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**Diversity and distribution of patellid limpets along the
southwestern African coast (Benguela current)**

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Abstract

Limpets are keystone species on the intertidal communities because of their constant grazing, but their diversity and taxonomy are poorly assessed, particularly along the African shores. In this study specimens of the Patellidae family collected along the southwestern coast of Africa and from north Atlantic were barcoded for the mitochondrial gene cytochrome c oxidase subunit I (COI). Genetic data and literature surveys were used to generate distribution maps of the identified species, to understand the range of each species within these regions. Phylogenetic analyses based on available GenBank sequences were also conducted to further explore species relationships. From the samples collected, 72 DNA sequences were obtained, of which 9 different species of the family Patellidae were identified. At least one species of each genus was recognized, in addition to 2 Siphonariidae and 1 Fissurellidae limpets. *Patella* was only found in the North Atlantic. *Cymbula* and *Helcion* were restricted to Southern Africa, with *Cymbula safiana* being the only species with a distribution reaching north of Africa. Only one *Scutellastra* was identified in Africa, but we cannot confirm through genetic data its widest distribution, documented to reach from South Africa to Australia. Multivariate analysis showed important regional differences in species composition along the Eastern Atlantic coast. Three different groups were recognized: 1) South Africa and Namibia; 2) Angola and Tropical Africa; and 3) Northeast Atlantic and Mediterranean Sea. Phylogenetic analyses are in line with previous studies. *Patella* genus forming a well-supported clade, with *Cymbula* and *Helcion* sharing the same branch as sister taxa, and *Scutellastra* subdivided in paraphyletic biogeographic lineages. One lineage formed a clade with *Cymbula* and *Helcion*. We can conclude that are still needed a revision on the taxonomy and distribution of the Patellidae family, particularly on the African coasts.

Keywords: Patellogastropoda, DNA barcoding, “true” limpets, phylogeny

Resumo

As lapas da ordem Patellogastropoda, ou verdadeiras lapas, são consideradas espécies-chave nas comunidades intertidais devido a diversos aspectos da sua ecologia, mas a alta plasticidade e convergência das características morfológicas, trazem desafios na taxonomia do grupo e identificação das espécies. Mesmo assim, os caracteres morfológicos foram o único método disponível por muitos anos para a identificação das espécies, mas sempre carente de resolução e consenso entre os autores. As primeiras monografias taxonômicas mundiais foram criadas usando uma combinação de morfologia de concha e caracteres da rádula, porém apresentando numerosas divergências. Com a consciência de que a morfologia simplesmente não era suficiente na taxonomia deste grupo, os estudos tentaram englobar múltiplas características físicas. Apesar de vários estudos mais recentes utilizando técnicas moleculares, como o “DNA barcoding”, é correto afirmar que é essencial para trazer mais esclarecimentos sobre o assunto, particularmente em zonas historicamente pouco estudadas como a costa africana.

Assim, os objetivos desta tese são melhorar o conhecimento relativamente à diversidade e distribuição de lapas na costa africana e comparar comunidades do Atlântico norte e sul. Espécimes da família Patellidae recolhidos ao longo da costa sudoeste da África e do Nordeste Atlântico foram analisados através de barcoding do gene mitocondrial do citocromo c oxidase subunidade I (COI). Foi realizada uma análise filogenética englobando todas as espécies identificadas desta família de lapas. Posteriormente, foram gerados mapas de distribuição das espécies identificadas, para entender a distribuição de cada espécie dentro dessas regiões. Através da extração de DNA e barcoding das amostras recolhidas foram obtidas 72 sequências de DNA. Foram identificadas 9 espécies diferentes da família Patellidae, bem como 2 espécies diferentes da família Siphonariidae e 1 da família Fissurellidae. Dentro da família Patellidae estavam representados os quatro géneros, com 3 espécies de *Cymbula*, 1 de *Helcion*, 1 de *Scutellastra* e 4 de *Patella*. Com base nos mapas de distribuição gerados, o género *Patella* só é encontrado no Atlântico Norte. *Cymbula* e *Helcion* restringem-se ao Sul de África, com *Cymbula* a chegar até Angola, sendo a *Cymbula safiana* a única espécie com uma distribuição atingindo o norte de África. Apenas uma espécie de *Scutellastra* foi amostrada, na costa da África Atlântica, portanto não podemos confirmar por meio de dados genéticos a sua distribuição mais ampla, que se sabe estender da África do Sul,

incluindo Angola, à Austrália. Em relação às espécies da família Siphonariidae apenas *Siphonaria capensis* apresentou resultados distintos dos já conhecidos. Foi identificada geneticamente nas Ilhas Canárias e Angola, locais onde não havia registo de ocorrências, tanto morfológica como geneticamente. Métodos de análise multivariada foram utilizados no sentido de compreender as diferenças em termos de composição de espécies ao longo da costa do Atlântico Leste. Foi compilada informação de base de dados sobre a presença/ausência das espécies em estudo. Foram detetados três grupos distintos: 1) África do Sul e Namíbia; 2) Angola e África Tropical e 3) Atlântico Norte e Mar Mediterrâneo.

Foi gerada uma análise filogenética da família Patellidae com base em todas as sequências do gene COI da referida família publicadas no GenBank. Estas sequências e as obtidas experimentalmente foram alinhadas e o alinhamento usado para reconstruir árvores filogenéticas, uma Bayesian e uma Maximum Likelihood (ML). Os resultados do ramo *Patella* são consistentes com os obtidos por outros estudos, com exceções. *Patella caerulea* é aqui apresentada como táxon irmão do grupo formado por *Patella candei* das Ilhas Canárias e Selvagens e *Patella lugubris* de Cabo Verde, e o ramo formado pelos três é depois grupo irmão da *P. candei* dos Açores. Enquanto em outros estudos a *P. candei* das Canárias e Selvagens aparece agrupada com a *P. lugubris*, mas aparecem irmãs da *P. candei* dos Açores, Madeira e Desertas e só então todas formam um ramo com a *P. caerulea*. O suporte para este grupo é alto na árvore Bayesian, mas menores na ML. Os géneros *Cymbula* e *Helcion* formaram um ramo com elevado valor de probabilidade posterior e “bootstrap”, em linha com outros estudos, e também o facto de ambos serem restritos do Sul de Africa. Relativamente ao género *Cymbula*, a espécie *Cymbula compressa* formou um grupo com a espécie *Nacella concinna*, que pertence a uma família diferente. A hipótese sugerida é que as sequências na base de dados GenBank estão mal identificadas. Noutros estudos que incluem sequências de genes diferentes, como o 12S e o 16S, a espécie aparece, como esperado, como irmã de *Cymbula miniata*. Portanto, as sequências COI identificadas como *C. compressa* provavelmente pertencem a alguma espécie da família Nacellidae e não da família Patellidae. O género *Scutellastra* pode ser dividido em três subgéneros parafiléticos, apoiados com valores de probabilidade posterior/“bootstraps” muito altos e também por outros estudos filogenéticos. Esses subgéneros são correspondências diretas à sua distribuição geográfica, que são: Sul de Africa, Austrália e Indo-Pacífico. É também aquele com variações entre os estudos filogenéticos e de difícil comparação, uma vez que nem todos os estudos incluem as

mesmas espécies e a maioria dos estudos realizados utilizaram os genes 12S e 16S. Mesmo assim, os nossos resultados corroboram os obtidos a partir dos genes 12S e do 16S, que mostram (*Scutellastra* I) *Scutellastra granularis* e *Scutellastra miliaris* formando um ramo irmão com *Scutellastra argenvillei*. Por outro lado, (*Scutellastra* III) *Scutellastra flexuosa* é claramente parafilética, aparecendo como táxon irmão de *S. optima*, num ramo que inclui igualmente *Scutellastra exusta*, e novamente como outgroup desta linhagem. Esta situação pode ser explicada pelo fato de serem de regiões diferentes e provavelmente pertencerem a espécies diferentes. Apesar de vários estudos mostrarem *Scutellastra* como um gênero parafilético, não foi realizado nenhum trabalho para alterar a sua taxonomia com objetivo a que esta seja correspondente às várias diferenças observadas geneticamente.

