Phylogenetic analysis of *Pisidium* (Bivalvia: Sphaeriidae) based on molecular mtDNA and nuclear sequence data

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Dissertação de Mestrado apresentada à Faculdade de Ciências da Universidade do Porto em Biodiversidade, Genética e Evolução

2013

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Biodiversidade, Genética e Evolução
Departamento de Biologia
2013

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Agradecimentos

Gostaria de agradecer as seguintes pessoas; sem elas não teria sido possível a realização da tese:

Especial agradecimento à Doutora Elsa Froufe por me ter guiado nesta jornada, acompanhando-me durante um ano, contribuindo em muito para o meu enriquecimento.

Agradeço ao Doutor Ronaldo Sousa pela ajuda prestada na realização desta Tese.

Um obrigado à Carina pela orientação e adaptação naquele novo espaço.

Obrigado ao Tio pela assistência técnica

Por fim, mas não o menos importante, um muito obrigado aos meus pais, avós e à Diana por me terem sempre apoiado para que esta jornada tivesse o melhor desfecho possível.

Esta tese foi realizada com o apoio do projeto da FCT: CONBI: Biodiversidade e Conservação de Bivalves – ecogeografia, genética e informação fisiológica (PTDC/AAC-AMB/117688/2010)

Resumo

Os ecossistemas de água doce estão sob constante ameaça devido a fatores como fragmentação do habitat, poluição, sobre-exploração de recursos, alterações climáticas ou devido à introdução de espécies invasoras, levando à extinção de várias espécies. Ao contrário de alguns grupos taxonómicos de água doce (ex. mamíferos), outros, têm recebido pouca atenção, especialmente invertebrados. Neste grupo poderão ser incluídos os bivalves que devido à sua capacidade de escavar, filtrar e produção de conchas têm um papel essencial nos ecossistemas de água doce. Apesar da sua importância no bom funcionamento do ecossistema, muito pouco é conhecido sobre a sua ecologia e genética. No presente estudo é dada especial atenção ao género *Pisidium* (Bivalvea: Sphaeriidae). A sistemática deste género tem sido desenvolvida essencialmente a partir de estudos morfológicos, sendo descritas 24 espécies de *Pisidium*. Em Portugal apenas sete foram descritas, nomeadamente *P. amnicum*, *P. casertanum*, *P. henslowanum*, *P. milium*, *P. nitidium*, *P. personatum* e *P. subtruncatum*.

Neste estudo foram recolhidas 36 amostras de 3 principais bacias de Portugal, Douro, Ave e Cávado com o objetivo de as caracterizar geneticamente. As amostras foram analisadas usando primers para os genes ITS1 e 16S. A partir da análise filogenética foram obtidas 25 amostras que podem ser classificadas como *P. casertanum*, 9 como *P. subtruncatum* e 2 como *P. personatum*. O valor das distâncias genéticas entre as novas amostras classificadas como *P. personatum* e as sequências do GenBank foi elevado, semelhante quando comparado com o de entre outras espécies. Este resultado poderá indicar a presença de uma nova espécie ou simplesmente não há nenhuma sequência disponível no GenBank para aquela espécie. Por outro lado, essas mesmas espécies que apresentam valores semelhantes poderão estar mal identificadas. Este estudo poderá ser usado como ponto de partida para a realização de novos trabalhos onde deverá incluir mais amostras por local e também de outros rios e bacias. É necessário investir neste tipo de estudos uma vez que em Portugal muitas espécies podem ainda nem ter sido identificadas e outras já estão ameaçadas. Deverá também ter-se em conta a realização de estudos populacionais com o objetivo de desenvolver estratégias de gestão e conservação.

Palavras-chave: água doce, *Pisidium*, *P. casertanum*, *P. subtruncatum*, *P. personatum*, Douro, Ave, Cávado, PCR, primers, ITS1, 16S, filogenia, haplotipo, distâncias genéticas, conservação

Abstract

Freshwater ecosystems are threatened due to loss and fragmentation of habitat, pollution, over exploitation of resources, climate change and introduction of non-native species, leading native species to extinction. If in one hand some studies about some freshwater taxonomic groups (e.g., mammals) have received special attention, others have almost no information, especially invertebrates. Bivalves, included in this group, have a central role in freshwater ecosystems such as burrowing, production of shells and filtering activity. Despite their functional relevance in freshwater ecosystems, a lack of knowledge about their basic ecology and genetics still exists. In the present study was given special attention to the genus *Pisidium* (Bivalvia: Sphaeriidae). Essentially, *Pisidium* systematics has been developed through morphological studies and 24 different species have been described. In Portugal only 7 are described, namely *P. amnicum, P. casertanum, P. henslowanum, P. milium, P. nitidium, P. personatum* and *P. subtruncatum*.

In this study, 36 samples were collected from three main Portuguese hydrological basins, Douro, Ave and Cávado in order to do molecular characterization of *Pisidium* in Portugal. These samples were analised for ITS1 and 16S genes. Through phylogenetic analysis, 25 samples may be classified as *P. casertanum*, 9 as *P. subtruncatum* and 2 as *P. personatum*. Genetic distances also showed some differences among samples which may indicate gene flow between rivers. Samples classified as *P. personatum* showed a high genetic distance from Genbank sequences, similar when compared values between some other *Pisidium* species. This may indicate the existence of a new species, or there is no correspondent sequence for this specie on Genbank. On the other hand those species that present similar values of genetic distance may be misidentified. The present study is a starting point for future research that should include more samples per site and from other basins and rivers. More genetic studies should be developed concerning this genus, since in Portugal some species could even be unknown while others are endangered. Furthermore population studies should be taken into account in order to develop management and conservation measures.

Keywords: Freshwater, *Pisidium*, *P. casertanum*, *P. subtruncatum*, *P. personatum*, Douro, Ave, Cávado, PCR, primers, ITS1, 16S, phylogeny, haplotype, genetic distances, conservation

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ALCI – ATP Citrate Lyase I

BI – Bayesian Inference

bp – base pair

COI – Cytochrome c Oxidase I

COII – Cytochrome c Oxidase II

DNA – Deoxyribonucleic Acid

ITS1– Internal Transcribed Spacer 1

km – Kilometer

ML – Maximum Likelihood

mtDNA – Mitochondrial Deoxyribonucleic Acid

ND4 – NADH Dehydrogenase Subunit 4

P. – Pisidium

PCR – Polymerase Chain Reaction

rDNA – Ribosomal Deoxyribonucleic Acid

U.S.A – United States of America

UV – Ultra violet

Introduction

It is already well established that declines in freshwater biodiversity are greater than in the most affected terrestrial ecosystems (Sala *et al*., 2000). It is pointed out by some authors that freshwater ecosystems may well be the most endangered ecosystems in the world (Dudgeon *et al*., 2006). In fact, aquatic habitats have higher diversity of plants and animals when compared with terrestrial ecosystems if we standardized our data by area (Dudgeon *et al*., 2006). Besides that, freshwater ecosystems provide goods and services very important for the global economy (Postel & Carpenter, 1997) as well as for the livelihoods of many people (Neiland & Bene 2008; Rebelo *et al*., 2009; Dugan *et al*., 2011). However, the exploitation of these resources for food, energy, transport, and water supply (Dudgeon *et al*., 2006), and the effects generated by climate change (Darwall *et al*., 2011) or introduction of non-native species (e.g. Bunn & Arthington, 2002; Koehn, 2004) have led to a high number of extinctions (Ricciardi & Rasmussen, 1999; Darwall *et al*., 2009).

Despite the important contribution of freshwater ecosystems to global biodiversity and the high threat that they are subject, conservation research is skewed toward terrestrial ecosystems and to more charismatic species groups, mainly birds and mammals (Clark & May, 2002). People are easily identified with those species, denominated flag-species (Dietz & Nagagata, 1985) and thus these faunal groups usually are the main targets of applied conservation measures. This view persists, despite several recent studies that demonstrate both a growing number of freshwater extinctions comprising fish, crustaceans and molluscs (Miller *et al*., 1989; Williams *et al*., 1993; Taylor *et al*., 1996; Neves *et al*., 1997) and their value on the ecosystem (Covich *et al*., 1999; Dudgeon *et al*., 2006). The lack of information in basic aspects such as distribution and conservation status of these freshwater organisms is maybe the main reason to not include them on general conservation plans (Grenyer *et al*., 2006; Rodrigues & Brooks, 2007). In one hand, both ecology and genetic studies on freshwater vertebrates are increasing and have captured some social and conservational attention nowadays; on the other, information about invertebrates is clearly insufficient (Strayer, 2006). Therefore, it is urgently necessary to increase the number of studies on freshwater invertebrates, which have a very important role on ecosystem processes and functions (Wallace & Webster, 1996; Cardinale *et al*., 2002; Jonsson & Malmqvist, 2003; Dangles *et al*., 2004). Among these groups of invertebrates we may include bivalves. Indeed, in some marine and freshwater systems, bivalves have some central roles in

ecosystem processes such as the production of shells and their burrowing and filter activities (Vaughn & Hakenkamp, 2001). They are essential, through removing particles from the water column (suspension or filter feeding), nutrients excretion and faeces and pseudofaeces biodepositition (Figure 1), to do the link between the water column and benthic compartments in freshwater habitats (Vaughn & Hakenkamp, 2001; Vaughn *et al*., 2008). This link may influence ecosystem functions and the entire food web (Vaughn *et al*., 2008).

