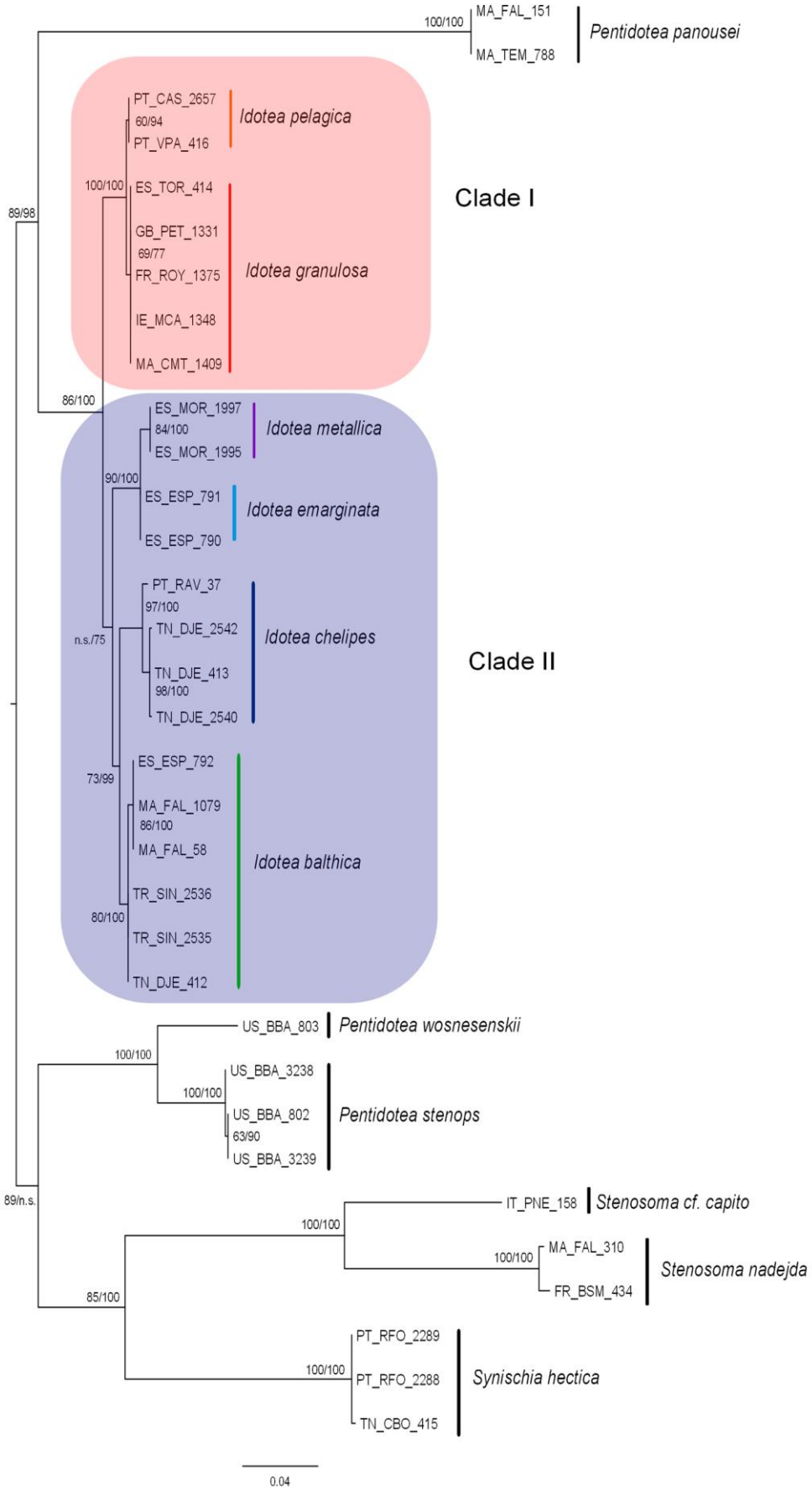

Fig. 5 - Maximum-Likelihood tree obtained for the 28S rRNA gene. Values of nodes correspond bootstrap support and to Bayesian posterior probability, respectively (n.s. indicates less than 50% support).The major clades of *Idotea* are depicted by different colours.