Com base nos resultados apresentados é necessário ainda uma profunda análise da família Patellidae utilizando métodos moleculares combinando diferentes genes e fazendo uma amostragem mais ampla de todas as regiões conhecidas com ocorrência destas lapas. Mesmo no caso do gênero *Patella* que é dos mais estudados tanto genética como morfologicamente ainda se encontra algumas divergências entre estudos continuando a sua caracterização incompleta. Depois ainda temos os gêneros *Cymbula*, *Helcion* e *Scutellastra* que se encontra muito pouco ou mesmo nenhum estudo focando todo o gênero, apenas algumas espécies. Devido á ampla distribuição de *Scutellastra* torna-o, talvez, o mais desafiante, mas que deveria ter também um foco maior no estudo da sua relação filogenética.

Palavras-chave: Patellogastropods, “DNA barcoding”, “verdadeiras” lapas, filogenética

Introduction

Patellogastropods, known as the “true” limpets, are one of the most abundant and significant gastropod groups in rocky shores around the world from tropical to polar regions (Branch, 1985; Nakano and Ozawa, 2004, 2007; Nakano and Sasaki, 2011; Kallouche *et al.*, 2018). They play an important role on intertidal ecosystems mainly for the grazing behavior, as it is a process that controls the growth and diversity of other species (Branch *et al.*, 1992; Gray and Hodgson, 1997; Dedola, 2011; Burgos-Rubio *et al.*, 2015; Henriques, Delgado and Sousa, 2016; Casal *et al.*, 2018). Limpets graze mostly on microalgae, diatoms and spores of macroalgae, defining and controlling the abundance of macroalgae and their space-competitors such as barnacles (Branch, 1971; Arrontes *et al.*, 2004; Marzinelli *et al.*, 2012; Burgos-Rubio *et al.*, 2015). Several studies show that removing limpets from the environment increase substantially the populations of the algae (Branch, 1971, 1975a; Marzinelli *et al.*, 2012), as the populations of barnacle decrease (Arrontes *et al.*, 2004; Burgos-Rubio *et al.*, 2015). Limpets are known to have gardens that causes an effect on productivity of the algae, enhancing it, but also reduces the biomass, because of the constant grazing that maintain the algae at a stage of early and rapid phase of growth (Branch *et al.*, 1992; Ridgway, Branch and Stewart, 1999; Casal *et al.*, 2018).

Limpets are sometimes used as shelter by many different organisms that habit the intertidal shores, such as fish, nemertines, polychaetas, crustaceans, mollusks, amphipods and isopods, because they retain water under the shells during low tide to protect themselves from desiccation (Branch, 1971, 1975a; Henriques, Delgado and Sousa, 2016). Most limpets have a ‘home scar’ to which they return after their normal activity, that can vary from species to species (Branch, 1971; Gray and Hodgson, 1997; Sebastián, Steffani and Branch, 2002). Is called ‘homing’ (Coleman, 2007) and can protect the individual against predators (Branch, 1978; Garrity and Levings, 1983; Iwasaki, 1992, 1993) and wave action (Branch, 1988; Gray and Hodgson, 1998), also used to prevent desiccation (Branch and Cherry, 1985; Iwasaki, 1994), reduction of the intraspecific competition (Mackay and Underwood, 1977; Iwasaki, 1995) and defense of the territory or for asserting dominance (Stimson, 1970; Branch, 1975c; Iwasaki, 1995).

Intertidal rocky shores are marine environments with variable and unpredictable conditions, creating an area of high speciation and zonation of the species habiting this

communities (Branch, 1971; Henriques, Delgado and Sousa, 2016). The constant abiotic changes in the environment influence the species structure (Branch *et al.*, 1987; Khouw, 2006a), that led to the morphological and biological characteristics observed (Henriques, Delgado and Sousa, 2016) and the vertical distribution of the individuals along the rocky shores (Branch, 1975b; Gray and Hodgson, 2004; Loqo, 2013). Studies show that the distribution of limpets varies due to physical factors (Gray and Hodgson, 2004; Loqo, 2013), such as temperature variation, salinity, humidity, availability of food, wave action and desiccation (Branch, 1971, 1975b; Khouw, 2006b, 2006a; Henriques, Delgado and Sousa, 2016). Similarly, predation plays an important role in this matter as also the competition between individuals (Branch, 1971; Khouw, 2006a; Loqo, 2013).

The order Patellogastropoda includes the limpets with the most primitive morphology, distinguishing them from the other gastropods. That is the reason why they were first placed on the basal branch of the extant Gastropoda (S. A. Ridgway *et al.*, 1998; Harasewych and McArthur, 2000; Nakano and Ozawa, 2007). They differ from other gastropods in morphological characteristics such as the shell, both in geometry and microstructures, the shape of the radula, morphology of the gills, alimentary system and other aspects of morphology (T. M. Ridgway *et al.*, 1998; Koufopanou *et al.*, 1999; Nakano and Ozawa, 2007). The high plasticity of morphological characteristics, coupled with convergence between species, lead to difficulties in the taxonomy of the group and identification of the species (Branch *et al.*, 1992; T. M. Ridgway *et al.*, 1998; Ridgway, Branch and Stewart, 1999; Ridgway, Stewart and Branch, 1999; Nakano and Ozawa, 2007; Nakano and Sasaki, 2011; Sanna *et al.*, 2011; Henriques, Delgado and Sousa, 2016). The shell form was the most recurrent and for many years the only aspect of identification, but it does not have great resolution and clarification due to high levels of diversity (T. M. Ridgway *et al.*, 1998; Nakano and Ozawa, 2004; González-Wevar *et al.*, 2011). So, the taxonomic studies started using additional morphologic characteristics, like radula structure, coloration of the foot and pallial tentacles, coloration of the eggs and sperm ultrastructure (Nakano and Ozawa, 2004).

The order Patellogastropoda was, at first, divided in three families: Acmaeidae, Patellidae and Lepetidae, based mainly to their simple shell morphology (Powell, 1973). In the following years with the development of the cladistic analyses, still based on morphological characters, Patellogastropod limpets suffered a re-classification into six families: Patellidae, Nacellidae, Lepetidae, Acmaeidae, Lottidae, Neolepetopsidae, but still holding some confusion and divergence between studies (Nakano and Sasaki, 2011).

Later the use of molecular phylogenetics made it possible a more clarified phylogenetic relations within the order and it was again re-classified into seven families: Eoacmaeidae, Lepetidae, Lottidae, Nacellidae, Neolepetopsidae, Pectinodontidae and Patellidae (Nakano and Ozawa, 2007; Nakano and Sasaki, 2011). The Patellidae family is one of the most biologically and morphologically studied (Branch, 1971, 1975a, 1975b, 1981, 1985; Branch *et al.*, 1992). Thiele (1929) initially divided this family in two genera: *Patella* and *Helcion*. This subdivision was based only in morphological characters and was accepted for the follow years (S. A. Ridgway *et al.*, 1998). Other studies on the taxonomy of Patellidae used a variety of characters, such as coloration of the foot and pallial tentacles (Evans, 1947; Fretter and Graham, 1976; Bowman, 1981; Cretella *et al.*, 1990), sperm ultrastructure (Hodgson and Bernard, 1988; Jamieson, Hodgson and Bernard, 1991; Hodgson *et al.*, 1996), configuration of the loops of the gut (Ridgway, 1994), chromosome number (Cervella *et al.*, 1988) and allozyme electrophoresis (Gaffney, 1980; Côte-Real, 1992; Sella, Robotti and Biglione, 1993; Côte-Real, Hawkins and Thorpe, 1996). Yet confusion continued to exist between them regarding the number of species that should be included within the family (S. A. Ridgway *et al.*, 1998). Jamieson, Hodgson and Bernard (1991) used data from the sperm morphology of the Southern Africa species to carried out the first cladistic analysis on the patellids. Later, Ridgway (1994) was the first to incorporate all known data from anatomy and shell ultrastructure in a cladistic analysis but focused on North Atlantic species.

