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Genetic structure of the American elephantfish (Chimaeriformes: *Callorhinchus callorynchus*) in South America

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Declaração de autoria

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Declaração de direitos de autor

Cornelia P. Erk

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Abstract

Understanding genetic population structure and connectivity is key to the establishment of effective species-specific management and conservation strategies. The American elephantfish, *Callorhinchus callorynchus*, is a year-round target of commercial and recreational fisheries in Chile and Argentina. However, the scarcity of studies on chimaeroids´ population structure and connectivity hampers adequate fisheries management of this highly vulnerable group. In this work, we explore the levels of genetic diversity and differentiation within *C. callorynchus* in South America at two widely used mitochondrial markers, the control region (CR) and the cytochrome oxidase I gene (CO1). Moreover, we assess levels of genetic diversity within, and divergence among the three valid extant species of the genus *Callorhinchus*, which exhibit allopatric geographical distributions in the southern hemisphere. Overall, sequence analyses of the mitochondrial CR and the CO1 revealed extremely low levels of sequence variation both within and among *Callorhinchus* species. Genetic homogeneity was found throughout the range of *C. callorynchus* coupled to low-frequency haplotype sharing across spatially distant locations in Chile and Argentina, suggesting gene flow along the South American coast. Moreover, our analyses support a scenario of recent population expansion of the species in South America. Given the absence of dispersive eggs or juvenile stages in chondrichthyans, gene flow is mainly mediated by actively swimming adults. Based on the available data, gene flow in callorhinchids appears to occur along continuous coastal regions, with deep oceanic waters serving as strong barriers, thus providing an important baseline for future research on dispersal and gene flow in other holocephalans.

Keywords — Chimaeras; genetic diversity; mitochondrial DNA; demographic history; Southern Hemisphere.

Resumo

As quimeras (Holocephali) constituem uma linhagem basal bem suportada entre os peixes cartilaginosos (Classe Chondrichthyes; Holocephali e Elasmobranchii). Em conjunto com os elasmobrânquios, as quimeras ocupam também uma posição filogeneticamente muito importante enquanto linhagem mais basal de vertebrados mandibulados, sendo por isso um grupo essencial no estudo da evolução de vertebrados. No entanto, este grupo continua pouco estudado e, dada a exploração pesqueira de algumas destas espécies, a escassez de estudos sobre a diversidade e estrutura genética de quimeras implicam lacunas significativas no conhecimento das unidades populacionais e na gestão adequada destes recursos.

O género *Callorhinchus* Lacepède 1998 (Chimaeriformes: Callorhinchidae) inclui três espécies válidas de quimeras de tamanho médio (até 125 cm de comprimento total), com distribuições geográficas mutuamente exclusivas de águas temperados no hemisfério sul. Especificamente, *C. callorynchus* (Linnaeus, 1758) está restrita à costa da América do Sul, *C. capensis* Duméril, 1865 é encontrada na costa da sul Africana, e *C. milii* Bory de Saint-Vincent, 1823 ocorre na Nova Zelândia e sul da Austrália. A aparência externa das três espécies de *Callorhinchus* é muito semelhante, não havendo ainda caracteres diagnosticantes para cada uma.

Callorhinchus callorynchus está amplamente distribuído ao longo da plataforma continental do Brasil, Uruguai, Argentina, Chile e Peru. Localmente conhecido como "pez gallo", está associado a fundos marinhos moles, a profundidades entre 10 e 481 metros. Esta espécie é alvo da pesca comercial e recreativa no Chile e Argentina ao longo de todo o ano, sendo também muitas vezes registada como captura acessória. No entanto, temos apenas um conhecimento rudimentar sobre o comportamento sazonal e padrões de distribuição dos indivíduos. Como a maioria dos peixes cartilagíneos, a diversidade e abundância das quimeras são altamente sensíveis a várias pressões humanas, nomeadamente à mortalidade pela pesca, devido às suas características de história de vida conservativas como crescimento lento, maturidade sexual tardia, baixa fecundidade e grande longevidade. No entanto, *Callorhinchus callorynchus* foi classificada como "Vulnerável" pela Lista Vermelha da IUCN, e são urgentemente necessários estudos sobre a variação genética e a estrutura espacial das populações para apoiar estratégias de gestão e conservação específicas para a espécie.

A nossa pesquisa visa contribuir para uma melhor compreensão da estrutura populacional e dos processos demográficos no *C. callorynchus*, avaliando os níveis e a distribuição da sua diversidade genética. Neste trabalho, analisámos sequências de dois

marcadores mitocondriais amplamente utilizados neste tipo de estudos, para explorar os níveis de diversidade e diferenciação de *C. callorynchus* ao longo da costa da América do Sul. Dadas as características ambientais e histórias geológicas distintas entre as costas atlântica e pacífica do continente sul-americano, a principal questão prende-se em saber se o *C. callorynchus* forma uma única unidade populacional ou se existem várias unidades populacionais ao longo da sua distribuição. As amostras foram recolhidas em vários locais das costas do Pacífico (Peru, Chile) e do Atlântico (Argentina). Além disso, amostras das espécies congéneres *C. milii* da Austrália e Nova Zelândia e *C. capensis* do sul de África foram incluídas nas nossas análises para avaliar os níveis de divergência intragenérica no género *Callorhinchus*.

Utilizando primers desenhados especificamente para *C. callorynchus*, realizei a amplificação por PCR de 506 pares de bases (pb) da região de controlo do genoma mitocondrial (CR) e de 458 pb do gene da subunidade 1 da citocromo oxidase (CO1). As análises revelaram níveis extremamente baixos de variabilidade nucleotídica, tanto dentro quanto entre as espécies de *Callorhinchus*, com níveis de diversidade extremamente baixos para *C. callorynchus*. Combinado com a homogeneidade genética geral ao longo da distribuição da espécie e a ausência de divergência genética significativa entre as amostras do Chile e da Argentina, os dados sugerem um fluxo genético substancial ao longo da costa sul-americana. Além disso, resultados das análises demográficas apoiam um cenário de expansão populacional recente de *C. callorynchus* ao longo da costa sul-americana.

Contudo, a costa sul-americana é caracterizada por vários fenómenos climáticos e variabilidades de mesoescala com importantes implicações para a divergência espacial e a heterogeneidade genética das espécies. Uma vez que o *C. callorynchus* é uma espécie ovípara sem ovos ou estádios larvares dispersivos, presume-se que o fluxo génico seja principalmente mediado pelo movimento/natação ativa na fase adulta. A homogeneidade genética observada ao longo da distribuição geográfica da espécie sugere que o fluxo genético não é significativamente limitado pela temperatura, salinidade e produtividade oceânica. Por outro lado, foi detectada uma divergência genética pronunciada entre os espécimes australianos e neozelandeses de *C. milii*, o que sugere que as águas oceânicas profundas provavelmente servem como barreiras significativas à dispersão e ao fluxo génico em *Callorhinchus*, devido à incapacidade dos adultos de atravessarem águas profundas. Isto é consistente com a distribuição geográfica mutuamente exclusivas das espécies do género, estando separadas por regiões extensas de oceano profundo. Estes resultados servem como base importante na avaliação da estrutura populacional de *Callorhinchus*, e como referência para estudos futuros sobre a dispersão e o fluxo genético noutros holocefalos.

O DNA mitocondrial (mtDNA) oferece uma abordagem acessível e rentável para estudar a diversidade e diferenciação genética e tem sido amplamente utilizado como marcador de diversidade molecular nas últimas décadas. No geral, parece que os taxa de holocefálicos exibem baixa variação genética em marcadores mitocondriais. Esta observação é consistente com os baixos níveis de evolução molecular detectados em espécies de condrictes. Assim, os resultados podem não representar a extensão total da diversidade genética devido aos marcadores escolhidos serem demasiado conservados. De facto, apesar da região de controlo mitocondrial ser amplamente reconhecida pela sua taxa evolutiva notavelmente rápida, a nossa comparação revelou uma variação genética ainda menor para a CR do que para o CO1, levando a uma separação incompleta de linhagens na nossa análise filogenética e limitando o nosso poder de avaliar a diferenciação genética intraespecífica. Assim, estudos futuros beneficiariam de técnicas moleculares alternativas, como RAD-sequencing ou ressequenciamento de genoma completo, para determinar de forma fiável o padrão de estrutura populacional e diversidade genética ao longo da distribuição da espécie que podem ser ignorados em estudos de locus único. Além disso, é necessário aumentar o equilíbrio no tamanho das amostras para melhorar a robustez e a fiabilidade das nossas análises e desenvolver uma compreensão mais abrangente da estrutura populacional da espécie. Estudos ecológicos que forneçam informações sobre a capacidade de dispersão e o comportamento de movimento do *C. callorynchus*, bem como de outros holocefalos, são também urgentemente necessários para apoiar os esforços de gestão futura, exploração sustentável e conservação.

Palavras-chave — Quimeras; diversidade genética; DNA mitocondrial; história demográfica; hemisfério sul.

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List of Abbreviations

AMOVA – analysis of molecular variance

ARG – Argentina

bp – base pairs

CHIL – Chile

CO1 – cytochrome oxidase subunit 1

CR – control region

df – degrees of freedom

gDNA – genomic DNA

h – haplotype diversity

H – number of haplotypes

HT – haplotype

IBD – isolation by distance

K2P – Kimura-2-parameter

mtDNA – mitochondrial DNA

PCR – polymerase chain reaction

PE – Peru

S – number of segregating sites

Sum Sq – sum of squares

π – nucleotide diversity

CHAPTER 1: General Introduction

Despite growing consensus and awareness about the impact of anthropogenic activities on marine ecosystems, the world's oceans continue to face significant threats that endanger the diversity of species and entire communities. In the face of rapid fisheries and global trade expansion, building a comprehensive understanding of population genetic diversity, structure and connectivity is a keystone in establishing effective species- and population-specific management. Management shortfalls remain particularly problematic in chondrichthyan fisheries, for which the extinction risk is substantially higher than for most other vertebrates (Dulvy *et al.*, 2014, 2021). Chondrichthyans are one of the most ancient, evolutionarily distinct, and ecologically diverse lineages within jawed vertebrates (Dulvy *et al.*, 2014; Stein *et al.*, 2018) and hold many crucial functional roles in marine ecosystem regulation (Stevens, 2000; Ferretti *et al.*, 2010). However, owing to their inherent conservative life-history characteristics (slow growth, late sexual maturity, low fecundity, and high longevity) and ecological susceptibility, their diversity and abundance are highly sensitive to various pressures, notably overfishing and habitat degradation, making them a group of high global conservation concern (Stevens, 2000; Ferretti *et al.*, 2010; Dulvy *et al.*, 2014, 2021).