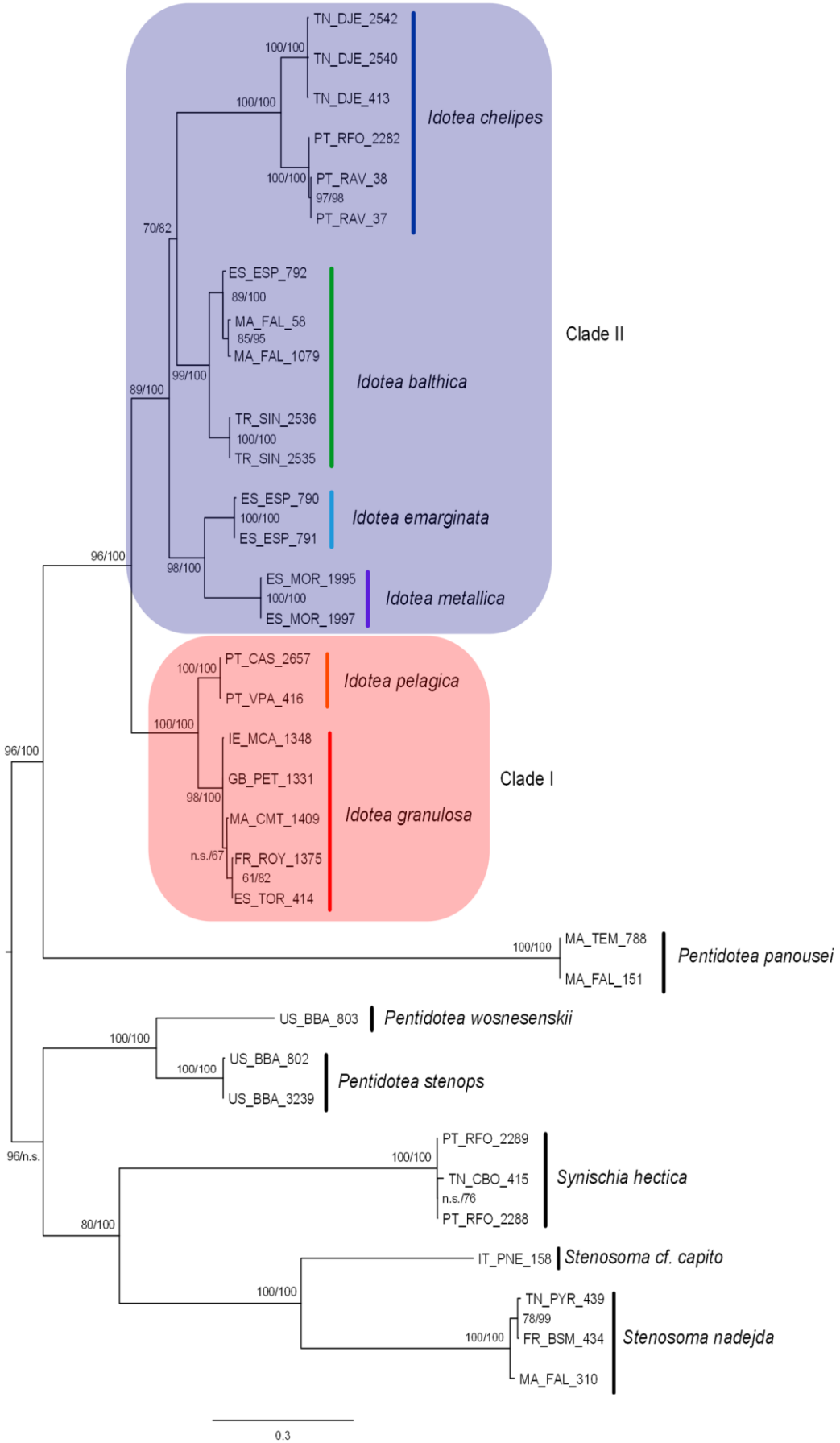

Fig. 6 - Maximum-Likelihood tree obtained for the concatenated dataset combining mitochondrial and nuclear genes (28S+COI+ND4). Values at the nodes correspond to Bootstrap support and Bayesian posterior probability, respectively (n.s. indicates less than 50% support). The major clades of *Idotea* are depicted by different colours.