Later, S. A. Ridgway *et al.* (1998) were the first to present a phylogenetic hypothesis constructed by a cladistic analysis of morphologic characters from 37 different species. They included morphologic aspects of the shell shape and microstructure, head foot, pallial complex, radula and sperm. These authors subdivided the family in four monophyletic clades which were treated taxonomically as genera: *Patella* Linnaeus, 1875, *Cymbula* H. and A. Adams, 1854, *Helcion* Monfort, 1810 and *Scutellastra* Quoy and Gaimard, 1834. *Patella* with 9 species in the northeastern Atlantic and Mediterranean; *Cymbula* with 8 species on Southern Africa, Eastern Atlantic and Southern Indian Ocean; *Helcion* with 4 species in Southern Africa; and finally, *Scutellastra* with 17 species in Southern and Southwestern Africa, Australia, Indo-West Pacific and Eastern Pacific (S. A. Ridgway *et al.*, 1998). This division of genera is still accepted by many authors (Koufopanou *et al.*, 1999; Sá-Pinto *et al.*, 2005; Henriques, Delgado and Sousa, 2016).

The use of morphological aspects in the study of the phylogeny of the patellid

limpets has been proven unsatisfactory, so with the advances in molecular techniques was a shift towards that to describe species diversity and relationships within Patellogastropods (Ridgway *et al.*, 2000; Borrell *et al.*, 2010; González-Wevar *et al.*, 2011). The DNA barcoding is the technique most used and the mitochondrial genes 12S rRNA, 16S rRNA and cytochrome c oxidase subunit I (COI). Due to the different levels of evolutionary rates is best to use mitochondrial genes (Nakano and Sasaki, 2011), and COI is considered the most effective in patellid limpet because appears to possess a greater range of phylogenetic signal than any other mitochondrial gene (Borrell *et al.*, 2010; Dedola, 2011; Lin, Kong and Li, 2016).

Currently the Patellidae family is represented by 49 species with its biogeography being described as: 10 species of the genus *Cymbula* distributed along Southern Africa, South-Eastern Atlantic and Mediterranean Sea; 4 species of *Helcion* in Southern Africa; 14 species of *Patella* exclusively found in North-Eastern Atlantic and Mediterranean Sea and 21 species of *Scutellastra*, the genera with the widest distribution reaching from Africa to the Indo-West and Eastern Pacific (Henriques, Delgado and Sousa, 2016).

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Diversity and distribution of patellid limpets along the Southwestern African coast (Benguela current)

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Keywords: Patellogastropoda, DNA barcoding, “true” limpets, phylogeny

Abstract

The focus of this work are the true limpets, organisms with crucial roles in coastal ecosystems around the world, here focusing only the shores of Eastern Atlantic. The high plasticity and convergence on morphological characteristics lead to difficulties in the taxonomy of the Patellogastropods. New molecular techniques, such as DNA barcoding, provide a powerful and more accurate approach to identify the species. Even so the classification and biogeography of the Patellidae, the most representative limpet family in the Atlantic, is not yet well resolved. Through DNA barcoding of the COI gene and phylogenetic analyses, this thesis aims to identify and refine the distributions of Patellid limpets. For this, samples were collected along the southwestern coast of Africa and North Atlantic. Barcoding data were complemented with literature surveys. Distribution maps were developed for each identified species, to understand the range of each species within this region. As a result of the DNA extraction 72 DNA sequences were obtained from Namibia, Angola, Mauritania, Macaronesia archipelagos and Portugal (mainland). The data shows that the distribution of the genus *Patella* is restricted to North Atlantic and *Cymbula*, *Helcion* and *Scutellastra* mostly found in Southern Africa. These regional differences were reflected on the multivariate analysis, that shows distinct species assemblages in: 1) South Africa and Namibia; 2) Angola and Tropical Africa; and 3) North Atlantic and Mediterranean Sea. Phylogenetic analyses revealed that *Patella* is sister to all other three genera, except from clade *Scutellastra* III. *Cymbula* and *Helcion* were sister genera, and *Scutellastra* was subdivided in three paraphyletic lineages matching distinct geographical regions. *Scutellastra* I was sister of the previous branch. These analyses show that a deep revision on the taxonomy and distribution ranges of the Patellidae family is still needed, especially on the West African coasts.

1. Introduction

“True” limpets, belonging to the order Patellogastropoda, have high importance in the rocky shores communities due to their grazing habits, controlling directly populations of algae and indirectly their space-competitors such as barnacles (Branch, 1985; Branch *et al.*, 1992; Gray and Hodgson, 1997; Arrontes *et al.*, 2004; Nakano and Ozawa, 2007; Dedola, 2011; Nakano and Sasaki, 2011; Marzinelli *et al.*, 2012; Burgos-Rubio *et al.*, 2015; Henriques, Delgado and Sousa, 2016; Casal *et al.*, 2018; Kallouche *et al.*, 2018).

Members in this order display high levels of morphological plasticity and convergence, complicating species identifications in the field. The shell form was the only method used on the taxonomy and identification of the species in the past, with very low resolution to distinguish species, even when coupled with others physical characters like radula structure, coloration of the foot and pallial tentacles, coloration of the eggs and sperm ultrastructure (Branch *et al.*, 1992; S. A. Ridgway *et al.*, 1998; T. M. Ridgway *et al.*, 1998; Ridgway, Branch and Stewart, 1999; Ridgway, Stewart and Branch, 1999; Nakano and Ozawa, 2007; González-Wevar *et al.*, 2011; Nakano and Sasaki, 2011; Sanna *et al.*, 2011; Henriques, Delgado and Sousa, 2016). This order as suffer, along the years some re-classifications at family level, until the use of molecular phylogenetics, that made possible a more clarified phylogenetic relations within the order and it was re-classified into seven families: Eoacmaeidae, Lepetidae, Lottidae, Nacellidae, Neolepetopsidae, Pectinodontidae, Patellidae (Powell, 1973; Nakano and Ozawa, 2007; Nakano and Sasaki, 2011). Within Patellogastropoda, the most biologically and morphologically studied family is the Patellidae (Branch, 1971, 1975b, 1975a, 1981, 1985; Branch *et al.*, 1992). The first studies on the taxonomy of this family, again, used morphological characters which proven to be very incongruent due to their high plasticity (Evans, 1947; Powell, 1973; Fretter and Graham, 1976; Bowman, 1981; Hodgson and Bernard, 1988; Cretella *et al.*, 1990; Jamieson, Hodgson and Bernard, 1991; Ridgway, 1994; Hodgson *et al.*, 1996; S. A. Ridgway *et al.*, 1998). S. A. Ridgway *et al.* (1998) were the first to present a classification of four genera, based on a study including 37 different species, that is still accepted to this day (Koufopanou *et al.*, 1999; Sá-Pinto *et al.*, 2005; Henriques, Delgado and Sousa, 2016). *Patella* Linnaeus, 1875, *Cymbula* H. and A. Adams, 1854, *Helcion* Monfort, 1810 and *Scutellastra* Quoy and Gaimard, 1834. Geographically, *Patella*, with 9 species in the northeastern Atlantic and Mediterranean; *Cymbula* with 8 species on

Southern Africa, Eastern Atlantic and Southern Indian Ocean; *Helcion* with 4 species in Southern Africa and last the *Scutellastra* with 17 species in Southern and Southwestern Africa, Australia, Indo-West Pacific and Eastern Pacific (S. A. Ridgway *et al.*, 1998).

Since the 00's there was a shift towards molecular approaches to describe species diversity and relationships within Patellogastropods (Ridgway *et al.*, 2000; Nakano and Ozawa, 2004; Borrell *et al.*, 2010; Dedola, 2011; González-Wevar *et al.*, 2011). Koufopanou *et al.* (1999) were the first to reconstruct a phylogeny based on molecular methods, sequencing the mitochondrial 12S rRNA and 16S rRNA genes. These authors confirmed the four genera that S. A. Ridgway *et al.* (1998) had proposed, but with some discrepancies between the studies (Nakano and Ozawa, 2004). They were also able to clarify with more detail the phylogenetic relationships and divergence times of geographical clades among the Patellidae in the Atlantic and Southern Africa (Nakano and Ozawa, 2004; Sá-Pinto *et al.*, 2005; Nakano and Sasaki, 2011).