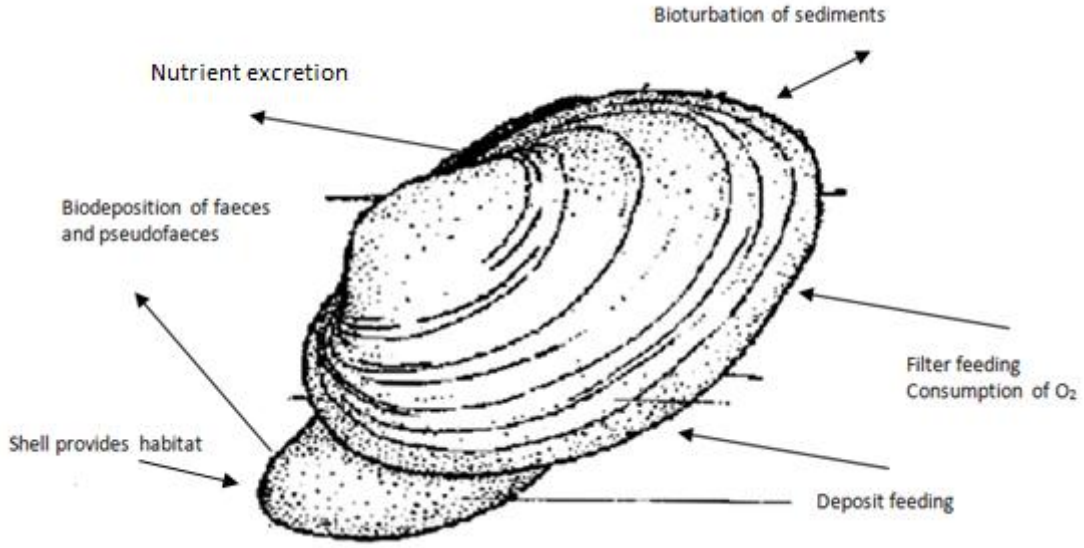

Figure. 1 Potential ecosystem functions performed by burrowing bivalves in freshwater ecosystems. (adapted from Vaughn *et al*., 2001)

Bivalves can also generate habitats for other species. Living bivalves or their empty shells offer habitat for other organisms through the provision of physical structure for colonization. This situation is particularly important in ecosystems where other hard substrata are unavailable (Gutierrez *et al*., 2003). The overall effects vary with bivalve abundance and range, community composition, environmental factors such as hydrologic conditions and temperature, as well with temporal scales (Vaughn *et al*., 2008). However, despite the relevance of bivalves in freshwater ecosystems, a lack of knowledge about their ecology and genetics still exists. It is necessary to improve the knowledge of their diversity in order to analyze and predict the effects of climate change, non-native species introductions and habitat loss on species and populations (Féral, 2002).

One way to estimate this biodiversity is using genetic markers. The interest of genetic markers rests on their ability to detect genetic variation, providing useful information

regarding phylogenetic relationships, population structure, gene flow, patterns of historical biogeography and the analysis of parentage (Sunnucks, 2000). DNA sequencing of nuclear and mitochondrial DNA genes is nowadays widely used. DNA sequencing of nuclear genes is a helpful tool to apply on genetic studies, due to their ability to detect polymorphisms and population structure (Avise, 1989). As they are easily assayed and have nonconserved spacer regions, these molecular markers are now classically used in population biology because it gives important insights in the establishment of phylogeography of a target species (Avise *et al*., 1987). In addition, mitochondrial DNA has been recently widely used in many taxonomic, population and evolutionary studies on bivalves (*eg*. Stepien *et al*., 1999; Matsumoto, 2003; Serb & Lydeard, 2003; Soroka, 2010). Their highly copy number, easily isolation and variability make them a valuable tool for many genetic studies (Lunt *et al*. 1996; Féral, 2002; Soroka, 2010). It is already known that the capacity to a species or populations to survive to natural or anthropogenic environmental changes depends on the level and kind of diversity that is available (Féral, 2002). Therefore, once the only way available to understand and study this diversity it is through genetics, it is clear the importance of molecular markers on conservation. Molecular markers enable to measure genetic differences among individuals within a species and provide important evidences about the history of species and details of its current population structure (Hilbish, 1996). Therewith, they are essential tools for better understand the diversity and which species or populations require special attention in their conservation status.

Although there is some progress and investment in genetic studies in bivalves, there is great ignorance concerning freshwater species mainly the ones from the Sphaeriidae family. The cosmopolitan species of the family Sphaeriidae, present in all kinds of lotic and lentic freshwater habitats (Burky *et al*., 2000), still have many uncertainties about their systematics. Over time, their systematics has been supported only by their life cycle, behavior and morphology, leading to an overestimation in the number of species and genera (Lee & Ó Foighil, 2003). Some authors chose to use a strikingly divergent system of classification based only on those principals where there are five different genera described: *Pisidium, Sphaerium, Musculium, Eupera,* and *Byssanodontu* (Cooley & Ó Foighil, 2000). Dreher-Mansur & Meier-Brook (1992, 2000) conducted the first cladistic analysis of sphaeriids where they propose two main clades: the Euperine, containing *Eupera* and *Byssanodonta*; and the Sphaeriine clade, with *Sphaerium, Musculium* and *Pisidium.* Still, there are many doubts of the intrageneric sphaeriinid

relationships and thus, many phylogenetic relationships have been proposed between the different genera and species (Cooley & Ó Foighil, 2000).

According to some authors (e.g. Hornbach *et al*., 1980; Dreher-Mansur & Meier-Brook, 1992; Dreher-Mansur & Meier-Brook, 2000) the Sphaeriidae family is divided into three primary genera: *Pisidium, Musculium,* and *Sphaerium*. The genus *Pisidium* contains 24 species; specimens are small (most species have less than 6 mm in shell length, with the exception of *P.amnicum* that can reach 11mm), and are cosmopolitan (Burky *et al*., 2000). The genus *Sphaerium* (10-30 mm shell length) and *Musculium* (can attain shell lengths > 20 mm) have seven and four species, respectively, and both are globally distributed (Burky *et al*., 2000; Sturm *et al.,* 2006). Species from this family are hermaphroditic and ovoviviparous. Whereas in species from *Pisidium*, juveniles are retained in marsupial sacs formed as outgrowths of the gill filaments and then released (Joyner-Matos *et al*., 2011), species from *Sphaerium* and *Musculium* have larval stages (Burky *et al.,* 1981). Therefore, broods of *Sphaerium* and *Musculium* are higher over a short period of time, when compared with *Pisidium*, requiring more time between broods (Burky *et al*., 1981). According to Holopainen and Hanski (1986) there is a high diversity both inter and intraspecific, regarding many life history traits, such as age at first reproduction, time of egg-laying, time of birth, litter size, number of litters per parent, and longevity. The most widespread and depicted species belong to the genus *Pisidium;* there are 21 species described in Europe, morphologically distinct from the other genera (*Sphaerium, Musculium*) (Holopainen & Hanski, 1986). On the other hand, morphology identification within species can be not so easy. The diversity of the environment (*e.g.* standing or current waters, size of the river, sediment composition, water chemistry, among others) contributes to the interspecific variation in shell morphology of many bivalves (Funk & Reckendorfer, 2008). In the case of *Pisidium* different studies suggest that morphology identification and taxonomy on this genus does not take into account shell shape variability within species caused by hydrologic or/and sediment composition (Funk & Reckendorfer, 2008).

In Portugal, and according to the last revision in *Pisidium* distribution conducted by Reis (2004), seven *Pisidium* species may exist. *P. amnicum* is the larger species, is rare and restricted to the Minho River (Sousa *et al.*, 2008). *P. henslowanum* is also a rare species and restricted to the Minho River. Both species require a special attention in terms of conservation since there are evidences of extensive decline in abundance and spatial distribution due to habitat degradation and effects generated by the introduction of the Asian clam *Corbicula fluminea* (Sousa *et al*., 2008). On the contrary,

P. casertanum is widespread and it is possible to find it in almost every freshwater habitat in Portugal. It can be observed preferentially in small rivers north of the Tejo River and does not raise any concern about its conservation status. *P. subtruncatum* is also widespread in rivers located north to the Tejo River. Both *P. subtruncatum* and *P. casertanum* have a short life cycle and can be easily dispersed (e.g., birds) which makes their colonization easier and faster. *P. milium* and *P. nitidium* are also well established and widespread, mainly in rivers located north to the Tejo River. *P. personatum* is easily misidentified with *P. casertanum* and can also be recorded in several rivers belonging to the Douro basin. Although widespread, this species has usually low abundance and possibly requires a special attention concerning its conservation status (Reis, 2004). It seems that all these *Pisidium* species preferentially colonize areas located north to the Tejo River; however, this situation may be a reflection of a much higher sampling effort in rivers located in the north of the country. In contrast with other bivalve species (mainly Unionidae), the Sphaeriidae family has not attained much attention on its conservational status, even while they may be facing a considerable extinction pressure. Hence, there is a great need to study such organisms, so that scientific information may contribute to reverse the decline presently shown by some species (Sousa *et al*., 2008). To fulfill this lack of information, given the great taxonomic and molecular uncertainties inside this group of bivalves and recognizing their conservational importance, the goal of the present study is to perform the molecular characterization of *Pisidium* in Portugal, as well as establish the phylogenetic relationships between species, within this genus. To accomplish this, both mitochondrial DNA (16S DNA) and nuclear sequence data (ITS1 DNA) were analyzed for the Portuguese *Pisidium* specimens collected from different populations, belonging to some of the main hydrological basins in the North of Portugal (Cávado, Ave and Douro). Therefore, we aim to fill the gap of knowledge on the status of the Sphaeriidae family in Portugal, in the wide context of biodiversity conservation.