The species tree topology coincided with the one obtained with nuclear and concatenated datasets (see for details Figures 5 and 6)

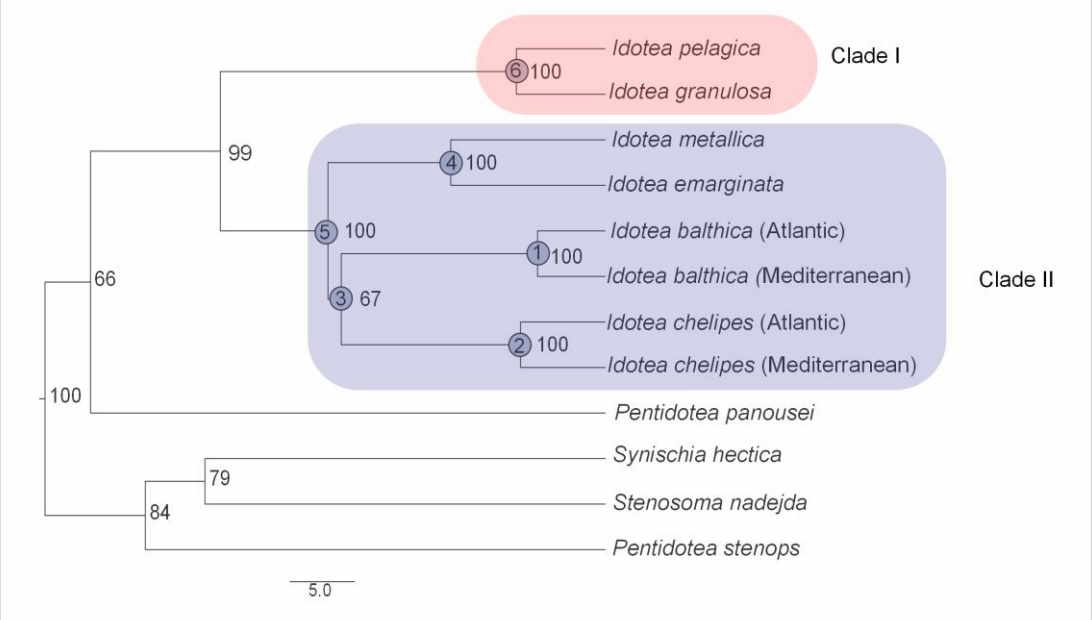

Fig. 6 - Species tree obtained with BEAST with datasets for both mitochondrial and nuclear genes. Values at the nodes correspond to Bayesian probability. The major clades of *Idotea* are depicted by different colours.

Estimates of TMRCA are detailed in Table 2. All splitting events are estimated to have occurred previously to the Messinian salinity crisis with the exception of the intraspecific divergence within *I. balthica* and *I. chelipes* that may have coincided with this period.

Table 2 - Estimated ages of nodes using Ketmaier et al. (2003) or Poulakakis & Sfenthourakis (2008) rates of substitution for the COI gene their 95% credibility intervals. Nodes are numbered according to Figure 6

Nodes	Ketmeier et al., 2003	Poulakakis & Sfenthourakis, 2008
1	5.99 Mya [2.72 - 9.82]	4.50 Mya [1.96 - 7.42]
2	7.64 Mya [3.64 - 11.88]	5.48 Mya [2.62 - 8.54]
3	24.06 Mya [16.12 - 32.32]	17.09 Mya [11.77 - 22.90]
4	13.58 Mya [7.67 - 19.65]	10.17 Mya [5.44 - 15.05]
5	25.20 Mya [17.49 - 33.51]	18.02 Mya [12.61 - 23.63]
6	8.12 Mya [3.91 - 12.79]	6.02 Mya [2.92 - 9.60]