Nakano and Ozawa (2004) constructed a molecular phylogeny for 86 extant species of the order Patellogastropoda by sequencing the 12S rRNA and 16S rRNA genes. They also described the geographical distributions. *Patella* diverged from the other genus and is found only in northeastern Atlantic, *Helcion* on South Africa, and *Cymbula* expanding further north on the West African coast. Sá-Pinto *et al.* (2005) used the COI and 12S rRNA and 16S rRNA genes to reconstruct a phylogeny on the genus *Patella* present on the Macaronesia Islands and in the Mediterranean Sea. Borrell *et al.* (2010) used the COI gene to identify and reconstruct the phylogeny of the patellids but simply from the Asturias. More recently, Kallouche *et al.* (2018) characterized the genus *Patella* along the Algerian coasts of Oran using the sequences of the COI gene.

Based on these studies, the systematics and taxonomy of the patellid limpets using molecular techniques, as DNA barcoding, was proven to be a tool essential to bring more clarification on this matter (Dedola, 2011; González-Wevar *et al.*, 2011; Nakano and Sasaki, 2011; Sanna *et al.*, 2011). Molecular-assisted identifications have the advantages of being more precise and efficient in species identification, require less taxonomic expertise, exhibit higher simplicity in operation and being less time-consuming (Lin, Kong and Li, 2016). Among the studies carried out with identification methods using DNA, the markers used have been the mitochondrial genes 12S rRNA, 16S rRNA and COI. Due to the different levels of evolutionary rates is best to use mitochondrial genes (Nakano and Sasaki, 2011), and COI is considered the most effective in patellid limpet because it appears to possess stronger phylogenetic signal than any other mitochondrial

gene (Borrell *et al.*, 2010; Dedola, 2011; Lin, Kong and Li, 2016).

Recently, the north-eastern Atlantic has been the target of studies of phylogeny for this family, as reviewed by Kallouche *et al.* (2018). Compared with other areas in the world, the south of Africa is where the patellid limpets seem to originate and from where they disperse to the Pacific Ocean, north of Africa, Indian Ocean and north Atlantic. Half of the known species are only found in south of Africa which led to a very high diversity (Branch, 1971; S. A. Ridgway *et al.*, 1998; Ridgway, Stewart and Branch, 1999; Ridgway *et al.*, 2000; Nakano and Ozawa, 2004; Mmonwa *et al.*, 2015, 2017; Henriques, Delgado and Sousa, 2016). The second largest area of diversity is the north-eastern Atlantic, whereas both the Indian and Pacific Ocean are regions with little diversity (S. A. Ridgway *et al.*, 1998; Koufopanou *et al.*, 1999). Currently the Patellidae family is represented by 49 species; 10 species of the genus *Cymbula* distributed along Southern Africa, South-Eastern Atlantic and Mediterranean Sea; only 4 species of *Helcion* in Southern Africa; 14 species of *Patella* exclusively found in North-Eastern Atlantic and Mediterranean Sea and 21 species of *Scutellastra*, the genera with the widest distribution reaching from Africa to the Indo-West and Eastern Pacific (Henriques, Delgado and Sousa, 2016).

Among the studies reviewed, it is clear that the focus has remained on the northern Atlantic and in the Mediterranean Sea, with many studies focusing on specific regions and a few species rather than the entire Patellidae family. In South Africa there are some studies, but they do not reach Namibia or Angola. For this reason, this work targets Patellidae of the southwestern zone of Africa, the Benguela current region, where many species are known to occur, but species identities and ranges are still insufficiently studied (T. M. Ridgway *et al.*, 1998; Koufopanou *et al.*, 1999; Nakano and Ozawa, 2004; Kallouche *et al.*, 2018). The Benguela Current Large Ecosystem (BCLME) is one of four world's major Eastern Boundary Upwelling Systems (EBUSs) affecting the Southwestern coast of Africa (Boyer, Cole and Bartholomae, 2000; Verheye *et al.*, 2016). This phenomenon covers the west coast of South Africa, the entire Namibian coast to the central coast of Angola, bordered by the Angola-Benguela Front in the north and the Agulhas Current in the south, roughly between 14°S and 37°S (Boyer, Cole and Bartholomae, 2000; Cochrane *et al.*, 2009; Hutchings *et al.*, 2009; Verheye *et al.*, 2016). Different from the other EBUSs this is limited by warm waters in the north and in the south, both displaying strong thermal fronts (Boyer, Cole and Bartholomae, 2000; Verheye *et al.*, 2016). This region is characterized by active and unpredictable upwelling cold waters that are constantly advected away from the shore, bringing large amounts of

nutrients providing a high productivity coastal ecosystem and species diversity (Branch *et al.*, 1987; Boyer, Cole and Bartholomae, 2000; Verheye *et al.*, 2016).

Currently, about all the species identified as belonging to the Patellidae family have available sequences in GenBank allowing confirmation of the species identifications already made based on morphological characters. These sequences also provide complementary information for a phylogenetic analysis of this group in relation to the region under study. The general aim of this thesis is to clarify species assemblages and refine species ranges and phylogenetic affinities of the Patellidae family, focusing on the southwestern coast of Africa, the region of the Benguela current. We expect to better understand the distributional range of the species along this geographical area, and if there is an overlap with Patellid communities of more tropical affinities and of the northern Hemisphere or if the tropics act as a natural barrier. These objectives will be achieved using a barcoding approach. Molecular data will be complemented with literature surveys to generate distribution maps of the identified species. Finally, a comprehensive phylogenetic analysis of the family Patellidae will be conducted to comprehend species relations and broad biogeographical patterns.

2. Material and Methods

2.1. Sampling

Limpets were collected in the intertidal rocky shores from five different countries making a total of 104 samples (see Table 2.1.). Specimens collected in the field were preserved in 95% ethanol and only a portion of tissue from the foot/mantle were dissected for DNA extraction. Some of the samples were identified in the field.

Table 2.1. Information of the sampling location, time of collection, number of individuals extracted, and sequences obtained.

Region	Sampling site	Date	Nº of individuals extracted	Nº of sequences	Collector
Namibia	Mile 4	Sep 2019	1	1	George Branch, Anja Kreiner
	Badewanne	Sep 2019	2	2	
	South	Sep/Out 2019	12	6	
	Diaz Point	Sep/Out 2019	2	1	
	Meob Bay	Sep/Out 2019	3	1	
	Rocky Point	Out 2019	16	11	
Angola	Luanda	Sep 2019	3	3	Ester Serrão
	Baia das Pipas	Sep 2019	20	14	
Mauritania	Cap Blanc	Feb 2020/Nov 2020	11/7	6/5	Ester Serrão
Portugal (mainland)	Arrábida	Aug 2020	4	3	Ester Serrão
	Praia Maria Luísa	May 2020	2	0	Rita Jacinto
Macaronesia	Canary Islands	Aug 2020/Set 2020	7	5	Gonçalo Calado
	Madeira		6	6	Francis Zino
	Azores	Dec 2019	8	8	Gonçalo Calado

2.2. DNA extraction, amplification and sequencing

In order to extract the genomic DNA from the samples a piece of the mantle/foot was cut and placed in an Eppendorf with silica during 48h, to dehydrate the cells. After this was necessary to disrupt the material to a fine dust, for that was used the TissueLyserII (Qiagen), at a frequency of 30 bits for 4 minutes, that through high-speed shaking can break multiple samples. Then the samples proceed to the extraction using a plant kit (NucleoSpin® Plant II protocol – Genomic DNA from plant). This plant extraction kit proved efficient since limpets have a mucus that makes extraction difficult

using other protocols.

The first step was the cell lysis. It starts with adding 400µL of buffer PL1, previously heated to resuspend the solution, plus 10µL of RNase A, and incubated for 30 min at 65°C. The next step was the filtration/clarification of crude lysate, the solution obtained in the step before is transferred to new eppendorfs with the NucleoSpin Filters, that will eliminate rests of cells. Centrifuged for 2min at 8000rpm and discharged the filter. By adding 450µL Buffer PC to the filtrated will make the DNA biochemically capable of binding to the next filter membrane, the vortex will help to mix the solutions. Next, transfer 700µL from the solution to a new set of tubes (Collection tubes 2mL) placed with the NucleoSpin Plant II Column and centrifuge during 1 min at 8000 rpm, discharging the liquid from the tube. Follows, washing and drying the silica membrane, for that it was added 400µL de Buffer PW1 to the filter and centrifuge for 1 min at 8000 rpm. On the second wash was added 700µL Buffer PW2 and centrifuge for 1 min at 8000 rpm. The third and last wash was to completely remove the buffer and dry the silica membrane, by adding 200µL Buffer PW2 and centrifuge for 2 min at 8000 rpm. To finish the extraction the DNA must be eluted from the membrane. In new Eppendorf's the filter was placed and added 100µL of Buffer PE, previously heated, and centrifuged for 1 min at 8000 rpm. The resulting liquid is the DNA sample. Then storage at -20°C.