Materials and Methods

Sampling

Individuals were collected from three main Portuguese hydrological basins: Douro, Cávado and Ave (Figure 2). In Douro basin, samples were collected from seven different rivers: Tâmega, Paiva, Ribeira das Tourinhas, Varosa, Balsemão and Fervença (Figure 2 and Table 1). In Tâmega basin were selected three different rivers, Olo, Beça and Terva, and from Paiva two sites, Borralhais and Malhada (Figure 2 and Table 1). In Cávado basin were collected samples from two rivers, Cávado River and Ribeira Salto (Figure 2 and Table 1). Finally, in Ave basin all the samples were collected from Ribeira de Moreira (Figure 2 and Table 1).

Study Area

Douro's hydrological basin is one of the biggest in the Iberian Peninsula, covering nearly 18 643 km ^{2} (Tockner *et al*., 2009). Douro River begins in Spain, Urbión, at about 2080 meters altitude and flows in Atlantic Ocean at Porto and Vila Nova de Gaia in Portugal.

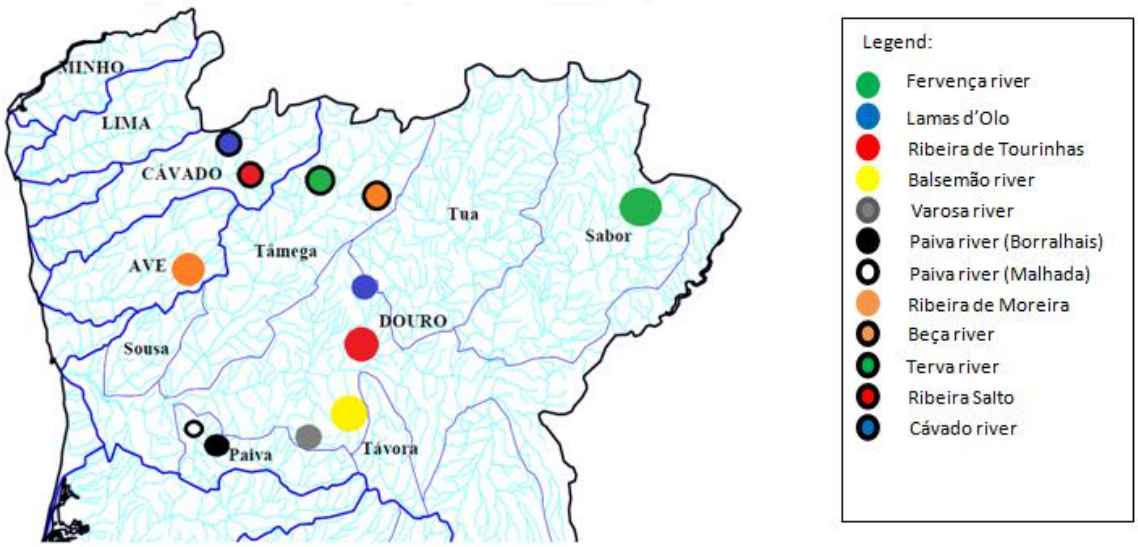

Figure 2. Hydrographic basins from North Portugal (adapted from Atlas do Ambiente Digital). Color circles represent the sites where samples were collected.

It has an overall length of 938 km splitting in three main segments: Spanish Douro (616 km), International Douro (122 km) and Portuguese Douro (200 km) (Ribeiro *et al.*,

1987). Douro has many tributaries and in this study, samples were collected from Tâmega, Paiva, Varosa and Fervença rivers. The Tâmega river rises in Ourense, Spain, flows in Entre-os-Rios and has a total length of 145 km (Fernandes, 1960) being the Rivers Beça and Terva important tributaries. The Paiva river starts at the Serra do Leomil, at approximately 1000 meters altitude (Oliveira *et al*., 1999) and has a total length of 108 km and a catchment area of 795 km2 entirely located in Portugal (Sousa *et al*., 2013). The Varosa River rises in Castro Daire and has approximately 45 km length. The Fervença River is located in Bragança, rises in the Serra da Nogueira (1300 m altitude) and has 14 km of total length. The Cávado hydrographic basin covers an area of 1600 km^2, ranging from Trás-os-Montes to Minho (Paredes, 1990); it has several important tributaries, such as the Homem River on the right bank and Rabagão River on the left bank, whose basins occupy 257 Km^2 and 246 Km^2, respectively. Almost with the same area as Cávado, nearly 1400 km^2, Ave hydrological basin has as main tributaries the Vizela River, where samples were collected, and Este River. This is one of the most polluted Portuguese rivers; however in the last years several management actions have been implemented in order to increase the overall water quality.

Molecular Techniques

Thirty-six individuals were chosen based on their variation in shell phenotype. The majority (twenty-eight) came from the Douro basin, whereas the remaining came from Ave and Cávado basins, four individuals for each basin (Table 1). Genomic DNA was extracted from ethanol (96%) preserved tissue. The whole animal body was processed with a JetQuick Spin - Kit Genomic P/DNA (Genomed Gmbh, Lohne). DNA fragments were amplified using eight different sets of primers (Table 2). The target fragments were amplified with Taq DNA Polymerase (Invitrogen) and a negative control (no template) was included in each amplification, under the conditions described on Table 2. Primers for ITS1 and 16S genes were used following Lee & Foighil (2003) once this was the most recent and complete genetic study on Sphaeriidae family. The remaining primers tested were available in the laboratory (Table 2) and have been previously used for other invertebrates. They were used to try to amplify different genes, in order to support the results. The PCR products were visualized on 2% agarose gels, under UV light, and the positive ones were sent for sequencing. Sequencing reactions were performed by Macrogen.

Table 1. Information about the collecting sites, with corresponding amplified genes and identification codes. Numbers between brackets indicate the ITS1 and 16S haplotypes.

River	Localization	Basin	Code	ITS1	16S
Paiva River	Gavião /Malhada	Douro	PS 136	X (4)	X (3)
Paiva River	Gavião /Malhada	Douro	PS 137	X (5)	X (4)
Paiva River	Gavião /Malhada	Douro	PS 138	X (4)	X (4)
Paiva River	Gavião /Malhada	Douro	PS 139	X (5)	X (4)
Paiva River	Gavião /Malhada	Douro	PS 140	X (2)	X (1)
Paiva River	Gavião /Malhada	Douro	PS 141	X (5)	X (4)
Paiva River	Borralhais	Douro	PS 302	X (4)	X (3)
Paiva River	Borralhais	Douro	PS 303	X (4)	X (2)
Paiva River	Borralhais	Douro	PS 304	X (2)	X (1)
Paiva River	Borralhais	Douro	PS 305	X (3)	X (4)
Paiva River	Borralhais	Douro	PS 306	X (4)	
Paiva River	Borralhais	Douro	PS 308	X (4)	X (3)
Paiva River	Borralhais	Douro	PS 309	X (4)	X (2)
Paiva River	Borralhais	Douro	PS 310	X (5)	
Bacia do Olo/Tâmega	Lamas de Olo	Douro	PS 449	X (4)	
Bacia do Olo/Tâmega	Lamas de Olo	Douro	PS 450	X (4)	
Balsemão River	Balsemão Penude	Douro	PS 475	X (5)	
Balsemão River	Balsemão Penude	Douro	PS 476	X (5)	
Ribeira das Tourinhas	Vila Real	Douro	PS 509	X (3)	
Ribeira das Tourinhas	Vila Real	Douro	PS 511	X (5)	
Varosa River	Tarouca	Douro	PS 546	X (5)	
Varosa River	Tarouca	Douro	PS 547	X (5)	
Fervença River	Bragança	Douro	PS 570	X (1)	
Fervença River	Bragança	Douro	PS 571	X (3)	
Beça River/Tamega	Canedo	Douro	PS 594	X (5)	
Beça River/Tamega	Canedo	Douro	PS 595	X (4)	
Terva River/Tamega	Boticas	Douro	PS 596	X (3)	
Terva River/Tamega	Boticas	Douro	PS 597	X (5)	
Ribeira de Moreira	Ponte de Pingue	Ave	PS 370	X (3)	
Ribeira de Moreira	Ponte de Pingue	Ave	PS 371	X (3)	
Ribeira de Moreira	Ponte de Pingue	Ave	PS 372	X (3)	
Ribeira de Moreira	Ponte de Pingue	Ave	PS 373	X (3)	
Ribeira Salto	Salto	Cávado	PS 592	X (5)	
Ribeira Salto	Salto	Cávado	PS 593	X (5)	
Cávado River	Frades	Cávado	PS 598	X (5)	
Cávado River	Frades	Cávado	PS 599	X (5)	

Table 2. Primers tested with respective amplification regions, sequences and PCR conditions.