Table 3 details the levels of inter specific divergence between *Idotea* species. For COI interspecific divergence ranged between 10-22%. For ND4 it varied between 12-26% and for 28S it ranged between 0.3-3.4%. For all gene portions, lowest levels of

divergence were found between *I. pelagica* and *I. granulosa*. Divergence levels of the two lineages found within *I. balthica* and *I. chelipes* was comparable to the levels of interspecific divergence found between *Idotea* species.

Table 3 – Pairwise distances between Idotea species, calculated for the three separated genes

	I.balthica (Med)	*I.balthicas* (Atl)	*I. chelipes* (Med)	*I. chelipes* (Atl)	*I. pelagica*	*I. granulosa*	*I. metallica*	*I. emarginata*	
I.balthica (Med)	-								COI
	-								ND4
	-								28S
I.balthicas (Atl)	0.072								COI
	0.106								ND4
	0.002								28S
I. chelipes (Med)	0.176	0.165							COI
	0.242	0.255							ND4
	0.018	0.020							28S
I. chelipes (Atl)	0.192	0.190	0.094						COI
	n.a.	n.a.	n.a.						ND4
	0.016	0.017	0.007						28S
I. pelagica	0.182	0.172	0.185	0.177					COI
	0.209	0.207	0.251	n.a.					ND4
	0.021	0.024	0.026	0.026					28S
I. granulosa	0.174	0.169	0.203	0.210	0.099				COI
	0.203	0.208	0.262	n.a.	0.115				ND4
	0.022	0.024	0.028	0.027	0.003				28S
I. metallica	0.181	0.172	0.183	0.216	0.196	0.193			COI
	0.192	0.215	0.260	n.a.	0.198	0.202			ND4
	0.025	0.027	0.032	0.034	0.030	0.031			28S
I. emarginata	0.143	0.146	0.182	0.188	0.187	0.166	0.154	-	COI
	0.168	0.173	0.205	n.a.	0.216	0.217	0.166	-	ND4
	0.023	0.024	0.031	0.032	0.031	0.033	0.006	-	28S

4. Discussion

4.1. Phylogenetic relations within the genus *Idotea*

Monophyly of *Idotea* was only supported by nuclear and concatenated datasets, and the species tree reconstruction. This is probably due to recurrent homoplasic events affecting mitochondrial genes due to their faster evolution relative to nuclear genes. Similar issues were found while reconstructing the phylogeny of the idoteid genus

Stenosoma (Xavier et al., 2012). Apparently, these issues extend beyond the molecular domain and are equally observed at the morphological level. In fact, the taxonomy of genera and species of the family Idoteidae can be seen as a case of a Wittgenstein "familial resemblance": taxa which are thought to be connected by one essential common feature are actually connected by a series of overlapping similarities, where no one feature is common to all (Ereshefsky, 2000). In Idoteidae, there are many cases where characters that allow the diagnosis of species within a genus are "shared" by otherwise distantly related genera.