For the amplification of the mitochondrial barcode gene, COI, it was used the primers LC01490 (5'-GGTCAACAAATCATAAAGATATGG-3') and HC0298 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.*, 1994), it was selected this gene because of his strong conservative coding. The PCR was performed in a total volume of 25µL, with the following reagents added: 10x GoTaq buffer, 2mM of each dNTP's, 2.5mM of MgCl₂, 10µM of the froward and reverse primers, 5u/µL GoTaq DNA Polymerase, 2µL of DNA samples, filled up with ultrapure sterile H₂O. The thermal conditions for PCR were as follows: one cycle of 94°C for 4 minutes; thirty cycles of 94°C for 30 seconds, 51°C for 30 seconds, 72°C for 30 seconds; one cycle of 72°C for 4 minutes.

To visualize the DNA amplifications and the DNA extractions, 1% agarose gels were used. For the DNA to be visible under UV light, GelRed (1:500) and 5x Green GoTaq Buffer were added to the DNA before loading in the gel. The GeneRuller 100bp Plus DNA Ladder has used as reference marker. The electrophoresis occurred at 120 volts for 20 min. ImageLab was the software used to obtain the image from the gels.

Sequencing was performed at CCMAR using the ABI PRISM capillary automated

sequencer 3130XL Genetic Analyzer (Applied Biosystems). Chromatograms were viewed and edited manually using the Geneious software (Geneious 4.8.5 (<https://www.geneious.com>)). Sequences were identified using the Blastn algorithm in https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome (2021), that lists the sequences in the GenBank database with highest similarity (Altschul *et al.*, 1990). Sequences were also compared directly in Geneious against all Patellidae sequences.

2.3. Distribution Maps

For the distribution maps of the species identified by the DNA extraction, it was used data from the GBIF (GBIF.org, 2021), that aggregates information from multiple sources, like museums, herbaria, collections of individual researchers, photo applications and from other databases, that are edited by very different people at different periods in time. In order to clean and eliminate problematic coordinates it was used the package *CoordinateCleaner* from Rstudio (R Core Team, 2021) and some additional packages that allowed the creation of the maps and the inclusion of my own data, like *tmap*, *tmaptools*, *sf*, *leaflet*, *ggplot2*, *ggmap*, *maps*, *mapdata*, *ggrepel*, *ggsn*, *readxl*. With the *CoordinateCleaner* package it was possible to automatically eliminate sea coordinates, coordinate - country mismatches, coordinates assigned to country and province centroids, coordinates within city areas, outlier coordinates and coordinates assigned to biodiversity institutions and even coordinates with low precision.

Distributional data was synthesized in a matrix of presence/absence of the Patellidae family considering several Atlantic regions from the south of Africa to Scandinavia. Using the program Primer Permanova (Anderson, Gorley and Clarke, 2008) a multivariate analysis was carried out with the objective of emphasize the difference on species composition between regions on the Western Atlantic coasts using the Bray-Curtis similarity.

2.4. Phylogenetic analysis

In addition to the DNA sequences obtained experimentally, all COI gene sequences of Patellidae available in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) (a total of 1314 sequences) were downloaded and aligned with the new sequences using the software Geneious (Geneious 4.8.5

(<https://www.geneious.com>)). Two sequences from the genus *Cellana*, *Cellana pricei* and *Cellana solida*, and one sequence from the genus *Nacella*, *Nacella concinna*, from the family Nacellidae were used as outgroups. In the alignment, 4 similar sequences were selected to represent distinct taxa. Some of the sequences had longer and uncertain bases at the end so the sequences were trimmed to a minimum common length.

Two types of analyses were carried out, Bayesian tree and Maximum Likelihood (ML). The best-fit model of DNA sequence evolution was selected using the jModelTest 2.1.10 (Darriba *et al.*, 2012) based on the Bayesian Information Criterion (BIC). The Bayesian analyses were ran with the program MrBayes 3.2.7 (Ronquist *et al.*, 2012). The parameters for the Markov Chain Monte Carlo (MCMC) search for trees were ngen=2000000, nruns=2, samplefreq=100 and printfreq=3000, until the Average Standard Deviation of Split Frequencies < 0.01. For the ML tree was used the online program IQ-Tree (Trifinopoulos *et al.*, 2016). Using 100 bootstraps. Resulting trees were viewed and edited with the software FigTree v1.4.4 (Rambaut, 2018).

3. Results

3.1. Species Identification

A total of 72 DNA sequences were obtained, 9 different species of the family Patellidae were identified, 2 different species of the family Siphonariidae and 1 species of the family Fissurellidae. The three species that do not belong to the family Patellidae can be explained by the difficulty of identifying limpets morphologically and because of that easily mistaken.

Inside the Patellidae family the four genera were represented, with 3 species of *Cymbula*, 1 species of *Helcion*, 1 species of *Scutellastra* and 4 species of *Patella* (see Table 3.1). Sequencing confirmed some field identifications, but for most samples identified there was a conflict between molecular and field identifications. For example, one of the samples from Namibia was first identified as *Cymbula compressa* and through molecular methods was identified as *Siphonaria capensis*, the same happened to two samples first identified as *Patella skeletonenses* and was genetically identified as *Cymbula safiana*, and one *Cymbula swalopumdenses* was identified as *Cymbula miniata*. In the Canary Islands two samples were identified as *Patella crenata* and one as *Patella piperata*, and molecularly one is *Patella candei* and two *Patella rustica*. *Cymbula miniata* only sampled in Namibia, as well as *Cymbula granatina*, *Helcion dunkeri* and *Fissurellidae sp.* *Cymbula safiana*, *Siphonaria capensis* and *Scutellastra granularis* were sampled in Namibia and Angola; the first was also found in Mauritania and the second in the Canary Islands. *Siphonaria pectinata* was only sampled in Mauritania. *Patella rustica* was identified both in Portugal (mainland) and Macaronesia archipelagos, for *Patella ulyssiponensis* and *Patella vulgata* they were sampled in Portugal (mainland). Finally, *Patella candei* only found in Macaronesia archipelagos.

Table 3.1. List of species identified and number of sequences in each region of sampling.

Region	Species Identified	N° of sequences
Namibia	<i>Cymbula miniata</i>	5
	<i>Cymbula granatina</i>	3
	<i>Cymbula safiana</i>	5
	<i>Helcion dunkeri</i>	2
	<i>Scutellastra granularis</i>	2
	<i>Siphonaria capensis</i>	1
	<i>Fissurellidae sp.</i>	5
Angola	<i>Cymbula safiana</i>	7
	<i>Scutellastra granularis</i>	5
	<i>Siphonaria capensis</i>	4
Mauritania	<i>Cymbula safiana</i>	9
	<i>Siphonaria pectinata</i>	2
Portugal (mainland)	<i>Patella rustica</i>	1
	<i>Patella ulyssiponensis</i>	1
	<i>Patella vulgata</i>	1
Macaronesia	<i>Patella candei</i>	16
	<i>Patella rustica</i>	2
	<i>Siphonaria capensis</i>	1

3.2. Distribution Maps

For each species identified from the DNA extraction a distribution map was created using the GBIF records (Fig. 3.2.1 to Fig. 3.2.11). This included all Patellidae limpets (Fig. 3.2.1 to Fig. 3.2.9), as well as the two species of the family Siphonariidae (Fig. 3.2.10 and Fig. 3.2.11).