1.1 Chimaeras (Holocephali)

Building a comprehensive understanding of population structure and dynamics is contingent upon biological, ecological, and evolutionary information. However, research efforts are often focused on charismatic or commercially important species, hindering the development of management strategies for the majority of the (less valued) exploited species and causing biases in conservation efforts (Cardoso *et al.*, 2011; Di Marco *et al.*, 2017; Troudet *et al.*, 2017; Prokop *et al.*, 2022; Caldwell *et al.*, 2024). Such paucity of information is reflected in chimaeras (Holocephali) (Venkatesh *et al.*, 2014), a well-supported ancient lineage within cartilaginous fish (Class Chondrichthyes) that has received very little attention so far. Similarly to their closest living relatives, the elasmobranchs (Order Elasmobranchii) (Nelson, 2006), chimaeras hold a phylogenetically very important position, providing a critical reference for our understanding of vertebrate evolution (Inoue *et al.*, 2010). They are characterised by the fusion of the upper jaw and the neurocranium; the anterior extension of the ethmoid region; and their complete, non-suspensory hyoid arch (Didier, 1995; Grogan *et al.*, 1999; Helfman *et al.*, 2009; Didier *et al.*, 2012). Chimaeras exhibit several distinctive traits from elasmobranchs such as their non-replaceable hypermineralised dental plates, opercular covers, and the frontal tenaculum on the forehead of male individuals in addition to the paired pelvic claspers (Helfman *et al.*, 2009; Farrell, 2011; Didier *et al.* 1994, 2012). The extant chimaeras are represented by the order Chimaeriformes and comprise only three families worldwide: the families Chimaeridae, Rhinochimaeridae, and the monogeneric Callorhinchidae (Inoue *et al.*, 2010; Catarino *et al.*, 2017).

The genus *Callorhinchus* Lacepède 1998 (Chimaeriformes: Callorhinchidae) includes three valid extant species with mutually exclusive geographical distributions in the southern hemisphere (Didier, 1995), that align with the pattern of elevated marine endemism of marine coastal realms (Spalding *et al.*, 2007). Specifically, *C. callorynchus* (Linnaeus, 1758) is restricted to the temperate South American realm, *C. capensis* Duméril, 1865 is found in the temperate waters of South Africa, *C. milii* Bory de Saint-Vincent, 1823 in temperate Australasia, including New Zealand and southern Australia (*sensu* Spalding *et al.* 2007). The external appearance of living Callorhinchidae is characterised by the plough-shaped snout extending forwards from the front of the head, which is the basis of their common name as plough-nose chimaera or elephant fish. While most chimaeras are known to be deep-water dwellers (i.e. occur below 200 m), the three species of the genus *Callorhinchus* also inhabit shallower coastal waters (e.g. 0-600 m; López *et al.*, 2000; Di Dario *et al.*, 2011). Physiological studies of *C. milii* revealed urea-based osmoregulatory mechanisms that allow survival in varying salinities (Hyodo *et al.*, 2007). These mechanims have been hypothesised to be an adaptation to the elephant fish's evolved littoral lifestyle (Hyodo *et al.*, 2007).

1.2 The American elephantfish (*Callorhinchus callorynchus*)

1.2.1 Geographical distribution

The American elephantfish (*C. callorynchus*) is widely distributed along the continental shelf of South America, namely off Brazil, Uruguay, Argentina, Chile, and Peru (López *et al.*, 2000; Chirichigno and Cornejo, 2001; Swing and Béarez, 2006; Di Dario *et al.*, 2011; Cousseau and Perrotta, 2013; Chierichetti *et al.*, 2017; Finucci and Cuevas, 2020). Locally known as "pez gallo", it exhibits a continuous distribution with its presence observed from ~ 23°S in the south-western Atlantic (Rio de Janeiro, Brazil), to ~4°S in the south-eastern Pacific (Peru) (López *et al.*, 2000; Di Dario *et al.*, 2011; Cousseau and Perrotta, 2013; Finucci and Cuevas, 2020). A single observation off Ecuador's coast was attributed to fluctuations in climatic conditions rather than an extended distribution range (Swing and Béarez, 2006).

1.2.2 Biology and Ecology

The American elephantfish is a medium-sized chimaeroid (up to 102 cm total length) (Di Giácomo and Perier, 1994), whose longevity is estimated to range around 14 years for males and 21 years for females (Bernasconi *et al.*, 2015a), with females being larger and heavier than males (Di Giácomo, 1992; Alarcón *et al.*, 2011; Bernasconi *et al.*, 2015a; Chierichetti *et al.*, 2017).

Like other chimaeroids, *C. callorynchus* is an oviparous species with internal fertilization and a mean ovarian fecundity estimated at ca. 8.4 per reproductive cycle (Di Giácomo and Perier, 1994; Chierichetti *et al.*, 2017). Both left and right ovaries are functional, enabling them to lay two eggs at a time (Dean, 1906; Di Giácomo and Perier, 1994; Didier *et al.*, 1998). Long egg incubation periods are relatively widespread among oviparous chondrichthyans in temperate waters that are reproductively active throughout the year (Wourms, 1977). Eggs of *C. milii* Bory de Saint-Vincent, 1823, the Australian and New Zealand representative of the Callorhinchidae, have incubation periods of 10 months (Coakley, 1971), suggesting similar long incubation periods for *C. callorynchus*. Catch compositions of *C. callorynchus* in the Gulf of San Matías, Argentina, have revealed discrete spawning seasons of the species, with the primary spawning season being from July to November (Di Giácomo and Perier, 1994). Size-at-birth was determined at 13 cm in total length (Finucci and Cuevas, 2020).

Callorhinchus callorynchus is associated with soft bottoms of continental and insular shelves at depths between 10 to 481 m, although it is most abundant below 200 m (Finucci and Cuevas, 2020). The species' diet is composed of various benthic organisms, mainly hard-shelled invertebrates, that they crush with their dental plates, i.e., three pairs of tooth plates in the roof of the mouth and lower jaw (Didier *et al.*, 2012; Roman *et al.*, 2020).

The trophic ecology, movement behaviour, and distribution patterns of individuals of the American elephantfish remain largely incomplete across its full geographical range. However, previous studies have indicated a discontinuous distribution of the species in coastal waters of the northern Argentinean Sea (Di Giácomo, 1992). The species' seasonal occurrence in winter and spring is suggested to be influenced by changes in prey availability in the studied area (Di Giácomo, 1992; Chierichetti *et al.*, 2017). Moreover, size-segregation in northern Patagonian waters has been observed with larger individuals occurring at depths greater than 50 m (Di Giácomo, 1992; Alarcón *et al.*, 2011; Bernasconi *et al.*, 2015a). This size-based distribution may be associated to an inshore migratory behaviour for reproduction and egg-laying, a pattern that has been demonstrated in the congener *C. capensis* Duméril, 1865 wherein adults move into deeper waters after breeding, while immature fish remain in inshore environments after birth (Freer and Griffiths, 1993).

1.2.3 Commercial importance and exploitation

The American elephantfish is a target of handline, demersal gillnet, trawl, and longline fisheries year-round throughout its distribution range, but mainly in Argentina and Chile (Alarcón *et al.*, 2011; Bernasconi *et al.*, 2013; Chierichetti *et al.*, 2017; Finucci and Cuevas, 2020). However, in Argentina, *C. callorynchus* is also known as an important resource for recreational and small artisanal fisheries (Cedrola *et al.*, 2011). Moreover, it is often recorded as bycatch in different commercial fisheries (e.g. Lamilla *et al.*, 2008; Góngora *et al.*, 2009; Hernandez *et al.*, 2010). Like other chondrichthyans, populations of the American elephant fish show low biological productivity and low capacity for density-dependent change (weak density-dependent compensation) due to late maturation, slow reproduction, and long gestation periods (Di Giácomo and Perier, 1994; Didier *et al.*, 1998; Bernasconi *et al.*, 2015b; Chierichetti *et al.*, 2017; Finucci and Cuevas, 2020). Therefore, the species should be monitored from a fisheries management and from a species conservation perspective.

1.2.4 Conservation status

Ontogenetic differences in spatial distribution of the American elephantfish, coupled to recent commercial interest and the particularities of its exploitation, determine the degree of a given population vulnerability (Bernasconi *et al.*, 2015b). Therefore, quantifying the impacts of fishing mortality, estimating stock abundance as well as the relationship between parental stock and recruitment success, are fundamental to the development of adequate conservation and management strategies (Hoenig and Gruber, 1990; Bernasconi *et al.*, 2015b). *Callorhinchus callorynchus* has been listed as "Vulnerable" showing decreasing population trends as reported by the IUCN 27/09/2024 16:03:00. Species-specific management implementations are currently limited to some gear restrictions, recreational bag limits, and daily catch limits in some regions of the Argentinean Sea (Venerus and Cedrola, 2017; Finucci and Cuevas, 2020). However, in Chile, the species is still unregulated and there are no catch limits (Aedo *et al.*, 2010).

1.3 Objectives

In this study, we aim to assess the levels and distribution of genetic diversity in the American elephant fish (*C. callorynchus*) using mitochondrial genetic markers, namely the mitochondrial control region (CR) and the cytochrome oxidase subunit 1 gene (CO1). Sequence analyses of samples from both the Pacific (Peru, Chile) and the Atlantic Ocean (Argentina) will provide new insights into genetic connectivity in *C. callorynchus* and inform species-specific management and conservation. We compare and contrast the patterns of population structure obtained in both molecular markers and discuss their suitability for population genetic studies of *Callorhinchus*. Moreover, we provide a first assessment of the levels of intrageneric genetic diversity and divergence in *Callorhinchus* by analysing samples of the congeners *C. milii* Bory de Saint-Vincent, 1823 from New Zealand/Australia, and *C. capensis* Dumeril, 1865 from South Africa/Namibia.