In a recent review of the Valvifera, Poore (2001) lists 27 good species of *Idotea,* which together occur on all major oceans, thus conferring a cosmopolitan status to this genus. This does not mean that the taxonomy of *Idotea* is completely established. Far from it, the current number of species results mainly from the legacy of a troubled taxonomy and not from an up-to-date knowledge of the species that belong to *Idotea*. In the redescription of the genus *Idotea* made by Poore and Lew Ton (1993), these authors recognized only *I. emarginata*, *I. metallica*, *I. pelagica*, *I chelipes* and *I. balthica* as belonging to this genus. They also admitted that, although they had not examined directly the remaining European and North Pacific species presently assigned to *Idotea*, many of them would be excluded from this genus given their own diagnosis. This taxonomic view implicitly places the center of diversification of *Idotea* in the NE Atlantic and the Mediterranean, because with the exception of *I. metallica* (a cosmopolitan obligatory rafter) and *I. balthica* (which reaches the Atlantic coasts of northern America), all other cited species are restricted to the former regions. Preliminary morphological analysis, coupled with the genetic similarity between *I. granulosa* and *I. pelagica,* allow us to add the former to the list of Poore and Lew Ton (1993). Two other species remain to be studied: *I. neglecta* and *I. linearis.* Although we had no access to individuals of either species, the former seems to conform to the previously mentioned taxonomic concept of the genus *Idotea*. Regarding *I. linearis*, it differs considerably from typical *Idotea* species, being much more slender and with larger antennas. Bate and Westwood (1868) depict two separate but contiguous penes in *I. linearis*, a characteristic of *Idotea sensu* Poore and Lew Ton (1993), but on the absence of any other detailed descriptions of its parts, it is not possible to ascertain if it belongs or not to *Idotea*.

Phylogenetic reconstructions based on the concatenated dataset and the species tree recovered two major clades within *Idotea,* one including strictly Atlantic species (Clade I: *I. pelagica* and *I. granulosa*), and the other including the remaining species (Clade II). Within Clade II, *I. emarginata* and *I. metallica* were always recovered as sister

taxa. However, depending on the analyses, they were more closely related with *I. balthica* or with a group including *I. balthica* and *I. chelipes*.

4.2. Cryptic species within the genus *Idotea*

Evidence of very divergent genetic lineages were found in two *Idotea* species: *I. balthica* and *I. chelipes*. In the case *I. balthica,* one of the two reciprocally monophyletic clades included exclusively Atlantic specimens, whilst the other included only Mediterranean (Tunisia) and Black Sea (Turkey) individuals. Uncorrected p-distances between this two linages (COI: 0.072; ND4: 0.106; 28S: 0.002) are below average values for other pairwise comparisons between *Idotea* species (Table 3). However, they are quite similar to the ones exhibited by *I. granulosa* and *I. pelagica,* two morphologically distinct species (COI: 0.099; ND4: 0.115; 28S: 0.003). Moreover, Xavier et al. (2012) showed that for other Idoteid species (*Stenosoma*) these differences can even be lower (COI: 0.049; ND4: 0.048; 28S: 0.019).

A considerable amount of morphological and genetic diversity has been described for *I. balthica* in European waters (e.g. Bulnheim and Fava, 1982; Legrand-Hamelin and Legrand, 1982; Bulnheim, 1984; Wares 2001). Hence, *I. balthica* is considered a polytypic species, including four sub-species or varieties: *I. b. basteri* (Mediterranean) *I. b. triscuspidata* (Atlantic), *I. b. balthica* (Baltic Sea), and *I. b. stagnea* (French Mediterranean coast). *I. b. basteri* dates back to the 19th century and was described as a good species by Audouin (1826). The present results, together with earlier molecular evidence supporting the differentiation of the four subspecies of *I. balthica* (see Wares et al, 2007), is probably enough to attribute the status of species to *I. b. basteri*.

Two divergent and reciprocally monophyletic lineages were also found in *I. chelipes.* While one was restricted to the Mediterranean (Tunisia), the other included individuals from the Atlantic (two locations in Portugal) but also an additional sequence from Italy (S. Sfenthourakis, *pers. comm.*) with GenBank accession number GQ302695. Hence, and contrary to *I. balthica*, the deep divergence between lineages of *I. chelipes* does not coincide with an Atlantic-Mediterranean break, as one of them is present in both basins. Again, uncorrected p-distances between this two linages (COI: 0.094; 28S: 0.007) are similar to the ones exhibited by *I. granulosa* and *I. pelagica,* the major difference being that it was not possible to sequence the ND4 gene for the Atlantic-Mediterranean linage (see results).