For the *C. granatina*, *C. miniata*, *P. candei* (*sensu lato*), *P. ulyssiponensis*, *P. rustica*, *P. vulgata* and *H. dunkeri* our distribution data is within the known range limits of the species. Two *Cymbula* species were only present in south Africa similar to *S. granularis* and *H. dunkeri*, and only *C. safiana* showing a more spread distribution along the coast of Africa. *Patella* species were restricted only to the northeast Atlantic. *P. candei* appeared only on the Atlantic islands, *P. ulyssiponensis* spreading from the Mediterranean to the British islands. *P. rustica* appeared on Mediterranean Sea and reached the north coast of Spain. Finally, *P. vulgata* although present in the Mediterranean Sea, was more abundant from the north coast of Iberian Peninsula to the north of Scandinavia.

On the southern hemisphere, our data brings new information for *C. safiana* and *S. granularis*. For example, *C. safiana* appears in Mauritania and two more points in Angola and Namibia. For *S. granularis*, their presence in Angola is first confirmed.

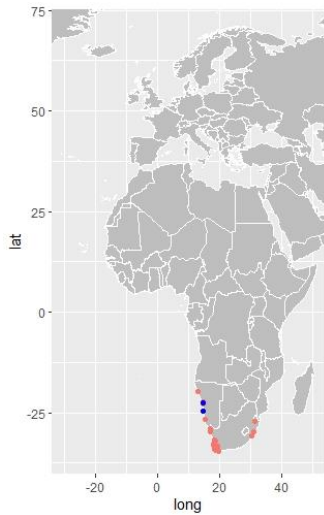


Fig 3.2.1. Map of the distribution of *Cymbula granatina* with GBIF records (pink) and original records (blue)

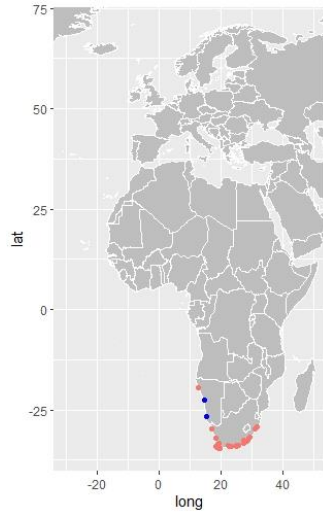


Fig 3.2.2. Map of the distribution of *Cymbula miniata* with GBIF records (pink) and original records (blue)

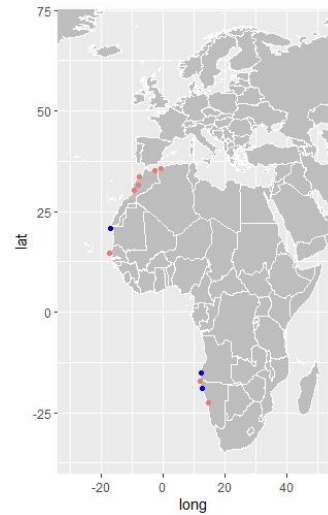


Fig 3.2.3. Map of the distribution of *Cymbula saffana* with GBIF records (pink) and original records (blue)

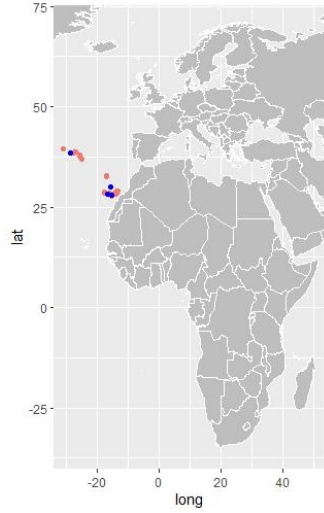


Fig 3.2.4. Map of the distribution of *Patella candei* (sensu lato) with GBIF records (pink) and original records (blue)

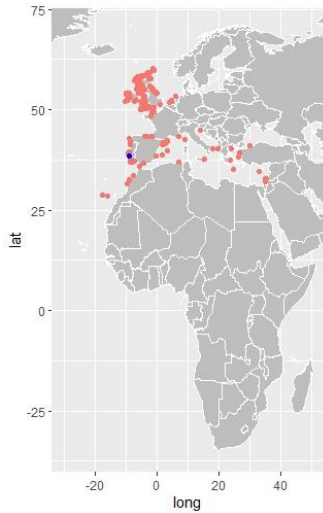


Fig 3.2.5. Map of the distribution of *Patella ulyssiponensis* with GBIF records (pink) and original records (blue)

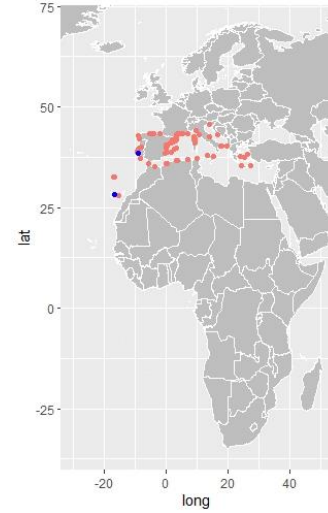


Fig 3.2.6. Map of the distribution of *Patella rustica* with GBIF records (pink) and original records (blue)

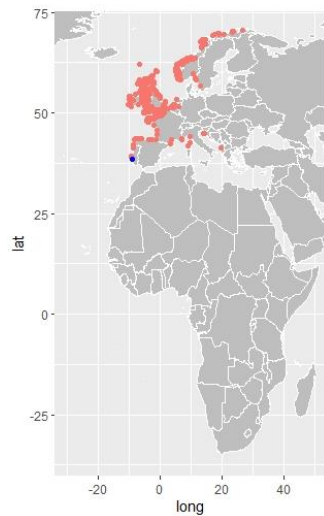


Fig 3.2.7. Map of the distribution of *Patella vulgata* with GBIF records (pink) and original records (blue)

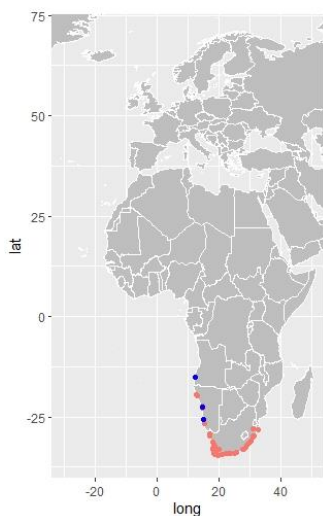


Fig 3.2.8. Map of the distribution of *Scutellastra granularis* with GBIF records (pink) and original records (blue)

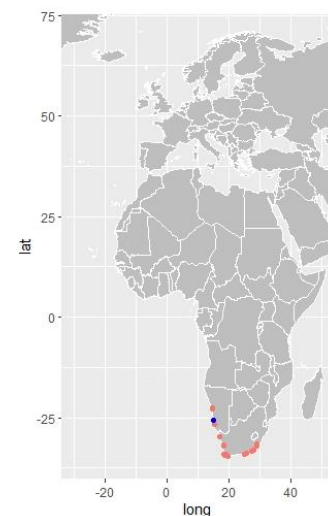


Fig 3.2.9. Map of the distribution of *Helcion dunkeri* with GBIF records (pink) and original records (blue)

The distribution of *S. capensis* (Fig. 3.2.10) shows that the new sequences place the species in Angola and in the Canary Islands. For the *S. pectinata* (Fig. 3.2.11) it placed them also in Mauritania.

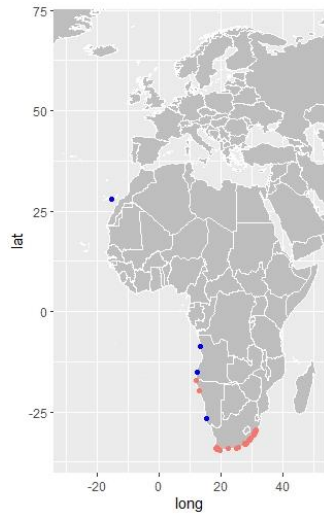


Fig 3.2.10. Map of the distribution of *Siphonaria capensis* with GBIF records (pink) and original records (blue)

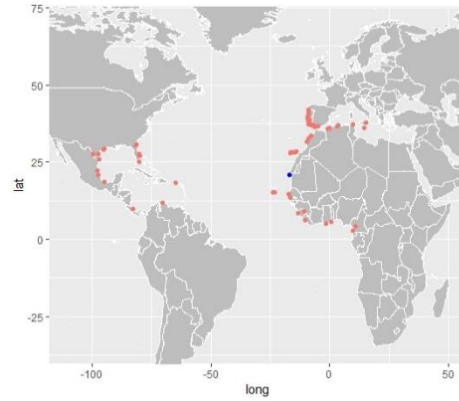


Fig 3.2.11. Map of the distribution of *Siphonaria pectinata* with GBIF records (pink) and original records (blue)

Cluster and Multidimensional scaling (MDS) plots show that there are important regional differences in patellid limpet assemblages. South Africa and Namibia have a similarity and move away from the other zones. In the case of Angola and Tropical Africa the data were few, but even so the results show a greater proximity between these regions than with the others. The North Atlantic forms a cohesive group different from the South, with only Scandinavia deviating a bit from the rest.