1.4 Approach

Sequencing-based approaches transformed the field of population genetics by unbiasedly sampling genetic variability and providing important data on genetic population structure (Pool *et al.*, 2010). Mitochondrial DNA (mtDNA) has been widely used as a marker of molecular diversity over the last decades due to several favourable properties that make it convenient in practical application: mtDNA's high-copy number within the cell facilitates amplification, saving time and resources compared to nuclear DNA (Galtier *et al.*, 2009). Furthermore, mtDNA amplification via the Polymerase Chain Reaction (PCR) benefits from the availability of "universal" primers (e.g. Cheng *et al.*, 2012), as well as from the amount of pre-existing mtDNA sequence data (Lupi *et al.*, 2010), facilitating new primer design and reducing the need for extensive primer optimisation. Adding to these practical issues, mtDNA's clonal inheritance without recombination simplifies the representation of intraspecific diversity analyses (Galtier *et al.*, 2009). Genetic studies at the population-level furthermore benefit from the high mutation rate reported for the mitochondrial genome; this is particularly so in vertebrates, although Chondrichthyes show a decreased rate of molecular evolution compared to tetrapods or teleost fish (Martin *et al.*, 1992; Martin and Palumbi, 1993; Martin, 1999; Wang *et al.*, 2008; Mulley *et al.*, 2009; Renz *et al.*, 2013). Therefore, we use two popular mtDNA markers to evaluate intraspecific genetic diversity and divergence among *C. callorynchus* individuals sampled in different locations in South America. The origin of heavy-strand replication, also called D-loop or control region (CR), and the cytochrome oxidase subunit 1

(CO1) both have been used in population-level genetic studies in a variety of organisms (Upholt and Dawid, 1977; Wilson *et al.*, 1985; Hebert *et al.*, 2003; Chu *et al.*, 2006; Srivastava *et al.*, 2015; Hikmawaty *et al.*, 2020; Shah *et al.*, 2020; Schroeder *et al.*, 2021). They have been of crucial value in the investigation of population-level genetic differentiation, and their importance and efficiency as a tool in DNA barcoding of fishes have been highlighted in the past (Ward *et al.*, 2005; Xie *et al.*, 2006; Hubert *et al.*, 2008; Hao *et al.*, 2013; Francisco *et al.*, 2022).

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CHAPTER 2: Genetic Structure of the American elephantfish (Chimaeriformes: *Callorhinchus callorynchus*) in South America

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Abstract

Understanding genetic population structure and connectivity is key to the establishment of effective species-specific management and conservation strategies. The American elephantfish *Callorhinchus callorynchus*, is a year-round target of commercial and recreational fisheries in Chile and Argentina. However, the scarcity of studies on chimaeroids´ population structure and connectivity hampers adequate fisheries management of this highly vulnerable group. In this work, we explore the levels of genetic diversity and differentiation within *C. callorynchus* in South America at two widely used mitochondrial markers, the control region (CR) and the cytochrome oxidase I gene (CO1). Moreover, we assess levels of genetic diversity within, and divergence among the three valid extant species of the genus *Callorhinchus*, which exhibit allopatric geographical distributions in the southern hemisphere. Overall, sequence analyses of the mitochondrial CR and the CO1 revealed extremely low levels of sequence variation both within and among *Callorhinchus* species. Genetic homogeneity was found throughout the range of *C. callorynchus* coupled to low-frequency haplotype sharing across spatially distant locations in Chile and Argentina, suggesting gene flow along the South American coast. Moreover, our analyses support a scenario of recent population expansion of the species in South America. Given the absence of dispersive eggs or juvenile stages in chondrichthyans, gene flow is mainly mediated by actively swimming adults. Based on the available data, gene flow in callorhinchids appears to occur along continuous coastal regions, with deep oceanic waters serving as strong barriers, thus providing an important baseline for future research on dispersal and gene flow in other holocephalans.

Keywords — Chimaeras, demographic history, genetic diversity, mitochondrial DNA, Southern Hemisphere

2.1 Introduction

Genetic diversity, i.e. the variability in the genetic code of living organisms, is the most basic and fundamental biodiversity level. Indeed, the genetic diversity harboured among populations of a given species determines its ability to adapt to future environmental or community changes (Reed and Frankham, 2003). But despite the growing awareness and consensus on the importance of protecting global biodiversity, our knowledge on the genetic diversity and distribution patterns of the vast majority of the species is still very limited and often focuses on charismatic, endangered, or commercially important species (Cardoso *et al.*, 2011; Troudet *et al.*, 2017; Di Marco *et al.*, 2017; Prokop *et al.*, 2022; Caldwell *et al.*, 2024). However, limited or no knowledge of the species´ population units can lead to ineffective conservation outcomes and potential overexploitation of certain units (Coates *et al.*, 2018). Building a comprehensive understanding of the distribution and connectivity of population units is therefore fundamental to the establishment of appropriate management strategies and conservation priorities.

Cartilaginous fish (Chondrichthyes) are the most ancient and most evolutionary distinct lineage of jawed vertebrates (Dulvy *et al.*, 2014; Stein *et al.*, 2018). As a chondrichthyan group, chimaeras (Holocephali) hold a phylogenetically very important position, providing a critical reference for our understanding of genetic evolution in vertebrates (Inoue *et al.*, 2010). However, research on holocephalan genetic diversity and structure is scarce. The genus *Callorhinchus* Lacepède 1998 (Chimaeriformes: Callorhinchidae) includes three valid extant species of medium-sized chimaeroids with mutually exclusive geographical distributions in the southern hemisphere (Didier, 1995). Specifically, *C. callorynchus* Linnaeus, 1758 is found in the temperate South American realm, *C. capensis* Duméril, 1865 in the temperate waters of southern Africa, and *C. milii* Bory de Saint-Vincent, 1823 is restricted to temperate Australasia, aligning with the pattern of elevated marine endemism of marine coastal realms (Spalding *et al.*, 2007). Callorhinchids' appearance is strongly characterised by a plough-shaped snout extending forwards from the front of their head, which is the basis of their common name as plough-nose chimaera or elephant fish.

Callorhinchus callorynchus, the American elephant fish, exhibits a continuous coastal distribution from Puerto López in Ecuador to the Brazilian state of Rio de Janeiro (López *et al.*, 2000; Chirichigno and Cornejo, 2001; Swing and Béarez, 2006; Di Dario *et al.*, 2011). *Callorhinchus callorynchus* is a year-round target of handline, demersal gillnet, trawl, and longline fisheries throughout its geographical range, but mainly in Argentina and Chile (Alarcón *et al.*, 2011; Bernasconi *et al.*, 2013; Chierichetti *et al.*, 2017; Finucci and Cuevas, 2020). Moreover, it is often recorded as bycatch in different commercial fisheries (e.g. Lamilla *et al.*, 2008; Góngora *et al.*, 2009; Hernandez *et al.*, 2010). However, as most cartilaginous fish, chimaeroids' diversity and abundance are highly sensitive to various pressures, attributable to their intrinsic conservative life-history characteristics such as slow growth, late sexual maturity, low fecundity, and high longevity (Stevens, 2000; Ferretti *et al.*, 2010; Dulvy *et al.*, 2014, 2021). Overall, it has often been reported as one of the most landed species of cartilaginous (e.g. Jaureguizar *et al.*, 2015; Ruibal Núñez *et al.*, 2018) and has been listed as "Vulnerable" by the IUCN Red List (Finucci and Cuevas, 2020).

This work constitutes the first investigation into the genetic diversity and distribution of *C. callorynchus*, aiming to contribute to a better understanding of population structure and demographic processes in the species. For this purpose, the genetic diversity at two mitochondrial genetic markers will be screened in sample collections obtained along the species´ distribution range. Based on the general trend of low genetic diversity reported for chondrichthyans (Martin *et al.*, 1992; Martin and Palumbi, 1993; Martin, 1999; Wang *et al.*, 2008; Mulley *et al.*, 2009; Renz *et al.*, 2013), we hypothesise that analyses of mitochondrial genetic markers will reveal low levels of genetic variation in samples of *C. callorynchus* from different geographical regions in South America. Given the distinct environmental features and geological histories of the Atlantic and Pacific coasts of the South American continent, shaping spatial divergence and genetic heterogeneity of species (Peterson and Whithworth, 1989; Camus, 2001; Acha *et al.*, 2004; Spalding *et al.*, 2007, Montecino and Lange, 2009; Miloslavich *et al.*, 2011; Meuser *et al.*, 2013; Artana *et al.*, 2019; Orúe-Echevarría *et al.*, 2021), the main question pertains to whether the American elephant fish forms a single population unit or, whether there are multiple population units throughout its distribution range. Moreover, we explore levels of intrageneric diversity and divergence in *Callorhinchus* by including samples of the congeners *C. milii* Bory de Saint-Vincent, 1823 from New Zealand and Australia, and *C. capensis* Dumeril, 1865 from South Africa and Namibia in our genetic analyses. We anticipate that the low levels of variation in mitochondrial sequences expected for *C. callorynchus* will be similarly low in the available samples of the congeners *C. capensis* and *C. milii*.

2.2 Material and Methods

2.2.1 Sampling

Tissue samples of *C. callorynchus* were collected from specimens obtained at different sampling locations off the Pacific and the Atlantic coasts of South America (Table 2.1, Table S1). The specimens were obtained year-round between 2021 and 2023 from catches of commercial fisheries. To visualise the sampling spatial distribution, QGIS software (v. 3.34; QGIS.org, 2024) was used to create a map with sampling locations (see Fig. 2.1). Furthermore, specimens of *C. milii* were caught off the coasts of New Zealand during May 2014 and 2021, and July of 2023, and of *C. capensis* were caught off South Africa during August 2018. All tissue samples were preserved in 100% ethanol and stored at -20°C until DNA extraction.

2.2.2 DNA isolation

Genomic DNA (gDNA) was isolated using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Mississauga, ON, Canada) following the manufacturer´s protocols. DNA isolation included RNase treatment using 4 µl of RNase A stock solution (10 mg/mL) per sample to remove contaminating RNA. Selectively bound gDNA was eluted from the DNeasy membrane in two elutions, to maximize DNA yield. To test the quality of gDNA extractions, both elutions were visualised upon electrophoresis on 0.8% agarose gel with GelRed (Biotium, Fremont, CA, U.S.A.), run on 0.5X TAE buffer at 300 V.

2.2.3 Mitochondrial DNA amplification

To analyse population genetic structure within *C. callorynchus* representatives in South America, we analysed two mitochondrial markers. Oligonucleotide primers were newly designed and used for the amplification of 506 base pairs (bp) of the control region (CR) and

458 bp of the cytochrome oxidase subunit 1 gene (CO1) using the polymerase chain reaction (PCR) (Table 2.1). To establish zones suitable for primer design, nucleotide sequences of the mitochondrial target genes and adjacent regions of all three *Callorhinchus* species were extracted from the NCBI nucleotide database (HM147135, HM147136, HM147137; available at https://www.ncbi.nlm.nih.gov/nucleotide/, accessed February 2024) and aligned using the software Geneious Prime 2023.2.1 (https://www.geneious.com, accessed February 2024). Primer characteristics were analysed *in silico* using the online tool OligoAnalyzer, available from Integrated DNA Technologies (accessible at https://www.idtdna.com/pages/tools/oligoanalyzer accessed February 2024) to exclude primer pairs leading to hairpins, self-dimers, or primer-dimers.