Like *I. balthica, I. chelipes* has been described as a polytypic species ((Charfi-Cheikhrouha, 1996) including three sub-species: *I. c. bocqueti, I. c. mediterranea,* and *I. c. chelipes* that occupy, respectively, the eastern Mediterranean basin (eastern Tunisia coasts), the western Mediterranean basin (north Tunisia and southern French lagoons) and the NE Atlantic coasts (from Morocco to France), North Sea and the Baltic. While the Atlantic specimens used in this work belong clearly to *I. c. chelipes,* Mediterranean specimens apparently belong to *I. c. bocqueti* from Tunisia. This sub-species was originally described as a good species by Rezig (1977). According to (Charfi-Cheikhrouha, 1996), genetic differentiation determined by allozyme data was enough to confer the species status to the three sub-species. The present results suggest that *I. bocqueti* Rezig (1977) is a good species. The reason why this taxonomic view has never been adopted by the scientific community is probably because of the absence of unambiguous diagnostic traits, an issue that is apparently frequent in idoteids (Santos et al, 2012).

Until now, no species of *Idotea* have been found exclusively in the Mediterranean Sea. However, phylogeographic studies focusing in other Idoteids, namely the genus *Stenosoma,* have already helped uncovering previously unrecognized species and revealed the existence of high levels of species diversity in the Mediterranean region. Currently, five endemics species of *Stenosoma* were described in this region (Xavier et al., 2011). Hence, the Mediterranean is clearly a center of diversification for some Idoteids. These high levels of diversity were related to the geological and climatic events that impacted the Mediterranean Sea region: the MSC and the Pleistocenic glaciations. In this study while most of the diversification within *Idotea* pre-dates the MSC, speciation within *I. balthica* and *I. chelipes* is presumed to have occurred during the MSC. The fact that the Mediterranean lineages were so far found in the eastern Mediterranean could be indicative of a Messinian refugium in this area that has allowed the survival of populations of *I. balthica* and *I. chelipes* inside the Mediterranean during this period.

However, in the absence of any reliable calibration points these times of divergence estimates should be interpreted with caution.

4.3. Phylogenetic position and classification of *Pentidotea panousei*

Pentidotea panousei was originally described as *Idotea (Pentidotea) panousei* (Daguerre de Hureaux, 1968), hence as an *Idotea* of the subgenus *Pentidotea,* following

the taxonomic concept of Menzies and Miller (1972). This classification is no longer in use, and the species was moved to the genus *Pentidotea*. Apart from the original description, *P. panousei* was only mentioned once on a species checklist (Menioui 1998), ranging from Rabat to Tarfaya. The present work extends its distribution up to the entrance of the Mediterranean. As Daguerre de Hureaux (1968) noted, this is the only *Pentidota* known from the Atlantic, apparently restricted to its eastern side, a surprising fact considering that all other 10 known species of *Pentidotea* occur in the Pacific Ocean.

None of the phylogenetic analysis used in this work grouped *P. panousei* with the other *Pentidotea* species (*P. stenops and P. wosnesenskii)*. In fact, in all analyses where individuals from these three species were present, *P. panousei was* always more related to *Idotea* species than to *P. stenops and P. wosnesenskii.* While mitochondrial data suggests a tricotomy between *P. panousei* and the two *Idotea* clades, the concatenated dataset and the species tree suggest that it is a sister taxon of *Idotea*. In the light of the present data it is clear that *P. panusei* does not belong to genus *Pentidotea*, thus corroborating the suspicions expressed by Daguerre de Hureaux (1968) in the original description of this species. However, whether *P. panousei* belongs to the genus *Idotea* or deserves another generic name remains inconclusive.

P. panousei shares with *Pentidotea* a character that is diagnostic of this genus: a maxillipedal palp with five articles, contrasting with *Idotea* that has a four-article palp. The five-article maxillipedal palp is considered a plesiomorphy within the Valvifera, being found in all families, including the Idoteidae (Poore 2001). The importance of this characteristic as a diagnostic character has varied over time. For example, Menzies and Miller (1972) noted that although adults of *Pentidotea* had a five-articulated palp, juveniles of *P. resecata* Stimpson 1857 only had four articles. Based on this variability they considered *Pentidotea* as a subgenus of *Idotea*. Brusca (1984) also noted that the reduction of the number of articles may occur either by loss or fusion, a feature which cannot be determined unambiguously in many species. In his revision of the families of Valfifera, Poore (2001) acknowledge the importance of this character but pointed its low phylogenetic value, especially above the genus level and discarded it from his analysis.