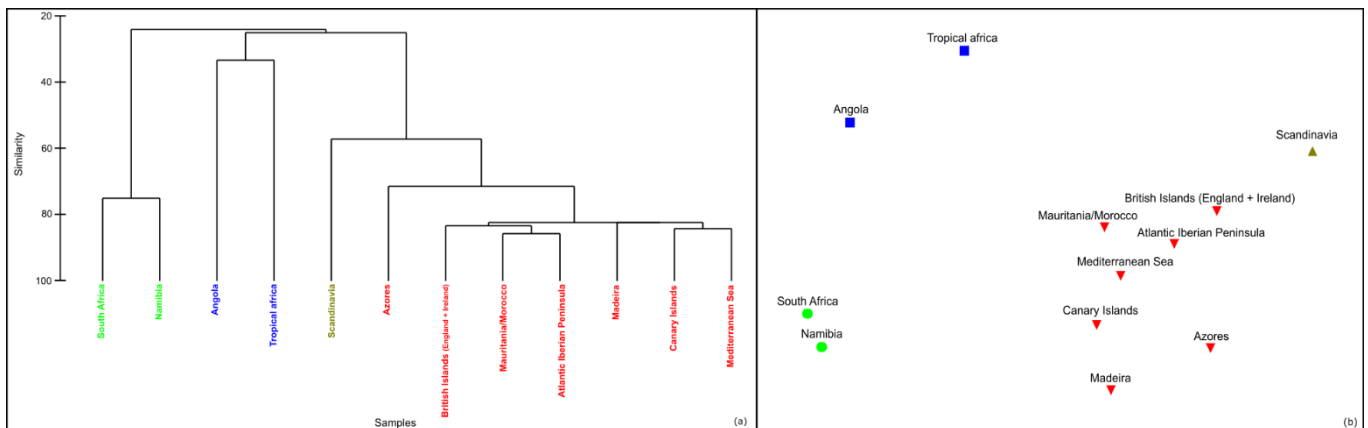


Fig. 3.2.12 (a) Dendrogram; (b) Multidimensional scaling (MDS) of Patellid assemblages based on presence/absence data of Patellid limpets. (Tropical Africa – refers to the region between Angola and Mauritania).

3.3. Phylogenetic analysis

The final alignment had 170 sequences and 509bp. Bayesian and ML trees were reconstructed using the model HKY+I+G and they showed similar topologies. Posterior probabilities and bootstraps values are shown above and below the respective nodes, respectively (shown on Fig. 3.3.).

The genus *Patella* formed a natural group with high bootstrap and posterior probability support. *P. candei* was divided by samples from Azores and from Canary Islands and Selvagens. The genus *Cymbula* formed a natural group sister to genus *Helcion*. *Scutellastra* could be divided in three different groups. *Scutellastra* I appeared as sister to the clade *Cymbula* and *Helcion*, and included the species *S. granularis/S. miliaris*, *S. natalensis*, *S. argenvillei*, *S. peronii* and *S. laticostata*, with the last two species forming separated clades. *Scutellastra* II included the species *S. barbara*, *S. longicosta* and *S. cochlear*, and was sister of the first *Scutellastra* group, of the *Cymbula* and *Helcion* and of the *Patella* clade. *Scutellastra* III was sister to all the above and included the species *S. flexuosa*, *S. optima*, *S. exusta*, with only one sequence of *S. flexuosa* grouping with *S. optima*, sister of *S. exusta*, and with other sequences of *S. flexuosa* forming other branch. *C. compressa* appeared to be related with the outgroup *Nacella concinna* and not, as expected, with the *Cymbula* clade.

Both species from the genus *Cellana* form a sister group with high posterior probabilities support. The *Nacella* species appears as the closest to the resulting tree also with a very high support value (100% on Bayesian tree and 73% on ML tree).

Overall, we obtained very high values of posterior probabilities for the Bayesian tree except for the clade that separates *P. ferruginea* and *P. rustica* from *P. vulgata* and *P. ulyssiponensis/P. aspera*, and for the node of *S. peronii* with the group of *Cymbula* and *Helcion* and *S. granularis/S. miliaris*, *S. natalensis* and *S. argenvillei*, that both have a probability less than 60%. For the ML tree the bootstrap support is, for the majority of the nodes, very low values, only four nodes above 90%.



Fig. 3.3. Bayesian tree obtained with 170 sequences of COI from 34 different species. Bayesian posterior probability values shown above the branches and Maximum Likelihood bootstraps values below the branches. Location from where the sequences were obtained follow the code: ● - Morocco, Mediterranean, Macaronesia, Iberian Peninsula, Mauritania; ● - Angola, Namibia, South Africa; ● - Southern Ocean; ● - Madagascar; ● - Australia, Japan, China, French Polynesia, Samoa.

4. Discussion

In the DNA amplification, 72 samples out of a total of 104 samples were amplified and successfully identified. This represents ca. 70% of the set of samples; it would perhaps be necessary to change the extraction protocol to obtain more sequences and thus a larger database, or perhaps the process of storing the samples was not carried out in the best way, which led to tissue degradation for extraction as more than 50% of the samples were successful in the extraction process used.

More genes could also be used in the extraction, such as 12S and 16S, for a greater comparison in terms of phylogeny, since the three genes are mostly used together in other identification and phylogeny studies of this same family. In terms of quality, 70% of the sequences obtained had HQ% above 80%. For identification in Blast, all sequences presented percentage of identity higher than 85%, which brings confidence in their identification. Most of the samples that were identified in the field did not correspond to genetic identification, often identified as a morphologically similar species. These discrepancies confirm the usefulness of molecular methods in identifying this morphologically conserved family.

9 different species of the Patellidae were identified in the samples analyzed, which for this family is not a big amount as there are 49 species documented worldwide (Henriques, Delgado and Sousa, 2016). The most frequent genus identified was *Patella* with 4 different species, and the least frequent were *Helcion* and *Scutellastra* with 1 species each. All recognized genera – *Patella*, *Cymbula*, *Helcion* and *Scutellastra* – were represented, and results confirm the general distribution described for each genus. *Patella* was only found in the North Atlantic. *Cymbula* and *Helcion* were restricted to Southern Africa, with *C. safiana* being the only species with a distribution spanning both hemispheres, reaching the north of Africa (Mauritania). Only one species of *Scutellastra* was identified, on the coast of Angola and Namibia. New genetic data cannot confirm its widest distribution, but occurrence records reach from South Africa to Australia. Although the general distribution of each genus is line with previous studies, barcoding results add new data on the geographic distribution of some species.

S. granularis is first confirmed from Namibia and Angola through genetic data and not just based on morphology. For the remaining species identified, although data does

not extend their geographic ranges, it confirms and provides more accurate information than just morphological observations that are often not reliable in Patellogastropoda. Surprisingly, *S. capensis* was detected in the Canary Islands and in two places in Angola. Previously, this species had only been documented in southern Africa, from Namibia to Mozambique. Two hypotheses emerge. Either the species is actually present further north and was never documented, or the specimen identified belongs to a closely related species. The second hypothesis is due to lack of genetic data in the GenBank, which would explain why Blast would identify these sequences as *S. capensis*. Looking at the percentage of similarity on Blast the values were above 95%, and comparing the sequences obtain in an alignment with other sequences there was many mutations. The second hypothesis is thus more likely to be correct. As far as *S. pectinata* is concerned, the place where it was identified is within the limits that would be expected to find this species.