Table 2.1 New primers (F: forward, R: reverse) designed for the amplification of 516 bp of the mitochondrial control region (CR) and 458 bp of the cytochrome c oxidase subunit I gene (CO1) in *Callorhinchus*. bp: Primer length in base pairs; GC: Guanine-cytosine content in percent (%); T_m: Primer melting temperature in degrees Celsius (°C); T_a: temperature of annealing in the PCR temperature profile.

Marker	Primer	Sequence 5’→3’	bp	GC	T_m	T_a
CR	CR-HF1	GYCCTGGTCTTGTAAACCARAG	22	50	60.3	62
	CR-HR1	RTGCGGAAACTTGCATGTGTAAG	23	46	59.8	62
CO1	CO1-CaF1	ATCATAAAGATATTGGCACCCTC	23	39	57.1	62
	CO1-CaR1	AGATTATACCGAAACCAGGTAGG	23	43	58.9	62

Target fragments of CO1 and CR were amplified via PCR consisting of an initial denaturation at 94°C for three minutes, followed by 35 cycles with 60 seconds of denaturation at 95°C, 60 seconds of primer annealing at 62°C and an elongation phase of 60 seconds for CO1 and 90 seconds for CR at 72°C, and a final extension step of five minutes at 72°C. The PCR reaction mix contained 2.5 µl autoclaved H_2O, 2.5 µl of MyTaq™ HS Mix (Bioline, London, UK), 0.2 µl of forward (L) and reverse (R) primer (10 µM), and 0.6 µl of gDNA adding up to a final volume of 5 µl. PCR products were loaded and visualised on 2% agarose gel upon electrophoresis (as described above) and purified with 1 µl of ExoSap-IT™ (Thermo Fisher Scientific, Waltham, MA USA) following the manufacturer´s instructions. Purified amplicons were processed for Sanger sequencing in both directions using the Big-Dye™ Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific, Waltham, MA USA) following the manufacturer’s instructions.

2.2.4 Sequence data analysis

The chromatograms obtained from Sanger sequencing were proofread in Geneious, manually edited, and the resulting nucleotide sequences were aligned using the built-in geneious algorithm. Publicly available nucleotide sequences of CO1 from all three *Callorhinchus* species were retrieved from the BOLD database (v. 4; Barcode of Life Data System; https://www.boldsystems.org, accessed 28th August 2024) (see Appendix, Table S2), and aligned with the sequences obtained through PCR. For subsequent statistical analyses, the homologous sequence alignments were saved using the FASTA format (Goldstein *et al.*, 2014, 2015) and read into R 4.3.1 (R Core Team, 2023) using the packages 'ape' (v. 5.7.1; Paradis and Schliep, 2019) and 'haplotypes' (v. 1.1.3.1; Aktas, 2023).

2.2.5 Genetic diversity analysis

Several genetic diversity indices were estimated for each *Callorhinchus* species, sampling location, and molecular marker, namely S: number of segregating sites, π: nucleotide diversity, *h*: haplotype diversity), using the 'pegas' package (v. 1.3; Paradis, 2010) in R. Overall mean genetic distances were calculated for each marker and species based on the Kimura-2 parameter (K2P) method in MEGA (v. 11.0.13; Kumar *et al.*, 2018). To explore divergence among species of the genus *Callorhinchus*, K2P distances were also calculated among *C. callorynchus*, *C. milii*, and *C. capensis* using CO1 data as well as the whole mitogenome. Whole mitogenome sequences were retrieved from GenBank® (www.ncbi.nlm.nih.gov/genbank/, accessed 28th August 2024; accession codes: HM147135, HM147136, HM147137).

The relationships among haplotypes in *Callorhinchus*, as well as their frequency and spatial distribution, were investigated for each molecular marker separately by the construction of haplotype networks using the TCS method (Templeton *et al.*, 1992) (Clement *et al.*, 2000) implemented in the software PopArt (v. 1.7; Leigh and Bryant, 2015). The online toolbox FaBox (v. 1.6; Villesen, 2007; https://users-birc.au.dk/~palle/php/fabox/index.php, accessed 12th June 2024) was used to create NEXUS files (Maddison *et al.*, 1997) with data and analysis settings included using the fasta2mrbayes tool. A GEOTAGs block was manually added to the NEXUS files as required by PopART to assign each sequence to the geographical sampling coordinates of the associated specimen. The final NEXUS files were imported into PopART. Final graphic editing was performed in the software Inkscape (v. 1.3.2; https://inkscape.org, accessed 13th September 2024).

2.2.6 Population genetic analysis

To estimate the levels of genetic differentiation between the different sampling collections of *C. callorynchus* based on haplotype diversity and frequency, a pairwise Phi-statistic (Φ_{ST}) was performed using the R package 'haplotypes' (v. 1.1.3.1; Aktas, 2023). Statistical significance for α=0.05 was evaluated upon correction for multiple testing using a strict Bonferroni correction (α/no. of pairwise comparisons; Bonferroni, 1936). To test the null hypothesis of genetic homogeneity between the American elephant fish from the two coasts of South American (Pacific: CHIL; Atlantic: ARG), an analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) was conducted using the 'poppr' package (v. 2.9.6; Kamvar *et al.*, 2014, 2015) in R. The significance of variance components and derived Φ-statistic were estimated over 1000 permutations using the 'ade4' R package (v. 1.7-22; Chessel *et al.*, 2004; Dray and Dufour, 2007; Dray *et al.*, 2007; Bougeard and Dray, 2018; Thioulouse *et al.*, 2018).

A scenario of isolation-by-distance (IBD) was tested using a generalised least square regression with correlated error structure between geographic and genetic distances using the R packages 'corMLPE' (v.1.0; Clarke *et al.*, 2002) and 'nlme' (v. 3.1-162; Pinheiro and Bates, 2000; Pinheiro *et al.*, 2024), to account for the non-independence of distance matrices. Pairwise genetic *p*-distances among sample collections were calculated in MEGA (v. 11.0.13; Kumar *et al.*, 2018). Geographic distances were calculated as least-cost distances between each pair of sampling sites in South America using the R packages 'marmap' (v. 1.0.10; Pante *et al.*, 2023) and 'gdistance' (v. 1.6.4; Van Etten, 2017), with a resolution of 10 minutes. The least-cost distances were restricted to a bathymetric range between 1-500 m of depth, representing the typical range inhabited by *C. callorynchus* to ensure that all computed paths followed alongshore routes above the 500 m isobath.

2.2.7 Demographic analysis

To test spatial or demographic expansion**,** Tajima’s *D* (Tajima, 1989), Fu’s *F*s (Fu, 1997), and Ramos-Onsins' & Rozas' R_2 (Ramos-Onsins and Rozas, 2002) statistics were estimated using the program DNAsp (v. 6.12.03; Rozas and Rozas, 1995; Rozas *et al.*, 2017). All parameters are expected to be equal to zero under the hypothesis of selective neutrality of genetic variation within the group, and significant deviations are interpreted in terms of historical shifts in effective population sizes. For instance, significant negative values can be interpreted as signatures of population expansion. The statistical significance of Tajima's *D* was tested for a significance level of α=0.05; Fu's *F*s and Ramos-Onsins' & Rozas' R_2 values were estimated by generating 1,000 random samples and the 95% confidence interval was calculated. All values were calculated based on an infinite-site model without recombination. Historical demographic patterns of both groups were further explored using mismatch distributions (Li, 1977) as implemented in DNAsp. Harpending's 'raggedness' index (*r*) (Harpending *et al.*, 1993) was used to quantify the smoothness of the distributions as an indicator of population expansion.

2.2.8 Phylogenetic relationship among haplotypes

To illustrate the evolutionary relationships among haplotype we first obtained a list of unique haplotypes in each dataset using the DNA haplotype collapser and converter in the online toolbox FaBox (v. 1.6; Villesen, 2007; https://users-birc.au.dk/~palle/php/fabox/index.php, accessed 12th June 2024), to then align unique haplotypes in each dataset using Geneious Prime 2023.2.1 (https://www.geneious.com, accessed February 2024). The NEXUS file of each alignment was imported into MEGA (Kumar *et al.*, 2018) to construct phylogenetic trees for each molecular marker separately, using number of differences as a distance measure and the neighbor-joining method (Saitou and Nei, 1987). Branch support was calculated via 1000 bootstrap replicates to assess the reliability of the phylogeny (Efron, 1982; Felsenstein, 1985). Trees were edited in Inkscape (v. 1.3.2; https://inkscape.org).

2.3 Results

In total, 68 sequences were generated for the mitochondrial control region (CR) of *C. callorynchus*. The primers that were specifically designed for the amplification of the CR (Table 2.1) performed well, yielding a distinct PCR product of uniform length. However, sequencing of the CR at full length was unsuccessful. Consequently, a fragment of the 5'-end of the CR was amplified and analysed (506 bp). For the cytochrome c oxidase subunit, I gene (CO1), 61 sequences were generated (458 bp). Analyses of CO1 were complemented by 86 sequences retrieved from the BOLD database (https://www.boldsystems.org, accessed 28th August 2024) (see Appendix, Table S2).

2.3.1 Genetic diversity analyses

Overall, the genetic diversity at each marker was very low, as indicated by the low values of nucleotide diversity (π), number of haplotypes (H) and segregating sites (S) (Table

2.2, Fig. 2.1). However, diversity levels were not equal among sampled sites: haplotype diversity (*h*) showed high location-dependent discrepancies for CR and CO1 (Table 2.2, Fig. 2.1). Despite the small sample size, the Peruvian sample (PE) showed the highest genetic diversity values compared to the Chilean (CHIL) or Argentinian samples (ARG), both in terms of π and *h*, while CHIL revealed the lowest genetic diversity at both markers.

Table 2.2 Genetic diversity indices for all sample collections of *C. callorynchus* based on sequence data of mitochondrial CR and CO1. Individuals are grouped into sample collections according to latitude of capture. N: number of individuals, H: number of haplotypes, S: number of segregating sites, π: nucleotide diversity, and *h*: haplotype diversity; PE: Peru, CHIL: Chile, ARG: Argentina.