Primers	Sequence (5'-3')	Amplified region	PCR conditions		
			94°C	3m	
		18s	94°C	30s	
ITS1-F [a]	GGTGAACCTGCGGAAGGATCAT	ITS1	65°C	1m	40
ITS1-R [a]	ACCCACGAGCCGAGTGATCC	5.8s	cycles		
			72°C	1m30s	
			72°C	10m	
LCo22 [b]	GGTCAACAAAYCATAARGATATTGG	COI			
HCo700 [b]	TCAGGGTGACCAAAAAAYC				
ATP-F [c]	AGCTTCTTCGACCAATTTATGAG	ACLI			
ATP-R [c]	TATGCGTGTGCTTGGTGTGCCA				
D-Loop-F [d]	CATACTCGGATTCTACCCTAGCA	D-Loop	94°C	3m	
D-Loop-R [d]	AAGGGGAACGTGTGGGCTATTTAGG		94°C	30s	
HCOI [e]	TAAACTTCAGGGTGACCAAAAAATCA	COI	45-60°C	40s-1m	35-
UCOII [f]	CAGTGGTATTGGAGGTATGAGTA		44 cycles		
HCOI [e]	TAAACTTCAGGGTGACCAAAAAATCA	COI	72°C	1m	
LCOI [e]	GGTCAACAAATCATAAAGATATTGG		72°C	10m	
MCOI20 [g]	GTCCCAATATCYTTATGRTTAGT	COII			
UCOII [f]	CAGTGGTATTGGAGGTATGAGTA				
ND4 [h]	CACCTATGACTACCAAAAGCTCATGTAGAA	ND4			
LEU [h]	CATTACTTTACTTGGATTTGCACCA				
			94°C	3m	
			94°C	30s	
16Sa [i]	ATGTTTTTGATAAACAGGCG		40-65°C	40s-1m	35-
16Sb [i]	ACGTGATCTGAGTTCAGACCGG	16S	44 cycles		
			72°C	1m30s	
			72°C	10m	

a - Gerke & Tiedemann, 2001
b - Walter *et al*., 2006
c - Giuffra *et al*., 1994
d - Unpublished
e - Folmer *et al*., 1994
f - Curole, 2004
g - Walter *et al*., 2007
h - Arevalo *et al*., 1994
i - Perez *et al*., 2005

Data analysis

Received sequences were analyzed and edited separately with Chromas Lite v.2.1.1 software (Technelysium Pty Ltd, Queensland, Australia). Each one was blasted in the NCBI database, on GenBank, to identify the most similar available sequences and to guarantee that the sequenced product was the intended. ClustalW software (Thompson *et al*., 1994) included in the program BioEdit (Hall, 1999) was used to perform the alignments. Sequences from ITS1 gene were obtained with ITS1-F primer and sequences from 16S gene with 16Sa primer (Table 2). Sequences of each gene

were aligned separately and their haplotype distribution analyzed using DnaSP v5 (Librado & Rozas, 2009).

All available GenBank sequences were downloaded and included in the analysis (Table 3). Samples for which were obtained sequences for both genes were used for an additional alignment. Firstly, two different alignments were created, one with the new ITS1 and GenBank sequences, and another with the new 16S and GenBank sequences (Table 3). Secondly, these two alignments were concatenated using Geneious 4.8.5 (Drummond *et al.*, 2009), creating a unique alignment for both fragments (i.e., ITS1+16S). For every alignment, sequences of *Eupera cubenis* and *Eupera platensis* were used as outgroups following Lee & Foighil (2003). The best-fitting models of nucleotide substitution for each alignment were chosen according to the Akaike Information Criterion (AIC), using jModelTest 0.1 (Posada, 2008). The methods chosen for phylogenetic analyses were the Bayesian Inference (BI), carried out in Mr. Bayes v.3.2 (Huelsenbeck & Ronquist, 2001), and Maximum Likelihood (ML) using RaxMl 1.3 (Silvestro & Michalak, 2012). Bayesian analysis was run for $1x10^7$ generations, saving one tree in each 1000 generations and burn-in 25%. Remaining trees were combined in a 50% majority consensus tree. ML analysis was performed with 20 runs and 500 bootstraps. To complement the ITS1 phylogenetic analyses, the ITS1 alignment was also used to create a haplotype network performed in TCS 1.21 (Clement *et al.,* 2000) software with the Maximum Parsimony method, coefficient limit of 95% and gaps treated as missing data.

Finally, the ITS1+16S alignment was used to calculate genetic distances, performed by MEGA version 5 (Tamura *et al.,* 2011) with the *p*-distance method and 500 bootstraps.

Results

Primers tested

All the PCR reactions performed with primer pairs LCO22 - HCO700, ATP-F - ATP-R, D-Loop-F - D-Loop-R, HCOI - UCOII, HCOI - LCOI and ND4 - LEU (Table 2) did not amplify any fragment. Several attempts were performed to optimize the PCR reactions by realizing several PCR reactions using different conditions, e.g., annealing temperatures, cycles, times in each step and different DNA and magnesium concentrations.

Table 3. GenBank sequences of *Pisidium* species used in the analysis with respective accession numbers for each gene and locality.

Species	GeneBank accession number		Locality	Species	GeneBank accession number		Locality
	ITS1	16S			ITS1	16S	
P. waldeni		EU559164 [a]		*P. kuiperi*		EU559123 [a]	
P. ventricosum		AY957837 [b]		*P. japonicum*	AY093532 [b]	AY093571 [b]	Japan
P. variabile	AY093530 [b]	AY957867 [b]	U.S.A	*P. insigne*		AY957838 [c]	
P. variabile (2)		AF152030 [b]	U.S.A	*P. hibernicum*	AY093522 [b]	AY093563 [b]	Germany
P. tenuilineatum		EU559163 [a]		*P. henslowanum*	DQ062584 [c]	DQ062622 [c]	
P. supinum	DQ062608 [c]	DQ062647 [c]		*P.henslowanum(2)*	DQ062583 [c]	DQ062621 [c]	
P. supinum (2)	DQ062606 [c]	DQ062646 [c]		*P. hallae*	AY093520 [b]	AY957829 [b]	Australia
P. supinum F	AY093529 [b]	AY093569 [b]	Germany	*P. hallae (2)*		AY093562 [c]	
P. subtruncatum	DQ062597 [c]	DQ062640 [c]		*P. globulare*		EU559114 [a]	
P. subtruncatum (2)	DQ062596 [c]	DQ062637 [c]		*P. ferrugineum*		AY957879 [c]	
P. subtruncatum G	AY093528 [b]	AY093568 [b]	Germany	*P. ferrugineum (2)*		AY957877 [c]	
P. sterkianum	AY093512 [b]		Argentina	*P. fallax*	AY093519 [b]	AY957816 [b]	U.S.A
P. sp.	AY093511 [b]		Ecuador	*P. fallax (2)*		AY093561 [c]	
P. pseudosphaerium		EU559147 [a]		*P. edlaueri*		EU559113 [a]	
P. personatum	DQ062595 [c]	DQ062633 [c]		*P. edlaueri (2)*		EU559111 [a]	
P. personatum (2)	DQ062594 [c]	EU559139 [a]		*P. dubium*	AY093533 [b]	AF152027 [b]	U.S.A
P. personatum G	AY093527 [b]	AY093567 [b]	Germany	*P. compressum*	AY093518 [b]	AF152029 [b]	U.S.A
P. parvum	AY093531 [b]	AY093570 [b]	Japan	*P. compressum(2)*		AY093560 [b]	U.S.A
P. obtusale		EU559138 [a]		*P. clarkeanum*		EU559105 [a]	
P. obtusale (2)		EU559137 [a]		*P. clarkeanum (2)*		EU559103 [a]	
P. nitidum	DQ062593 [c]	DQ062631 [c]		*P. cf. casertanum*		EU559102 [a]	
P. nitidum (2)	DQ062592 [c]	DQ062630 [c]		*P.cf.casertanum(2)*		EU559098 [a]	
P. nitidum F	AY093526 [b]	AY093566 [b]	Germany	*P. casertanum*	DQ062581 [c]	DQ062619 [c]	
P. nipponense	AY093525 [b]	AY093565 [b]	Japan	*P. casertanum (2)*	DQ062576 [c]	DQ062615 [c]	
P. nevillianum		EU559132 [a]		*P. casertanum G*	AY093515 [b]	AY093557 [b]	Germany
P. moitesserianum	DQ062591 [c]	DQ062628 [c]		*P. casertanum NA*	AY093516 [b]	AY093558 [b]	U.S.A
P.moitesserianum(2)	DQ062589 [c]	DQ062626 [c]		*P. annandalei*		EU559090 [a]	
P. milium	DQ062588 [c]	DQ062625 [c]		*P. amnicum*	DQ062574 [c]	DQ062610 [c]	
P. milium (2)	AY093524 [b]	AF152028 [b]	U.S.A	*P. amnicum (2)*	DQ062573 [c]	DQ062609 [c]	
P. milium F	AY093523 [b]	AY093564 [b]	Germany	*P. amnicum F*		AY093572 [b]	
P. maasseni		EU559131 [a]		*P. adamsi*	AY093548 [b]	AY093556 [b]	U.S.A
P. maasseni (2)		EU559128 [a]		*P. adamsi (2)*	AY093513 [b]	AF152031 [b]	U.S.A
P. lilljeborgi	DQ062587 [c]	DQ062624 [c]		*E. cubensis*	AY093501 [b]	AY093549 [b]	Cuba
P. lilljeborgi (2)	DQ062586 [c]	DQ062623 [c]		*E. platensis*	AY093502 [b]	AF152026 [b]	Argentina
P. lilljeborgi F	AY093521 [b]		Russia				