The Dendrogram and MDS plots (Fig. 3.2.12.) summarize what is observed on the maps. These analyses group the different geographic zones in relation to the species present in each region, revealing biogeographical patterns, but are dependent on the amount and accuracy of occurrence data. For example, some regions along the West coast of Africa, like in the Namibian desert and some tropical countries, are so remote that it is difficult to collect information. On the contrary, Europe and South Africa are regions with good access and consequently well surveyed. Also, we have the problem of these species being of very difficult morphological identification what can led to miss-identifications. The accuracy may not be as ideal as it should. In any case, these analyses clearly separate South Africa and Namibia from the northern Atlantic and Mediterranean Sea. Angola and Tropical Africa are also retrieved as a separate group, but the information in the database was insufficient, with only 4 recognized species in Angola and 1 in all region of Tropical Africa. These species are shared with South Africa and/or Namibia, what lead to the separation of this region from the others. Anyway, it is clear a genus separation from south Africa and north Atlantic, with *Patella* only present in Europe, Mediterranean Sea and Macaronesia archipelagos and *Cymbula*, *Helcion* and *Scutellastra* along the African coast. Koufopanou *et al.* (1999) study the antitropical distribution of patellids and because the lack of a good fossil records it is still unclear what was the process that led to the origin of the observed geographical distribution. Even so, although most patellids have pelagic larvae they are not widely dispersed. In the case of isolated islands, the appearance

of patellids is more due to the fact that adults migrate attached to macroalgae (Koufopanou *et al.*, 1999). Looking from a species diversity perspective between the two hemispheres, we have a total of 21 different species in the south hemisphere and 12 different species in the north hemisphere. Although the southern hemisphere has more variability it is not so big of a difference from a place where it is considered a diversity hotspot.

Phylogenetic analyses of Patellidae based on COI gene sequences (new and available from GenBank) were in line with previous studies, either based on morphological characters or molecular analysis based on this and other markers (S. A. Ridgway *et al.*, 1998; Koufopanou *et al.*, 1999; Nakano and Ozawa, 2004, 2007). Specifically, the four genera always appear forming the same exactly clades that are shown on Fig. 3.3. *Patella* genus was a very well supported clade, *Cymbula* and *Helcion* shared the same node as sister taxa, and *Scutellastra* was divided in three lineages separated by geography. One was composed by species identified from South Africa, as *Cymbula* and *Helcion*.

Inside the *Patella* clade, *P. pellucida* formed a distinct and very well supported branch from all others *Patella* species (100% for both trees, Bayesian and ML). *P. ferruginea* formed a group with *P. rustica* with a very high support, and together they were sister species of the clade *P. vulgata* and *P. ulyssiponensis/P. aspera* but not so well resolved with only 57% for Bayesian and 48% for ML. Even in the sister group *P. vulgata* and *P. ulyssiponensis/P. aspera* are not well resolved. These results are consistent with the ones obtained by Sá-Pinto *et al.* (2005). One exception is *P. caerulea* that was retrieved as sister to the group formed by *P. candei* from Canary Islands and Selvagens and *P. lugubris*. This clade formed by the three species was then sister to *P. candei* from Azores. In Sá-Pinto *et al.* (2005), *P. candei* from Canary Islands and Selvagens also appeared as sister of the *P. lugubris* but appear sisters to *P. candei* of Azores, Madeira and Desertas and only then all are shown forming a clade with *P. caerulea*. The support for this node is high in Bayesian tree but lower in the ML. Despite this difference the clade formed by these species grouped with *P. depressa*, as in Sá-Pinto *et al.* (2005). Sá-Pinto *et al.* (2005) these authors have suggested that *P. candei* appears to be paraphyletic because of the genetic differences between the specimens found in Azores, Madeira and Desertas from ones found in Canary Islands and Selvagens, also supported by our results. They proposed that this could be explained by an introgression between *P. candei* from Canary Islands and

Selvagens and an ancestor of *P. lugubris*. Another hypothesis, presented in the same study, is the occurrence of homoplasy or similar selective pressures within the two groups (Sá-pinto et al. 2005). Regarding *P. ulyssiponensis*, there is well resolved differences between continental and Macaronesian samples reported in some studies (Koufopanou et al., 1999; Sá-Pinto et al., 2005; Weber and Hawkins, 2005; Nakano and Sasaki, 2011) and in some *P. ulyssiponensis* is restricted to continental limpets and *P. aspera* for the Island form. Nakano and Sasaki (2011) suggested a taxonomic reconstruction, and the fact that in the phylogenetic trees their separation is poorly supported, the branch was collapsed.

Nakano and Sasaki (2011) also mention that both genera, *Cymbula* and *Helcion*, are endemic to South Africa, apart from *C. safiana* whose distribution spreads from West African coast from Namibia to the coast of Algeria. The tree presented by Nakano and Ozawa (2007), based on the 12S, 16S and COI genes, supports the tree reconstructed in this study, where *Cymbula* and *Helcion* appear forming a single clade with a high support values (100% for Bayesian and 78% for ML in this study). Inside the *Helcion* genus, *H. concolor* was the sister taxa of the *H. dunkeri* and *H. pruinosis*, the two forming a well-supported node (100% for Bayesian and 90% for ML). The variance between the two trees sits on *Cymbula* clade; the reconstructed tree shows *C. miniata* forming a branch with *C. safiana*, that then forms a node with *C. oculus* and *C. granatina*, but with a very low support, 74% for the Bayesian tree and 43% for the ML tree. As in Nakano and Ozawa (2007), *C. safiana* appears the most external taxon of the genus and with a very resolved support. On the other hand, they did not include the species *C. granatina*. Other major difference between these studies is the fact that *C. compressa* in the reconstructed tree appears as sister of *N. concinna*, a member of the family Nacellidae. Considering other studies, such as the ones present by Nakano and Ozawa (2004, 2007), the most likely hypothesis is that the sequences in the GenBank database are mis-identified, as other samples sequenced for different genes place the species as sister of *C. miniata*. So, the COI sequences identified as *C. compressa* most likely belong to some other species within the Nacellidae family.

Scutellastra was subdivided in three different clades with very high support values, as in other phylogenetic studies (Nakano and Ozawa, 2004, 2007; Lindberg, 2007; Nakano and Sasaki, 2011). These clades match very closely their geographical distribution, with *Scutellastra* I distributed in South Africa and Australia, *Scutellastra* II distributed in South Africa, and *Scutellastra* III distributed in Indo-West Pacific, from

South Africa through Indo-Pacific and Australia (Nakano and Sasaki, 2011). Koufopanou *et al.* (1999), were the first to conclude that *Scutellastra* is paraphyletic through molecular analyses, but these findings were later confirmed by other authors (Nakano and Ozawa, 2004, 2007; Lindberg, 2007; Nakano and Sasaki, 2011). Comparisons with earlier phylogenetic studies are difficult because not all studies included the same species, and most are based on the 12S and 16S genes. Even so, the results obtained support the ones obtained with 12S and 16S by Nakano and Ozawa (2004), that *S. granularis* and *S. miliaris* is sister to *S. argenvillei*. Nakano and Ozawa (2007) also used COI and obtained the same result that *S. optima* are sister taxa related to *S. exusta*. The *S. flexuosa* sequences analyzed were paraphyletic, with one group of sequences likely corresponding to a distinct species. This could be explained by the fact that they are even genetically differentiated within the French Polynesian archipelagos (Nakano and Sasaki, 2011). Some authors proposed the division of *Scutellastra* into several subgeneric or even generic ranks (Nakano and Sasaki, 2011), but the taxonomy of the group still does not reflect these different lineages. In general, this genus requires further phylogenetic and barcoding work to clarify species boundaries, affinities and distributions, and ultimately their taxonomy.

Globally, our data brings new insights on some species geographic distribution through genetic evidence and confirms previous work in showing the general distribution of each genus of the Patellidae family. Highlights the gap on the description of the patellids on the southwestern African coast and their lack of phylogenetic resolution. It is needed a deeply study on their taxonomy, using more than one genetic marker and from a geographical range wide enough to cover all territory of the Patellidae, in order to clarify their difference as species level and where each can be found.

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To my father, Albano. To my mother, Lucrecia. To my brother, César.

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