		N	H	S	π	*h*
CR		**68**	**8**	**8**	**0.0016**	**0.4096**
PE (12°S)		7	4	6	0.0047	0.8095
CHIL	overall	29	3	2	0.0003	0.1355
	33°S	15	2	1	0.0003	0.1429
	39°S	14	2	1	0.0003	0.1429
ARG (43°S)		32	4	3	0.0012	0.3810
CO1		**147**	**19**	**18**	**0.0013**	**0.4483**
PE (12°)		7	2	2	0.0025	0.5714
CHIL	overall	98	16	16	0.0010	0.3987
	29°S	9	5	4	0.0019	0.7222
	33°S	20	3	2	0.0008	0.3579
	36°S	9	3	2	0.0013	0.5556
	39°S	10	2	2	0.0009	0.2000
	NA	67	7	6	0.0009	0.4419
ARG	overall	42	9	8	0.0012	0.4901
	37°S	3	3	2	0.0029	1.0000
	43°S	32	7	6	0.0012	0.4839
	46°S	7	2	1	0.0006	0.2857

The haplotype networks for the CR (Fig. 2.1A) and CO1 (Fig. 2.1B) showed a star-shaped conformation, with a predominant central haplotype shared by the majority of individuals across the different sample collections with several low-frequency derived haplotypes (Fig. 2.1). Both molecular markers revealed low levels of haplotype divergence, generally characterised by one to two substitutions between haplotypes, with the maximum being four.

In total, the CR network comprised eight distinct haplotypes (Fig. 2.1A) and showed higher haplotype diversity in the small Peruvian sample collection (N=7; see Table 2.2). Except for the two specimens sharing HT2 with one specimen from CHIL and two from ARG, all specimens from PE had exclusive haplotypes (HT3, 6, 8). They are separated from the most common haplotype (HT1) by one to four mutations while other haplotypes in the network are separated from the central haplotype by one or a maximum of two mutations. In the CO1 network (Fig. 2.1B), we can observe 19 haplotypes in total. They all differ from the central haplotype by one or a maximum of two mutations. Unlike in the CR network (Fig. 2.1A), some individuals from PE share the most common haplotype (HT1) with the rest of the sample collections. All locations display low-frequency derived haplotypes. Additionally, six of these derived haplotypes are shared between CHIL and ARG (HT4, 5, 6, 10, 11, 16).

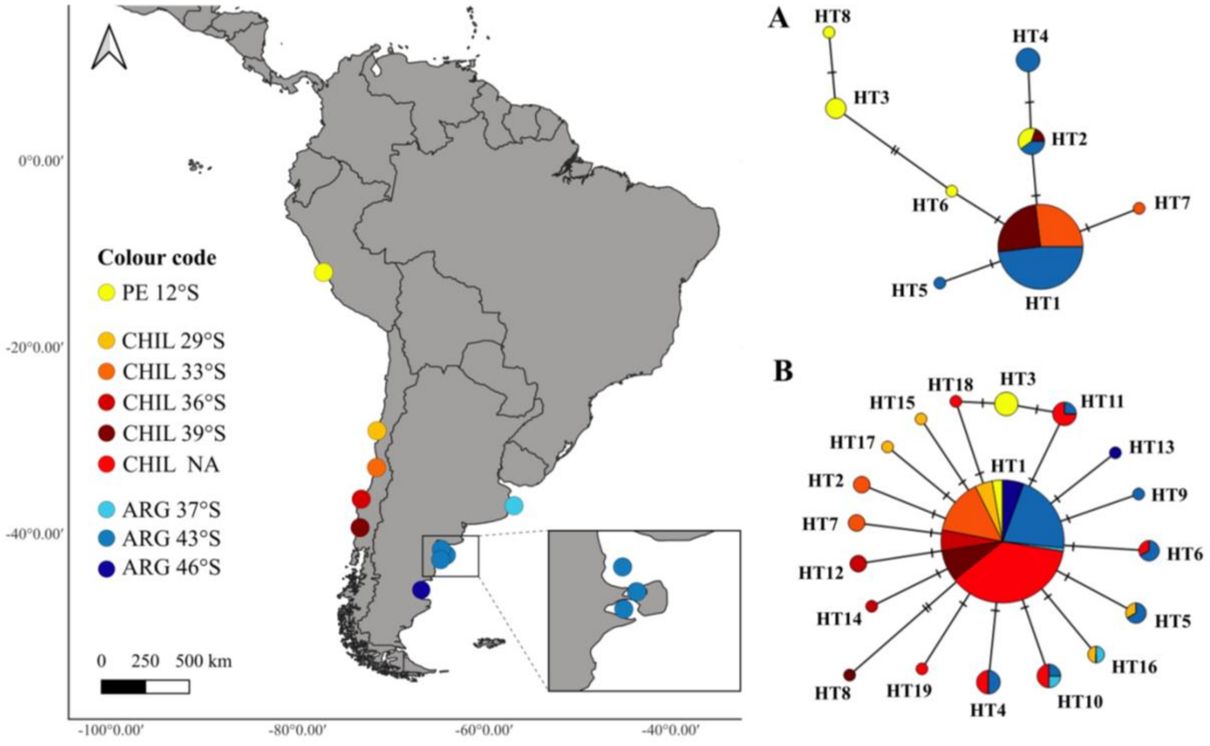

Figure 2.1 Map of South America with sample locations and TCS haplotype networks of *Callorhinchus callorynchus* (Linnaeus, 1758) based on A) 506 bp of the CR (N=68), and B) 458 bp of the CO1 gene (N=147). Numbering of haplotypes (HT) is independent for each genetic marker (see Appendix, Table S2). Circle sizes depict frequencies of haplotypes across all samples; numbers of mutations between haplotypes are visualised with hatch marks. Colour codes correspond to sampling locations: PE: Peru (latitude: 12°S), CHIL: Chile (latitudes: 29°S, 33°S, 36°S, 39°S), ARG: Argentina (latitudes: 37°S, 43°S, 46°S).

2.3.2 Population genetic analysis

The pairwise comparison of the genetic diversity between the Chilean (CHIL) and Argentinean (ARG) sample collections based on Φ_{ST} (Table 2.3) revealed no significant genetic differentiation among sampled sites of *C. callorynchus* upon correction for multiple tests.

Table 2.3 Pairwise Phi-statistics (Φ_{ST}) based on mitochondrial sequence data from four sample collections of *C. callorynchus*. Above diagonal: CR, below diagonal: CO1; no value was significant upon strict Bonferroni correction for multiple tests ($P < 0.005$).

	CHIL 29°S	CHIL 33°S	CHIL 36°S	CHIL 39°S	ARG 43°S
CHIL 29°S		NA	NA	NA	NA
CHIL 33°S	0.0479		NA	0.0002	0.0690
CHIL 36°S	0.0357	0.0925		NA	NA
CHIL 39°S	0.0045	0.0279	0.0549		0.0129
ARG 43°S	0.0080	0.0300	0.0587	0.0012	

For the AMOVA, sample collections were grouped into two regions corresponding to the Atlantic (East) and Pacific coasts (West) to test the null hypothesis of genetic homogeneity between the two continental margins of South America (Table 2.4). Due to small sample size (n=7) the Peruvian samples were excluded from the AMOVA. The differences within sample collections constitute the main source of variation in our data (variance %). Genetic differentiation between the West (CHIL) and East (ARG) coasts is not statistically significant for either of the two markers, corresponding to $\Phi_{ST} = 0.043$ and -0.004 for the CR (P=0.1) and CO1 (P=0.59), respectively.

Table 2.4 Analyses of molecular variance (AMOVA) based on CR and CO1 sequence data between *C. callorynchus* of the West (Chilean) and East (Argentinean) coasts of South America. df: degrees of freedom, Sum Sq: Sum of Squares.

Marker	Source of variation	df	Sum Sq	Variance component	Variance %
CR	Between regions	1	0.624	0.012	4.275
	Within regions	59	15.606	0.265	95.725
CO1	Between regions	1	0.331	-0.002	-0.379
	Within regions	138	58.769	0.426	100.379

Additionally, the IBD analysis based on least-square regression of distance matrices (Fig 2.2) indicated a tendency for genetic distances to increase with geographic distances among sampling locations based on the CO1 marker. However, this relationship was not significant as the 95% confidence interval of correlation estimate included zero (estimate: 0.33; 95% CI: -0.48 to 1.14).

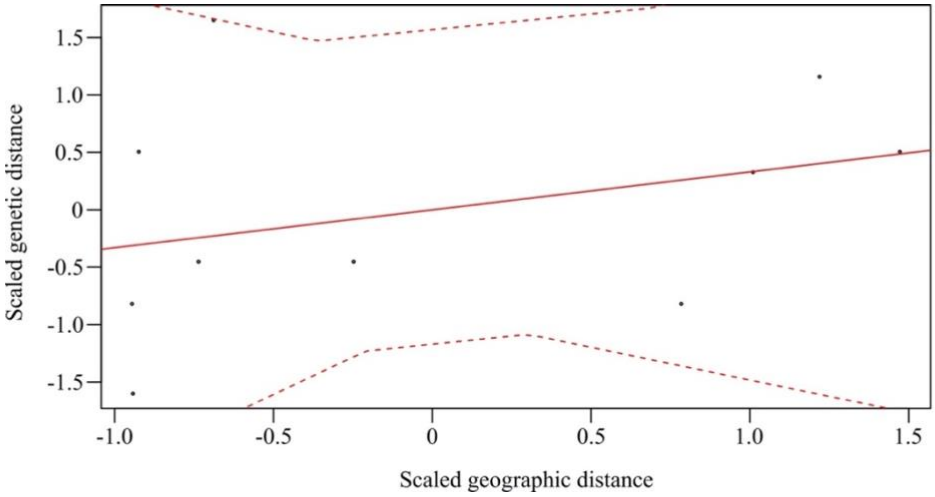

Figure 2.2 Least-square regression model of distance matrices for *Callorhinchus callorynchus* (Linnaeus), 1758. x-axis: scaled pairwise genetic p-distance values based on CO1 sequences; y-axis: scaled least-cost distances over 500 m between pairs of sampling locations.

2.3.3 Demographic analysis

Given the genetic homogeneity among sample collections as described above, the results of Tajima’s *D*, Fu’s *Fs*, and Ramos-Onsins' & Rozas' R_2 tests were calculated for a group including all samples of *C. callorynchus*, with exclusion of the Peruvian samples (n=7). Estimates of Tajima's *D* and Fu's *Fs* were negative for both molecular markers (Table 2.5); however, only the values of Tajima´s *D* for CO1 and Fu´s *Fs* for CR were statistically significant. Ramos-Onsins' & Rozas' R_2 statistics, which is more robust for small sample sizes, showed statistically significant positive values for both markers, pointing towards population expansion.

Table 2.5 Estimated demographic parameters Tajima's *D*, Fu's *Fs*, and Ramos-Onsins & Rozas' R_2 based on mitochondrial sequence data (CR, CO1) of *C. callorynchus* (including all samples from Chile and Argentina together); asterisks indicate significant values.