a - Shultheiss *et al*., 2008

b – Lee & Foighil, 2003

c – Steiner, 2005

Although all the efforts, it was impossible to amplify any fragments. PCR reactions performed with primer pair 16Sa-16Sb (Table 2) only amplified sequences of twelve individuals from Paiva River, six from Borralhais and six from Malhada sites. Despite many attempts to overcome this problem, using different PCR conditions, once again, it was not possible to solve the overall problem.

ITS1

The ITS1 alignment consisted of seventy-five sequences of *Pisidium* specimens and two outgroup specimens (Table 1 and 3). Aligned sequences had a total length of 433 bp, with seven polymorphic and seven parsimony informative sites and relatively high levels of nucleotide variability (Hd = 0.716, π = 0.0056, within *Pisidium* new sequences). The best model used in this data set was GTR+G. One consensus tree was recovered for each analysis (ML and BI). Since the results from BI and ML were congruent, only BI is represented in Figure 3. The new sequences clustered inside three main Groups: A, B and C (Figure 3). Group A, moderately well supported for the BI (78%), is divided in two groups, one containing PS 141 and 304 and other including all *P. personatum* sequences from the GenBank. Group B had no support and it contains the GenBank sequences of *P. casertanum*, *P. adamsi, P. variable, P. fallax, P. compressum and P. hallae* and the vast majority of the new sequences (n=28) that further cluster into groups 1, 2 and 3 (Figure 3). Moreover, group 1 and 3 formed two well supported different clusters (91% and 81%, respectively; Figure 3) with no association to any GenBank sequences. Finally, the remaining nine new sequences represented for visualization purposes by group 3 in the figure, do not form any supported cluster.

The relationships of the new haplotypes are shown in Figure 4, where the six new haplotypes retrieved correspond to haplotypes 1-6. Individuals corresponding to group 2 in the phylogeny (Figure 3) and GenBank sequences of *P. casertanum* 2 and *P. casertanum* G all share the same haplotype (number 1). Individuals corresponding to groups 1 and 3 in the phylogeny (Figure 3) correspond to haplotypes number 2 and 3, respectively. These two haplotypes differ only in one mutation from haplotype 1 (Figure 4). Individuals from group 4 in the phylogeny (Figure 3) and GenBank sequences of *P. subtruncatum* share the same haplotype, represented by number 4. On the other hand,

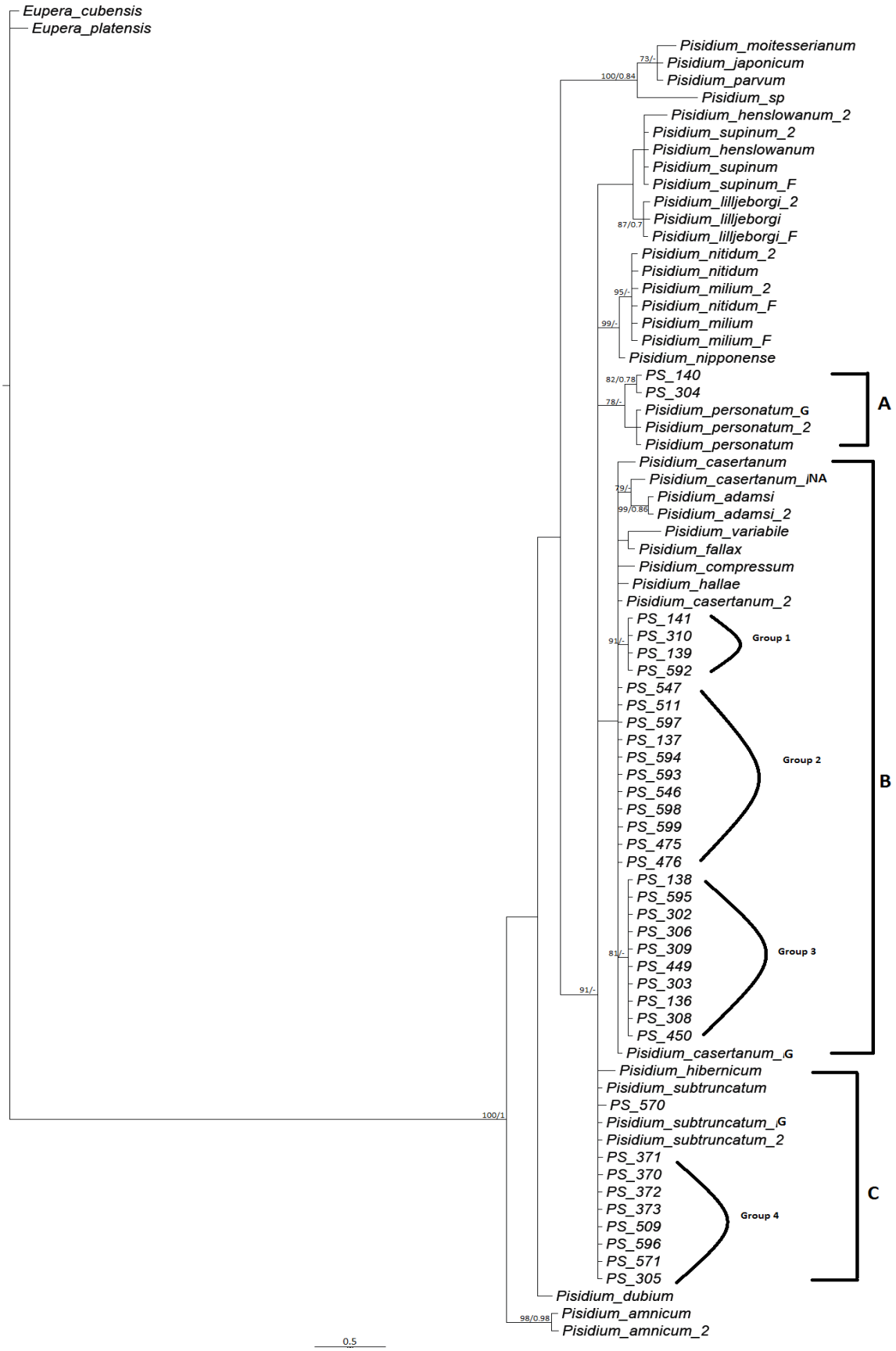

Figure 3. Phylogenetic tree obtained by Bayesian Inference of the ITS1 fragments. Support values (%) are given as Bayesian posterior probability/Bootstrap support. Maximum Likelihood support values are given as 0 to 1. Bootstrap support < 0.7; Bayesian posterior probability < 95%.