Contrary to *P. panousei,* the two Pacific species, *P. stenops and P. wosnesenskii,* were always recovered as a monophyletic clade. It is worth mentioning that all the attempts to amplify the ND4 gene fragment have failed for *P. panousei*, despite the success regarding the remaining *Pentidotea* species. This involved a vast range of PCR conditions experimented (i.e. different hybridization temperatures, $MgCl_2$ concentration, quantity of DNA, nº of cycles of PCR). Interestingly, *P. panusei* seems to be endemic to the Atlantic Moroccan coasts, and this region is a known hotspot of genetic diversity for

other Idoteids, such as *S. nadejda* (Xavier et al., 2011), *Idotea granulosa* (Natal, 2013), or other crustacean species (e.g. decapods and amphipods, unpublished data). In the case of S. *nadejda,* because no physical or ecological barriers were evident, Xavier et al. (2011) hypothesized that only historical isolation during Pleistocenic glaciations could account for the observed differences.

4.4. The taxonomic status of *Synischia hectica*

Synischia hectica (Pallas 1772) was formerly in the genus *Idotea*, until Poore and Lew Ton (1993) transferred it to the genus *Synischia*, based on its resemblances with *S. levidensis* Hale 1924, in the complete absence of dorsal coxal plates and the presence of a mid-dorsal ridge. However, other characters do not match, as *S. hectica* pleotelsonic formula is 2+1 (similar to *Idotea*) and that of *S. levidensis* is 0+3. Interestingly, the maxillipedal palp of *S. hectica* has its fifth article fused (so a four-article palp as in *Idotea*) although the articles are of similar proportions to those in *S. levidensis*. These inconsistencies prompted Charfi-Cheikhrouha (2000) to re-describe the species moving it again to *Idotea*. However, the current phylogenetic analysis support the removal of *hectica* from the genus *Idotea*, but doubt remains on whether the placement into the genus *Synichia* was correctly made. Because no genetic data exists for *S. levidensis,* and it was not possible to obtain specimens for this study, it is currently impossible to conclude if they belong effectively to the same genus. Their antipodal distribution (NE Atlantic-Mediterranean for *hectica*, southern Australia for *levidensis*) makes this scenario very unlikely.

5. Conclusions

The results from the present work are another example showing that even well studied regions such as the Mediterranean Sea may hide high levels of unknown endemic diversity, and that genetic analysis of species with low dispersal abilities, such as Peracarid crustaceans, are an important contribution to the knowledge of Mediterranean biodiversity (e.g. Xavier et al 2011, Xavier et al 2012).

Specifically, the results from the present work, revealed that although phylogenetic reconstruction generally agrees with the current taxonomy of the genus *Idotea* generally there are two new species within *I. balthica* and *I. chelipes*. Although a thorough morphological analysis is needed, we anticipate these are likely cryptic species, as no morphological differences were found so far. As in both cases type

localities are located in the Northeast Atlantic, these new species seem to be Mediterranean endemics. According to the dating of the species tree, it is also likely that these species originated through allopatric isolation during the Messinian Salinity Crisis.

Data from the present work also supports the removal of *I. hectica* from this genus, and raise doubts to whether *P. panusei* is in fact an *Idotea*, as mitochondrial data revealed a tricotomy between this species and the two main *Idotea* clades and concatenated dataset and the species tree place it as sister to *Idotea*.

Finally, the present work sets the basis for resolving the taxonomy of idoteids. However, further sampling of idoteid species and genetic information from more genes is needed to shed more light regarding the diversity, taxonomy and also aid determine the true distribution of these organisms.

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