Marker	Tajima's *D*	Fu's *Fs*	Ramos-Onsins' & Rozas' R_2
CR	-1.16582	-2.491*	0.0608*
CO1	-2.3221*	-24.020	0.0182*

The mismatch distributions (Fig. 2.3) of the CR (Fig. 2.3A) and CO1 data (Fig. 2.3B) revealed a mostly smooth distribution of pairwise nucleotide differences, characteristic of recent population expansion, in line with the results from neutrality tests (Table 2.5). The observed mismatch distribution based on the CR (Fig 2.3A) showed a slight deviation from the neutrality expectation. However, the raggedness statistic of mismatch distribution analyses (*r*) was low for both markers, confirming the fit of the data to a unimodal distribution.

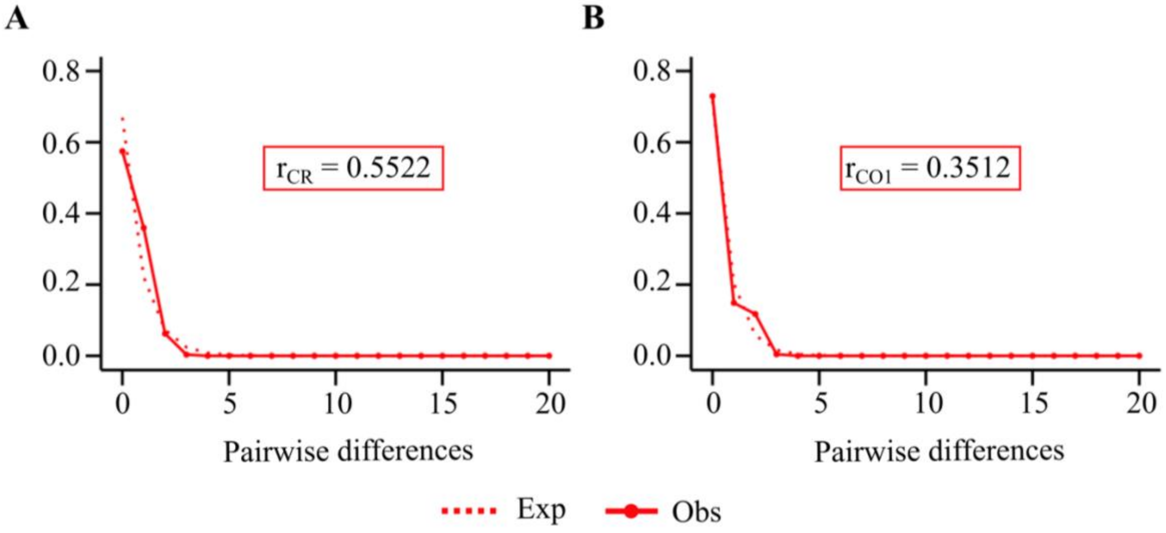

Figure 2.3 Mismatch distributions and Harpending's raggedness index (*r*) of *Callorhinchus callorynchus* (Linnaeus, 1758) (including all samples from Chile and Argentina together) based on A) CR, and B) CO1. x-axis shows number of pairwise differences; y-axis shows the frequency of the pairwise comparisons. Exp: Expected frequencies under the population expansion model; Obs: Observed frequencies.

2.3.4 Phylogenetic relationships among haplotypes

The phylogenetic trees based on CR (Fig. 2.4A) and CO1 (Fig. 2.4B) showed the existence of two main clades of *Callorhinchus* haplotypes. However, while CR haplotypes of *C. capensis* cluster together (bootstrap support (BS): 69%), *C. milii* haplotypes cluster in the same clade as haplotypes of *C. callorynchus* (Fig. 2.4A), pointing towards incomplete lineage sorting in the CR data. Moreover, two haplotypes of *C. milii* (HT11, HT12) form a visible subgroup with one haplotype of *C. callorynchus* (HT4, BS: 54%). The haplotypes exclusive to PE (HT3, HT6, HT8) form a separate cluster within *C. callorynchus*, albeit with low bootstrap support (BS: 50%). The above pattern seen for the CR is distinct from the one observed for CO1 (Fig. 2.4B), where haplotypes from each species of *Callorhinchus* form monophyletic clades, albeit with differing bootstrap support values. The *C. callorynchus* clade had high bootstrap support (BS: 94%) but no spatial structure was evident in the tree; indeed, relationships were not resolved except for two subgroups, represented by two haplotypes each (HT1 and HT2, BS: 100%; HT3 and HT18, BS: 52%). *C. capensis* forms a well-supported subgroup (BS: 78%) while the *C. milii* clade had the lowest support (BS: 51%). Interestingly, *C. milii* haplotypes were spatially structured: those exclusive to Australia (HT27, 28) formed a highly supported subgroup (BS: 99%), nested within the haplotypes represented by individuals from New Zealand (BS: 63%).

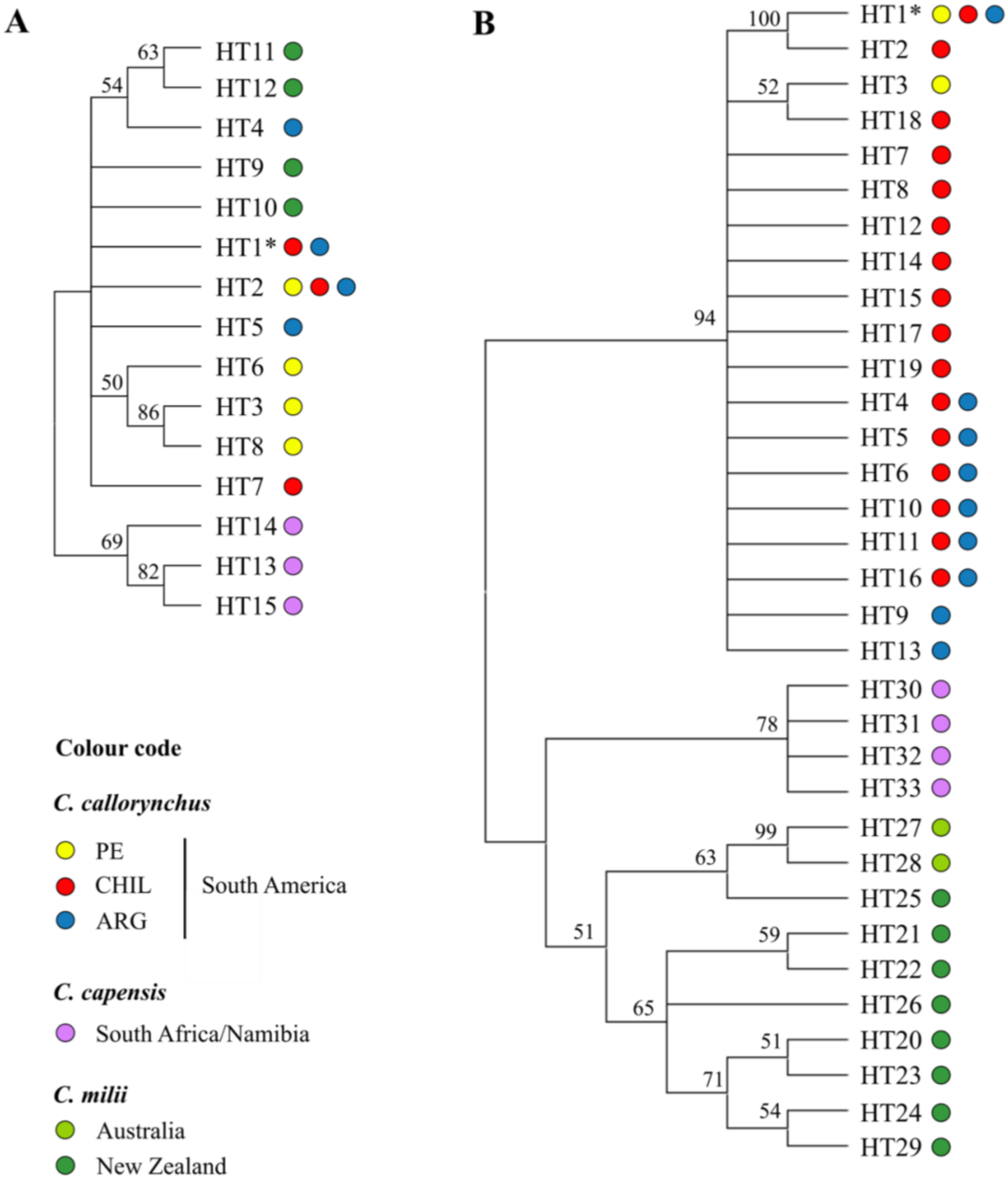

Figure 2.4 Neighbour-joining trees of *Callorhinchus* Lacepède, 1798 based on A) CR (506 bp, N=72); and B) CO1 (458 bp, N=147). Colour code describes sampling location: PE: Peru, CHIL: Chile, ARG: Argentina. Haplotype numbering and colour code of sampling locations correspond to haplotype networks in Fig. 2.1; asterisks indicate the most common haplotype; bootstrap support values >50 are shown over their respective nodes.

2.3.5 Interspecific comparisons

Sequence data for CO1 shows higher genetic diversity in *C. capensis* and *C. milii* compared to *C. callorynchus,* both in terms of higher haplotype (*h*) and nucleotide diversities (π), and despite their considerably smaller sample sizes (Table 2.6). The higher diversity in the CO1 dataset of *C. milii* was particularly evident in the comparison among congeners.

Table 2.6 Genetic diversity indices for the three species of *Callorhinchus* based on sequence data of the mitochondrial CO1 gene. N: number of individuals, H: number of haplotypes, S: number of segregating sites, π: nucleotide diversity, and *h*: haplotype diversity.

Species	N	H	S	π	*h*
C. callorynchus	147	19	18	0.0013	0.4483
C. capensis	10	4	6	0.0032	0.5330
C. milii	23	10	16	0.0137	0.7708

The K2P overall mean distances (Table 2.7) between individuals within *C. callorynchus* equalled 0.16% and 0.13% for CR and CO1 respectively, reflecting the low intraspecific diversity observed before (Table 2.1). Overall mean distances in *C. milii* and *C. capensis* were considerably higher compared to *C. callorynchus*, confirming the results of Table 2.6. *Callorhinchus milii* showed the highest diversity between individuals, with K2P distances being equal to 0.20% for CR and 1.53% for CO1. Among species, K2P distances ranged between 1.92-2.62% based on CO1 and 1.34-1.82% based on the mitogenome, demonstrating the overall low levels of diversity between each pair of species within *Callorhinchus*.

Table 2.7 Summary of Kimura-2-parameter (K2P) genetic distances (%) calculated for different markers and taxonomic levels.