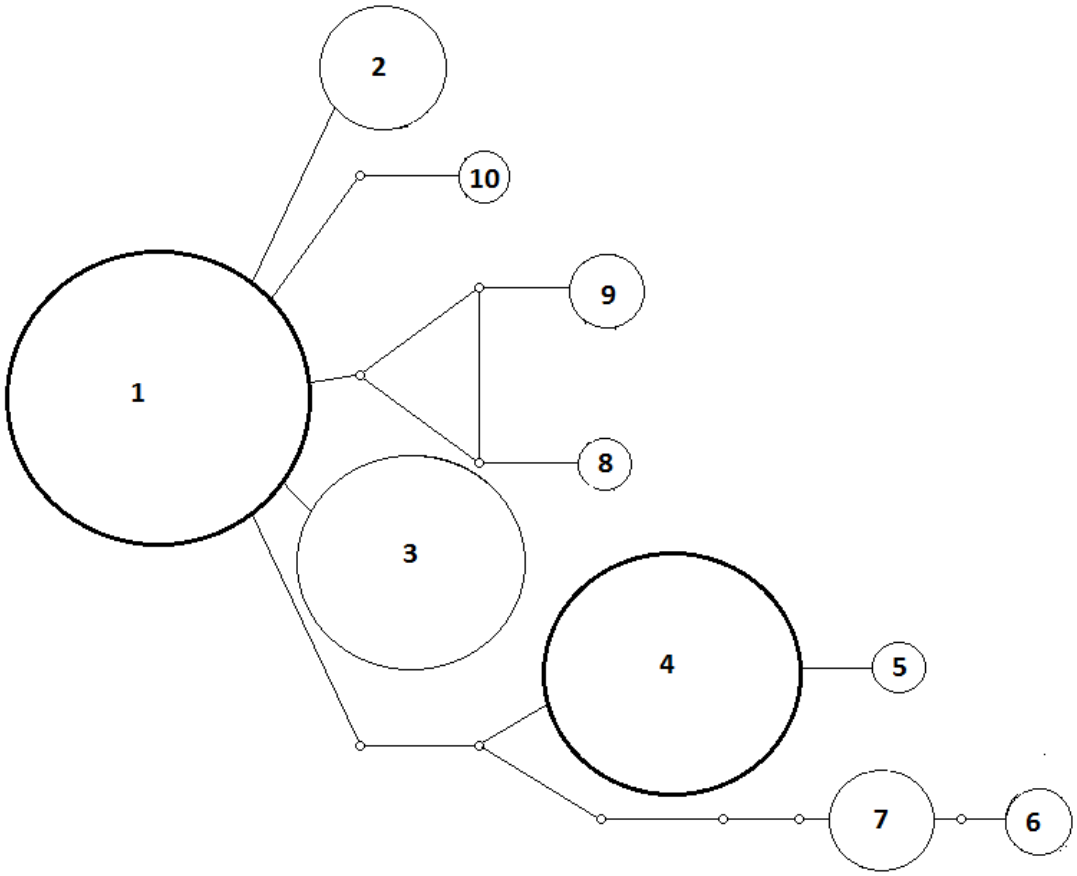

Figure 4. Haplotype (Maximum Parsimony) network done with 95% connection limit. Circle sizes are proportional to the number of individuals sharing the same haplotype. Dashes represent mutated positions. New *Pisidium* sequences are represented by numbers 1 to 6.

PS 570 is a unique haplotype, represented by number 5, with one mutation from haplotype 4. PS 140 and PS 304 correspond to haplotype number 6 that differ in a single mutation from *P. personatum* haplotype number 7. Haplotype number 9 corresponds to GenBank sequences of *P. adamsi* and haplotypes number 8 and 10 correspond to *P. casertanum* NA and *P. casertanum*, respectively.

16S

The 16S alignment consisted of seventy-six sequences from *Pisidium* specimens and two outgroup specimens (Table 1 and 3). Aligned sequences had a total length of 443 bp, with forty six polymorphic and thirty one parsimony informative sites and high levels of nucleotide variability (Hd = 0.924, π = 0.031, within *Pisidium* new sequences). The best-fit model used in this data set was GTR+I+G model. Once more, only one consensus tree was recovered for each analysis (ML and BI). As the results from BI

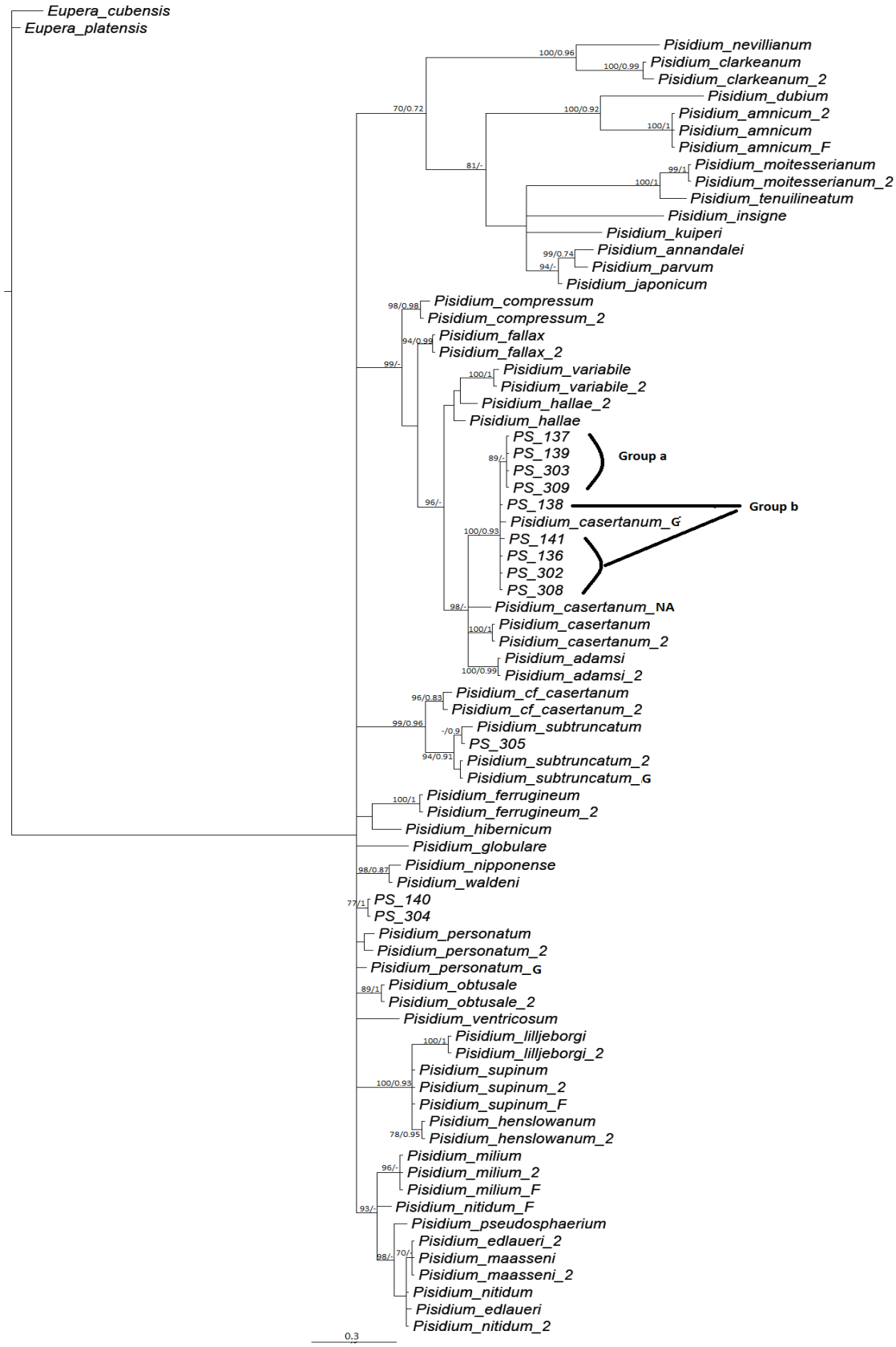

Figure 5. Phylogenetic tree obtained by Bayesian analysis of the 16S fragments. Support values (%) are given as Bayesian posterior probability/Bootstrap support. Maximum Likelihood support values are given as 0 to 1. Bootstrap support < 0.7; Bayesian posterior probability < 95%.

and ML were congruent, only BI was represented (Figure 5). The new *Pisidium* sequences PS 140 and PS 304, formed a single moderately well-supported group (77%; 1) with no closer affinity to any GenBank sequence. On the other hand, all the remaining new *Pisidium* sequences formed a highly supported clade (100%, 0.93) with *P. casertanum* G. Moreover, these sequences cluster into two groups, a and b (Figure 5), where b has a high support (BI 89%). Finally, PS 305 cluster inside a well-supported clade (94%, 0.91) composed by all the *P. subtruncatum* individuals retrieved from GenBank.

Combined (ITS1+16S)

The ITS1+16S alignment consisted of forty-nine sequences of *Pisidium* specimens and two outgroup specimens (Tables 1 and 3). Aligned sequences had a total length of 1041 bp and the best model used in this data set was GTR+G+I. One consensus tree was recovered for each analysis (ML and BI) and once again, since the results from BI and ML were congruent, only BI is represented (Figure 6). New *Pisidium* sequences PS 140 and PS 304 and all *P. personatum* GenBank sequences formed a well-supported clade (92%, 0.76). This clade is further divided in two well supported branches, one with PS 140 and PS 304 (100%; 0.99) and the other with *P. personatum* GenBank sequences (97%). Regarding *P. subtruncatum*, they all clustered together with PS 305 in a well-supported clade (100%, 1). All the remaining new sequences formed a highly supported clade (99%, 0.94) that also included *P. casertanum* G from GenBank. Moreover, the new sequences are divided in groups I, II and III, but only two were well supported (Figure 6). Samples from group I formed a well-supported one (100%; 0.96) whereas group II formed a well-supported clade in BI (91%). Pairwise genetic distances (*p*-distances) showed high similarity between samples from groups I, II and III and *P. casertanum* G, with values from 0 to 0.9% (Table 4). Values of pairwise genetic distances were higher when compared *P. casertanum* G and the new sequences with the remaining *P. casertanum* sequences and *P. adamsi*, over 1.8% (Table 4).

Pairwise genetic distances for *P. subtruncatum* are shown in Table 5; low values were depicted between PS 305 and GenBank sequences of *P. subtruncatum*. Moreover these values were lower between PS 305 and *P. subtruncatum* 2 and *P. subtruncatum* G, 0.2% (Table 5), than between PS 305 and *P. subtruncatum* sequences.