Taxonomic level of comparison	Taxon name	Marker	K2P
Within species	*C. callorynchus*	CR	0.16
		CO1	0.13
	C. capensis	CR	0.29
		CO1	0.35
	C. milii	CR	0.20
		CO1	1.53
Between species	*Callorhinchus*	CO1	1.92-2.62
		Full mitogenome	1.34-1.82

The *Callorhinchus* CO1 haplotypes revealed four distinct haplogroups separated by six to seven mutations each, and corresponding to the three currently accepted species (Fig. 2.5). Haplotypes of *C. milii* were further divided into two geographically distinct groups: one consists of haplotypes found exclusively in Australia, while the other includes only haplotypes from New Zealand specimens. The divergence observed within *C. milii* is marked by a minimum of seven mutations, which is therefore as pronounced as the separation between *C. callorynchus* and *C. capensis* haplotypes.

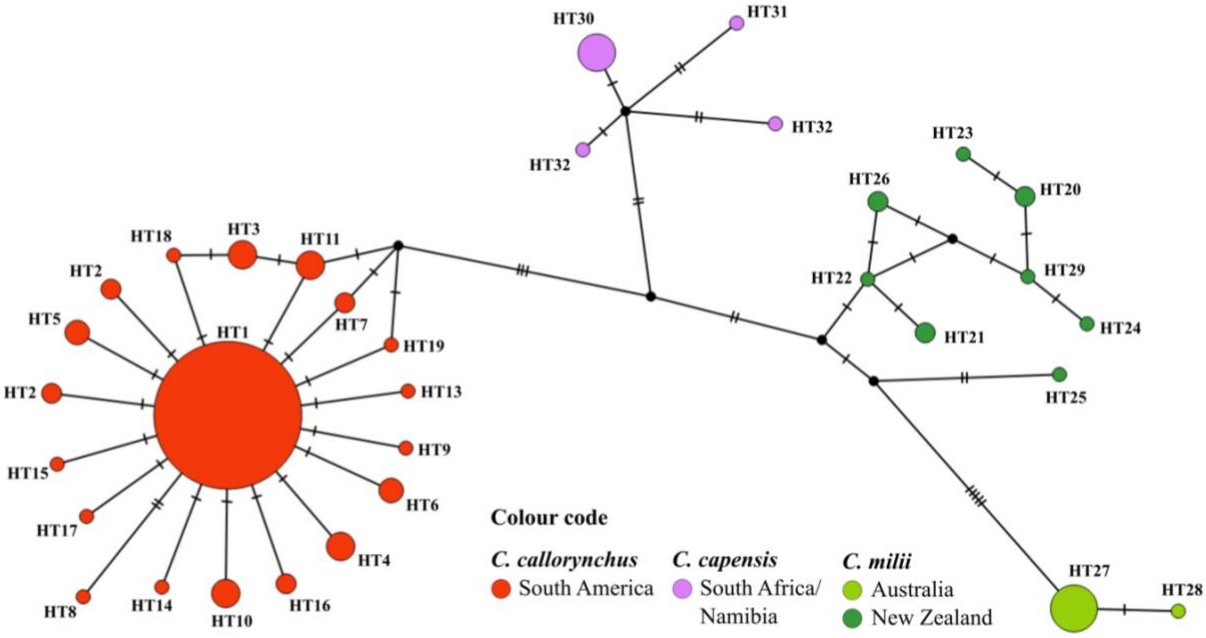

Figure 2.5 TCS haplotype network based on CO1 sequence data of *Callorhinchus* Lacepède, 1798. Circle sizes depict frequencies of haplotypes; numbers of mutations between haplotypes are visualised with hatch marks. Haplotype numbering of *C. capensis* and *C. milii* corresponds to tree in Fig. 2.4B; colour codes correspond to sampling locations.

2.4 Discussion

2.4.1 Low genetic diversity and weak population structure in *C. callorynchus*

This study is the first to provide insight into the patterns of population genetic diversity and differentiation of the vulnerable American elephantfish across its known distribution range, based on analyses of two mitochondrial markers. Our results show remarkably low genetic diversity in *C. callorynchus*, as shown by the low number of differences among sequences and low nucleotide diversity in both the CR and CO1 (see Table 2.2). The Chilean sample consistently showed the lowest level of diversity for each marker, both in terms of nucleotide and haplotype diversities, while the highest values were observed in the Peruvian sample

(Table 2.2). However, the high diversity levels in the Peruvian sample should be interpreted with caution in light of the limited sample size (n=7). We therefore decided to exclude it from further analyses to avoid potential biases in the results. Future studies should aim to adequately sample locations on the northern-most edges of the species´ distribution on both coasts of South America, as they may exhibit unique genetic diversity and distinct population units. Nevertheless, overall variation in the mtDNA of *C. callorynchus* is evidently very low, even among *Callorhinchus* as further supported by the low mean K2P distances (see Table 2.3). The results of our AMOVA (Table 2.4), the observed haplotype sharing among all sampling locations (Fig. 2.1), and the unresolved phylogenetic relationships among haplotypes (Fig. 2.4), moreover suggest that the distribution of the genetic variation among samples of *C. callorynchus* is not spatially structured. Most importantly, it suggests the absence of significant genetic differentiation between the samples from the Atlantic and Pacific coasts.

2.4.2 A phylogeographic perspective on *C. callorynchus* population structure

Previous studies collectively underscored the complex interaction of various ecological and environmental factors demarcating marine biogeographic provinces along the South American coast (e.g. Camus, 2001; Spalding *et al.*, 2007), that have important implications for spatial divergence and genetic heterogeneity of species within these realms. Since the American elephantfish is an oviparous species, depositing and attaching eggs on bottom substrates, it is likely that dispersal and gene flow is mediated by actively swimming adults, as is also the norm for Elasmobranchs. Thus, the genetic homogeneity observed in *C. callorynchus* throughout its distribution range in temperate and austral South America, suggests that soft environmental barriers, such as climatic phenomena and mesoscale variabilities affecting ocean temperature, salinity, and productivity, do not significantly restrict gene flow. Comparison of these results with those of other marine organisms with similar distributions are difficult as marine biogeographical studies throughout the area are limited, and most species analysed have a planktotrophic dispersal stage in their life cycle (e.g. Hernández *et al.*, 2005; Moreno *et al.*, 2006; Cárdenas *et al.*, 2009; Lancellotti and Vásquez, 2009; Brante *et al.*, 2012; Barahona *et al.*, 2019; Lara *et al.*, 2019). Moreover, previous studies carried out at the population genetic level did not find consistent patterns of species distributions and population dynamics across regions.

2.4.3 Recent demographic expansion in *C. callorynchus*

The extent of genetic structure of a species is not solely determined by the amount of past and/or current gene flow but by the impact of historical and demographic factors. Our genetic data exhibits several key characteristics that suggest a recent and rapid demographic expansion of *C. callorynchus* in South America. The star-like topology of the haplotype networks (Fig 2.1) highlights a central most common haplotype surrounded by many low-frequency derived variants. While the sharing of the central haplotype across all sampled locations of both the Atlantic and Pacific coasts suggests a common widespread ancestral population, the many low-frequency derived haplotypes are consistent with a recent evolutionary origin from the common one during a population expansion event. This scenario is also supported by the smooth and unimodal pattern of the mismatch distributions (Fig 2.3), and the neutrality tests (Table 2.5). The lack of statistical significance in some of these tests, combined with discrepancies between test results between the two markers, are potentially attributed to the few nucleotide differences among haplotypes noted in the previous diversity assessments. However, Ramos-Onsins and Rozas (2002) demonstrated that the power of R_2 is comparably higher when the number of segregating sites is low. Taken together, the integration of these results supports a scenario of a recent expansion of a single widespread population along the South American coast.

2.4.4 Identifying barriers to gene flow in holocephalans

The sharing of low-frequency derived COI haplotypes among distant sampling locations (Fig. 2.1), and the absence of genetic differentiation between regions in the AMOVA (Table 2.4), indicate the presence of gene flow in *C. callorynchus* along the South American coast ranging from Argentina to Chile. In short, *C. callorynchus* seems to be capable of dispersing over long distances alongshore. On the other hand, the results showed a tendency for genetic distances to increase with geographic distances (Fig. 2.2), although the signal was not strong enough to allow definitive conclusions. Future studies are needed to ascertain if this relationship is robust by adding more sampling locations and possibly larger sample sizes.

In contrast, the intraspecific divergence observed between Australian and New Zealand specimens of *C. milii* suggests that the deep oceanic waters in the Tasman Sea likely serve as significant barrier to dispersal and gene flow, as it has been shown in several coastal marine species (Grewe *et al*., 1994; Ward and Elliott, 2001). This is consistent with the distinct species distributions in the genus, with each species inhabiting mutually exclusive geographic regions. As noted before, *C. callorynchus* is confined to the South American coasts, *C. capensis* is found solely along the South African and Namibian coasts, and *C. milii* is exclusive to the coasts of southern Australia and New Zealand. These regions are all separated by deep open ocean waters, that are not traversed by shallow coastal species of small- to medium- sizes, such as *Callorhinchus*. These observations highlight the role of deep oceanic waters as barriers to gene flow, driving species divergence in *Callorhinchus*.

Studies from other holocephalan relatives occurring in deep waters, such as *Chimaera monstrosa*, suggested that the shallow water regions like the Strait of Gibraltar may act as a strong barrier to gene flow (Catarino *et al.*, 2017; Carugati *et al.*, 2024). These studies revealed marked intraspecific genetic heterogeneity attributed to geographical isolation with no shared haplotypes between the Atlantic Ocean and the Mediterranean Sea, as well as significant spatial divergence within the Tyrrhenian basin (Catarino *et al.*, 2017; Carugati *et al.*, 2024). Consequently, distribution of *C. monstrosa* is confined to deeper waters, with shallow areas acting as natural barriers to gene flow.

Overall, this suggests that shallow-water coastal species like *Callorhinchus* are limited in their ability to cross deeper oceanic zones but may move long distance along continuous suitable coastal habitat, while deep-water holocephalans, such as *C. monstrosa*, cannot traverse shallow water regions. To identify barriers to gene flow in holocephalans, it is therefore imperative to consider the distinct characteristics pertaining to the ecology of the species.

2.4.5 Callorhinchids exhibit low genetic diversity at mitochondrial markers

All species of *Callorhinchus* exhibited low genetic diversity values (Table 2.3), with *C. milii* showing slightly higher diversity compared to the *C. callorynchus* and *C. capensis*. Such low levels of intraspecific genetic diversity are accompanied by low interspecific genetic divergence among *Callorhinchus* congeners. Indeed, the haplotypes of the three recognised extant species of *Callorhinchus* exhibit overall high genetic similarity, with a low number of nucleotide differences separating them (Fig. 2.5) and low overall mean K2P distance among species (1.92-2.62% and 1.34-1.82% based on CO1 and the whole mitogenome respectively; Table 2.3). These observations are remarkable as the three congeners are separated by vast expanses of open ocean waters that are unlikely to be traversed by *Callorhinchus*.