Finally, the pairwise genetic distances for *P. personatum* are shown in Table 6. When comparing the samples PS 140 and PS 304 with GenBank sequences of *P.*

personatum, the lowest value of genetic distance was obtained with GenBank sequence *P. personatum* G, 1.7% (Table 6). Genetic distances between *P. personatum* F and the other two *P. personatum* sequences were lower than 0.6% (Table 6). Genetic distances between GenBank sequences of *P. casertanum* and *P. adamsi* and *P. nitidium* and *P. milium* (Table 7) were the lowest: 1.7% and 1.6%, respectively; while genetic distances between the remaining GenBank sequences were higher than 3.6% (Table 7).

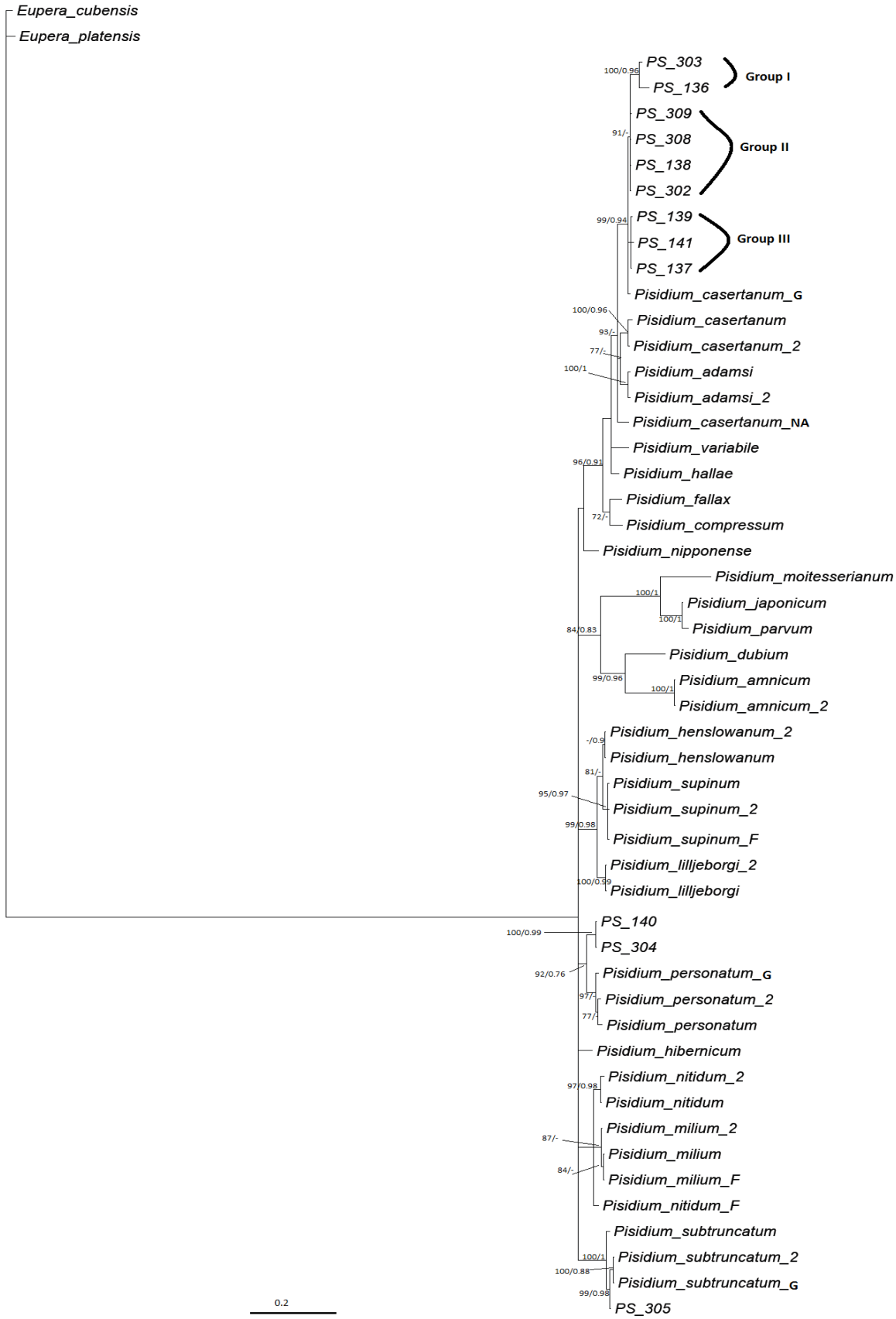

Figure 6. Phylogenetic tree obtained by Bayesian Inference of ITS1+16S fragments. Support values (%) are given as Bayesian posterior probability/Bootstrap support. Maximum likelihood support values are given as 0 to 1. Bootstrap support < 0.7; Bayesian posterior probability < 95%.

Table. 4 Pairwise genetic distances (*p*-distances) (%) between new *Pisidium* sequences and GenBank sequences of *Pisidium casertanum* and *Pisidium adamsi*.

	PS 136	PS 303	PS 138	PS 302	PS 308	PS 309	PS 137	PS 139	PS 141	*P. casertanum* G	*P. casertanum* NA	*P. casertanum*	*P. casertanum*2	*P. adamsi*	*P. adamsi*2
PS 136															
PS 303	0.3														
PS 138	0.6	0.5													
PS 302	0.6	0.5	0												
PS 308	0.6	0.5	0	0											
PS 309	0.8	0.4	0.1	0.1	0.1										
PS 137	0.8	0.6	0.3	0.3	0.3	0.2									
PS 139	0.9	0.8	0.4	0.4	0.4	0.3	0.1								
PS 141	0.9	1	0.4	0.4	0.4	0.5	0.3	0.2							
P. casertanum G	0.9	0.8	0.2	0.2	0.2	0.3	0.3	0.4	0.4						
P. casertanum NA	2.5	2.4	1.8	1.8	1.8	1.9	1.9	2	1.8	1.8					
P. casertanum	2.7	2.6	2	2	2	2.1	2.1	2.3	2	2	1.9				
*P. casertanum*2	2.4	2.3	1.7	1.7	1.7	1.8	1.8	1.9	1.7	1.7	1.6	0.3			
P. adamsi	2.5	2.4	1.8	1.8	1.8	1.9	1.9	2	1.8	1.8	1.8	1.8	1.5		
*P. adamsi*2	2.5	2.4	1.8	1.8	1.8	1.9	1.9	2	1.8	1.8	1.8	1.8	1.5	0.2	

Table. 5 Pairwise genetic distances (*p*-distances) (%) between new *Pisidium* sequence PS 305 and GenBank sequences of *Pisidium subtruncatum*.

	PS 305	*P.subtruncatum*G	*P.subtruncatum*	*P.subtruncatum*2
PS 305				
*P.subtruncatum*G	0.2			
P.subtruncatum	0.5	0.7		
*P.subtruncatum*2	0.2	0	0.7	

Table. 6 Pairwise genetic distances (*p*-distances) (%) between new *Pisidium* sequences and GenBank sequences of *Pisidium personatum*.

	PS 140	PS 304	*P. personatum*G	*P. personatum*	*P. personatum*2
PS 140					
PS 304	0				
*P. personatum*G	1.7	1.7			
P. personatum	2	2	0.6		
*P. personatum*2	2	2	0.5	0.5	

Table. 7 Pairwise genetic distances (*p*-distances) (%) between six species on GenBank.

	P.casertanum	*P.adamsi*	*P. personatum*	*P.subtruncatum*	*P.nitidium*	*P.milium*
P.casertanum						
P.adamsi	1.9					
P. personatum	3.9	4				
P.subtruncatum	5.1	5.2	3.5			
P.nitidium	4.3	4.5	3.5	4.8		
P.milium	4.7	4.9	3.6	4.5	1.6	

It is important to refer that eight samples from the Minho River were also used in this study. However, and despite positive PCR reactions (with ITS1 primers; Table 2) none of the sequences were possible to analyze. Different PCRs with different temperatures, cycles, time in each step, and DNA concentrations were performed but the problem remained. Even when the PCR bands were cut directly from the agarose gel, the sequences were not good enough for the inclusion in the analyses.

Regarding the COI fragment, when the primer pair MCOI20 – UCOII (Table 2) was tested, more than one fragment was amplified resulting in more than one band observed on agarose gel under UV light. More PCRs were conducted with different annealing temperatures, cycles, times in each step and different DNA and magnesium concentrations. Although all the efforts it was not possible to obtain any sequence.

Discussion

Genetic studies on the genus *Pisidium* are scarce (e.g. Dreher-Mansur & Meier-Brook, 1992 and 2000; Cooley & Foighil, 2000; Lee & Foighil, 2003) with the present study including for the first time the Portuguese populations. As a preliminary study, this work aims to promote future research on *Pisidium* in Portugal and to draw some conclusions about the number of species that occur in three main Portuguese hydrological basins. The results showed that all the new samples analyzed belong to species described by Reis (2004). In more detail, the results obtained by the ITS1+16S analysis (consensus tree shown in Figure 6) show that all the samples corresponding to groups I, II and III are associated to *P. casertanum*. Although only twelve samples were successfully sequenced for both gene regions (Table 1), these results seem to also be valid for the remaining individuals, only sequenced for ITS1, and that formed groups 1, 2 and 3, as seen in both ITS1 gene tree (Figure 3) and haplotype network (Figure 4). Thus, all the samples corresponding to groups 1, 2 and 3 do seem to belong to *P. casertanum*. Moreover, the pairwise genetic distances revealed further interesting results; unfortunately, it is only possible to know the collected localities from two of the four published available *P. casertanum* sequences: *P. casertanum* G is from Germany and *P. casertanum* NA from North America. As expected, Portuguese samples are more similar to the German individual than to the American one, due to their geographic proximity, as seen by the genetic distance values (Table 4). Additionally, there was no relation between haplotypes and populations (rivers) or basins among the new Portuguese *Pisidium* individuals (Figure 4 and Table 1), which may indicate the presence of gene flow between rivers. This lack of geographic concordance may be explained by the great dispersal capacity of *P. casertanum* (Mackie, 1979). Indeed, juveniles can be dispersed through aquatic insects, amphibians, and also survive the ingestion by aquatic birds, which may allow their high dispersion (Burky *et al*., 2000). Moreover, their ability to survive in a great variety of habitats, with all types of standing and running waters with variable conditions (Saunders & Rung 1990) and the short life cycle with high reproduction rate (Burky *et al.,* 1981), makes the specie's global distribution possible (Burky *et al*., 2000). In fact, this *Pisidium* species is probably the most widespread species inside this genus occurring throughout Eurasia, Africa, Australia, and South, Central and North America (Clarke, 1973). Also in Portugal, *P. casertanum* is described as the most common and widespread species (Reis, 2004); the new results presented here are consistent with this information as 69.4% of all

samples turned out to be *P. casertanum* with this species being only absent in the Ave basin (probably due to the low number of specimens analyzed in this basin).

Regarding *P. subtruncatum*, unfortunately, PS 305 was the unique sample for which both genes were sequenced and that came associated to *P. subtruncatum* (Figure 6). However, through ITS1 gene consensus tree (Figure 3) and haplotype network (Figure 4) it is possible to infer that sample PS 570 and all the other ones belonging to group 4 (Figure 3), may be classified as *P. subtruncatum*. Assuming this, from the nine samples analyzed of this species for the ITS1, only two different haplotypes were retrieved (Figure 4). Due to the small number of samples collected and their geographical locality, the characteristics of the gene sequenced, among others, it is not possible to make more assumptions regarding the Portuguese *P. subtruncatum*. In other hand, it is not unexpected to observe that the second most represented species in the present study is *P. subtruncatum*, since this species is also one of the most widespread in Portugal (Reis, 2004). As *P. casertanum*, *P. subtruncatum* is one of the most tolerant (i.e. abiotic factors such as conductivity and dissolve oxygen) bivalve species in Europe and may live in a high variety of aquatic environments (Kuiper & Wolf 1970; Funk & Reckendorfer, 2008). The comparison of genetic distances (Table 5) between PS 305 and the *P. subtruncatum* GenBank sequences reveals that the lower value is obtained between *P. subtruncatum* G and *P. subtruncatum* 2. Sadly, only the locality of *P. subtruncatum* G is known: Germany. However, as the genetic distances between *P. subtruncatum* G and *P. subtruncatum* 2 is 0% (Table 5) it may be inferred that the *P. subtruncatum* 2 sequence could be from the same or a near location.

Regarding *P. personatum*, our results show that samples PS 140 and PS 304 may be classified as *P. personatum* (Figure 6) which again is in accordance with Reis (2004) that has described the species in rivers from the Douro basin. However, when looking in more detail to both the phylogenetic analysis and genetic distances values for this species (Table 6 and 7), conclusions may be different. PS 140 and PS 304 form a well-supported group within the species clade (Figure 6), with genetic distance values ranging from 1.7% to 2% between these and the GenBank sequences (Table 6). Again, information on the locality of the GenBank sequences is only available for one of them: *P. personatum* G that was collected from Germany. Moreover, when comparing genetic distances values between related species such as *P. casertanum* and *P. adamsi* or *P. nitidium* and *P. milium,* values are very similar: 1.9% and 1.6%, respectively (Table 7). PS 140 and PS 304 have a value of genetic distance of 1.7% when compared with the

closest *P. personatum* individual (Table 6). This situation may indicate that the Portuguese samples are actually a new species related to *Pisidium personatum* or being not new to science, there are not yet available GenBank sequences for this species. On other hand, this result may be explained by the misidentification of the *P. personatum* GenBank sequences. Sequences of *P. casertanum* and *P. adamsi* could actually belong to a unique species and the same may be true for *P. nitidium* and *P. milium*. Several (morphological) forms have been published as distinct species, but in most cases, they are probably merely ecological forms (Kuiper *et al.,* 1989). Actually, a study developed by Funk & Reckendorfer (2008) corroborates that conclusion as their shell morphological work on *P. subtruncatum* populations demonstrates that shell characters are related to abiotic factors such as hydrology, sediment composition and water chemistry. In this scenario, PS 140 and PS 304 could correspond in fact to *P. personatum* presenting a high value of genetic distance from the other *P. personatum* due to the geographic distance (Portugal/Germany). In fact, the Iberian Peninsula is well known for its endemisms (e.g. Gómez-Campo *et al*., 1984; Carranza & Amat, 2005; Gómez & Hunt, 2006) as it was one of the most important Pleistocene glacial refugia in the European subcontinent (Hewitt, 1999 and 2001). Many authors verified the existence of many endemism in both Iberian plants and animals (e.g. Gómez-Campo *et al*., 1984; Doadrio, 1988; Saiz *et al*., 1998; Ribera, 2000; García-Barros *et al*., 2002; Reis & Araujo, 2009), indirectly indicating long-term survival and differentiation (Gómez & Hunt, 2006), leading to high genetic differentiation between Iberian species from the rest of Europe.

The present study is pioneer, since nothing has been done in this topic in Portugal; even, a very small number of studies are available worldwide. As such, much more work is necessary to obtain better and more assertive conclusions. Unfortunately, it was not possible to obtain amplified fragments with 16S primers for all the samples, which for sure would allow more confident conclusions. Samples from the Minho basin were also subject to several 16S PCR attempts without success; with those results would be possible to cover all the basins where *Pisidium* were described and so corroborate, or not, Reis (2004) study. Additionally, would be important to obtain more samples per site, mainly to better understand the dispersion mechanisms of different *Pisidium* species and to understand their populations' genetics. In fact, it is questionable that only seven *Pisidium* species are present in Portugal, as in many other European countries more species have been described. For example, in France at least eleven *Pisidium* species were described (Mouthon & Charvet, 1999), eleven in

Iceland, twenty three in Sweden (Kuiper *et al*., 1989), fourteen in Ireland and sixteen in Great Britain (Anderson, 2005).

A curious result was acquired: after many attempts to get positive PCR amplifications for cytochrome c oxidase complex genes it was only possible to achieve a result when using MCOI20-UCOII primer pair (Table 2) that amplifies the COII gene. Despite the lack of good sequences obtained in this study (from those PCRs) it can serve as a starting point to explore and better optimize the PCR conditions in order to get good sequences for future analysis. The study of mitochondrial DNA sequences has become the main method for a wide range of studies including taxonomy and evolution. Many aspects of the structure and evolution of mitochondrial DNA have made it a valuable evolutionary tool (Lunt *et al*., 1996), such as its ease of isolation, lack of recombination, high copy number, and the diversity of mutational rates in different regions within the molecule, contrasting with the conserved ones (Moritz *et al*., 1987; Harrison, 1989; Simon, 1991; Wolstenholme, 1992).

Overall, the abundance of *Pisidium* species has been declining in Europe and North America (Vaughn *et al.,* 2008; Sousa *et al.,* 2008). Their importance on the ecosystems is high and their management and conservation should be taken in account in future studies. Their burrowing and filtering activity makes these individuals essential for important ecosystem functions that may influence the entire food web (Vaughn & Hakenkamp, 2001; Vaughn *et al*., 2008). Genetics can be a great tool for management and conservation issues. Maintenance of biodiversity is one of the most important current concerns of humankind to guarantee their survival (Frankham *et al*., 2002). As genetic diversity is the basis of evolutionary potential of a species to respond to environmental changes, this becomes an essential keystone in conservation genetics (Toro & Caballero, 2005). Thereby, it is important to go further on the study of these species, comprehend their spatial and temporal distribution, and monitoring and developing genetic studies on the different populations. Since genetics studies at both the family (Sphaeriidae) and genus (*Pisidium*) level are at an early stage, they should also be combined with morphologic studies. This approach could give more supportive conclusions about each species and prevent misidentification. Moreover, and since *Pisidium* species are declining in Portugal (and Europe) due to human threats that include loss of habitat, pollution, climate change and the introduction of invasive species (Reis, 2004; Sousa *et al.* 2008), an intervention to counter this tendency is urgently needed.

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