In comparison, other chondrichthyans that show genetic divergence across ocean basins in the southern hemisphere (e.g. *Dasyatis brevicaudata*, see LePort and Lavery, 2012; *Galeorhinus galeus*, see Hernández *et al.*, 2015; Bester-van der Merve *et al.*, 2017) exhibit similarly low numbers of differences between mtDNA haplogroups. However, for these species, the observed genetic differences are considered to represent distinct regional populations within the same species, whereas in *Callorhinchus*, speciation has occurred even with relatively limited genetic divergence.

Recent isolation and speciation may lead to low genetic differentiation among species, including incomplete lineage sorting (Maddison and Knowles, 2006). When examining the mitogenome tree presented in Inoue *et al.* (2010), the three *Callorhinchus* species exhibit notably short branches compared to other holocephalans, suggesting recent divergence of the species. Indeed, recent speciation is also supported by the phylogenetic tree based on the CR showing incomplete lineage sorting between *C. callorynchus* and *C. milii* (Fig. 2.4A). Given the current disjunct distribution of *Callorhinchus* species in South America, southern Africa, and New Zealand & Australia, one could hypothesise that speciation was due to allopatric isolation associated with separation of continental landmasses in the southern hemisphere. However, the timing of such geological events would point to a relatively old speciation, ~50-130 mya, which appears inconsistent with the low levels of interspecific divergence observed among *Callorhinchus*.

One alternative explanation may be the extremely slow mutation rate of the mitogenome in *Callorhinchus* compared to other holocephalans. In fact, low genetic diversity at mitochondrial genes appears to be a feature of the family Callorhinchidae: *C. milii* has been noted to have the slowest-evolving genome of vertebrates (Venkatesh *et al.*, 2014). Our results indicate that the mitogenome of *C. capensis* and *C. callorynchus* is likely to exhibit similarly low mutation rates given the observed low interspecific genetic divergence, as well as the overall genetic homogeneity within *C. callorynchus*. On the other hand, similarly low levels of genetic variation in the CO1 gene were found for another Holocephalan, *Chimaera monstrosa* Linnaeus, 1758 (Catarino *et al.*, 2017; Carugati *et al.*, 2024), albeit focusing on a smaller geographical range.

The bulk of the evidence gathered so far suggests that Holocephalan taxa may exhibit low genetic variation at mitochondrial markers. This observation is consistent with the low levels of molecular evolution detected in chondrichthyan species (Martin and Palumbi, 1993). Indeed, Elasmobranchs, the closest living relatives of holocephalans, are well known for their especially low mitochondrial nucleotide substitution rates limiting the power to recover robust phylogenetic relationships among taxa (Martin *et al.*, 1992; Martin, 1999). This feature underlines the necessity of other sources of information to accurately reconstruct phylogenies, such as morphological, distributional, and ecological data (Ward *et al.*, 2007, 2008; Naylor *et al.*, 2012).

2.4.6 Considerations for future studies

This work is the first to date using the mitochondrial CR for population genetic analyses in any species of chimaera, providing a comparative analysis between two widely used mitochondrial markers (CR and CO1). The ability to discern the genetic population structure can be highly affected by the diversity of the markers used and the historical demography of the species, with impacts on precision (Pascual, 2019). In general, the CR yielded lower genetic diversity values than CO1, with higher discrepancies between diversity values observed among sampling sites. Moreover, CR haplotypes of *C. milii* cluster in the same clade as haplotypes of *C. callorynchus* in the phylogenetic tree (Fig. 2.1A), pointing towards incomplete lineage sorting in the CR data. However, while the CR is widely recognised for its notably fast evolutionary rate (Meyer, 1993; McMillan and Palumbi, 1997) and polymorphic nature (Ghatak *et al.*, 2016) in comparison to the rest of the mitogenome, this observation is not consistent with our findings for *C. callorynchus*. The surprisingly low variability in our sequence data of the CR in *Callorhinchus* makes it a less powerful marker for intraspecific and intrageneric analyses of genetic differentiation and may cause to overlook key elements of population structure. Therefore, despite the mtDNA's ease of use as a molecular tool, combining it with other approaches seems appropriate to enhance the power of molecular data for testing of hypotheses. Future studies should aim to screen a large number of nuclear markers, e.g. via RAD-sequencing or whole genome shotgun sequencing, to increase the power of detecting genetic differentiation in a potentially low diversity group of taxa.

Moreover, the results shown here may have suffered from incomplete geographic coverage and small sample sizes; to increase the statistical power of future studies, larger and more balanced sample sizes covering the whole geographic range of the species should be pursued to enhance the robustness and reliability of the analyses. Due to the limited availability of genetic data for *Callorhinchus*, our analyses based on the CO1 gene yielded a much larger number of samples with higher spatial resolution than the CR. This should be taken into account when designing sampling strategies in the future. In addition, ensuring a more equal representation by balancing numbers of the different geographical groups would be crucial for a more comprehensive understanding of the species' population structure.

Finally, future studies should address the observed spatial structure in *C. milii* between Australian and New Zealand specimens. This result raises questions on the possible existence of markedly diverged populations of *C. milii*, or even the existence of an undescribed cryptic species since the intraspecific divergence is as pronounced as interspecies differences between the other two congeners (Fig. 2.5).

2.5 Conclusions

This work provides the first step towards a comprehensive understanding of population structure in *C. callorynchus* that will be essential for the development of the species' management strategies in the future. It also contributes important information on the patterns of population genetic diversity and divergence in a data-deficient group of Cartilaginous fish, the Holocephali. Overall, our findings reveal extremely low genetic diversity levels in the mitochondrial genome, both within and among *Callorhinchus* species, which limits the power to detect intraspecific genetic differentiation. However, analyses of the CO1 gene in *C. callorynchus* support a scenario of a recent population expansion in South America. Moreover, the observed uniformity in haplotype distribution and the lack of distinct genetic separation between regions suggest gene flow across the geographical range of *C. callorynchus*, that seems unaffected by changes in temperature, salinity, and biological productivity. This gene flow is mainly mediated by actively swimming adults that disperse along coastal regions but are limited in their ability to traverse deep oceanic waters. This suggests that coastal connectivity is crucial for maintaining gene flow in shallow-water holocephalans such as *C. callorynchus* while previous studies have shown that shallow water regions act as significant barriers to dispersal and gene flow in deep-water holocephalans such as *C. monstrosa*. This provides an important baseline for future research on dispersal and geneflow in other holocephalan taxa.

However, even though mitochondrial markers offer an accessible and cost-effective approach to studying genetic diversity and differentiation, it seems appropriate to address to what level our results may be affected by potential undiscovered diversity due to the markers chosen being too conserved. Moreover, it has been shown that analyses of mtDNA alone can produce significant bias when inferring the demographic and/or evolutionary history of groups, as it only represents the maternal ancestry and does not account for paternal contributions (Ballard and Whitlock, 2004; Hurst and Jiggins, 2005). Therefore, future studies would benefit from alternative molecular techniques, such as RAD-sequencing or whole genome resequencing, to reliably determine the pattern of population structure and genetic diversity throughout the species' distribution that may be missed by single locus studies. Furthermore, ecological studies providing information on the dispersal ability and movement behaviour of the American elephantfish as well as other holocephalans are highly needed to support future management efforts, sustainable exploitation, and conservation.

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Appendix

Table S1 Summary table with sampling data of *Callorhinchus*. N: number of specimens; TL: minimum to maximum total length in cm; PCL: minimum to maximum pre-caudal length in cm; sex ratio is expressed as number of males per females; estimated depth of capture in meters.

Location	N	TL (min-max)	PCL (min-max)	Sex ratio	Fisheries	Gear	Depth
Callao, Peru	8	47.8-76.4	33.6-63.2	2	Artisanal	Gillnets	NA
Higuerillas, Chile	80	42.3-92.5	28.1-65.3	3.71	Artisanal	Gillnets	20-40
Queule, Chile	19	59.5-108.3	40.3-77.8	1.38	Artisanal	Gillnets	20-40
Golfo San Matías, Argentina	5	NA	48-60	0.25	Fishing tournament	Rod from coastline	4-10
Golfo Nuevo, Argentina	20	NA	37-63	0.43	Recreational Field work	Rod from coastline Long line	4-10 40
Golfo San José, Argentina	9	NA	34.5-59.5	1.25	Field work	Long line	40
West Coast, South Island, New Zealand	8	34.1-88.6	NA	0	Field work	Bottom trawl	27-31; 120
Wellington Harbour, North Island, New Zealand	2	80-86	NA	0	Recreational	Rod and reel	NA
St Joseph, South Africa	3	56-73	NA	NA	NA	NA	NA

Table S2 Summary table of all *Callorhinchus* samples used in molecular analyses. Specimens are organised by species and listed with sampling locations and haplotype identities for CR: the mitochondrial control region, and CO1: the cytochrome c oxidase subunit I gene.

Species	Sample ID	Sampling location	Haplotype identities	
			CR	CO1
C. callorynchus	PE11	Callao, Peru (12°S)	3	3
C. callorynchus	PE16	Callao, Peru (12°S)	3	3
C. callorynchus	PE17	Callao, Peru (12°S)	2	1
C. callorynchus	PE18	Callao, Peru (12°S)	3	3
C. callorynchus	PE19	Callao, Peru (12°S)	6	1
C. callorynchus	PE20	Callao, Peru (12°S)	2	1
C. callorynchus	PE40	Callao, Peru (12°S)	8	3
C. callorynchus	FCHIL433-07	Coquimbo, Chile (29°S)	NA	15
C. callorynchus	FCHIL434-07	Coquimbo, Chile (29°S)	NA	1
C. callorynchus	FCHIL435-07	Coquimbo, Chile (29°S)	NA	16
C. callorynchus	FCHIL436-07	Coquimbo, Chile (29°S)	NA	1
C. callorynchus	FCHIL437-07	Coquimbo, Chile (29°S)	NA	1
C. callorynchus	FCHIL438-07	Coquimbo, Chile (29°S)	NA	5
C. callorynchus	FCHIL439-07	Coquimbo, Chile (29°S)	NA	1
C. callorynchus	FCHIL440-07	Coquimbo, Chile (29°S)	NA	17
C. callorynchus	FCHIL441-07	Coquimbo, Chile (29°S)	NA	1
C. callorynchus	CCM643	Higuerillas, Chile (33°S)	1	NA
C. callorynchus	CCM645	Higuerillas, Chile (33°S)	1	1
C. callorynchus	CCM651	Higuerillas, Chile (33°S)	1	2
C. callorynchus	CCM654	Higuerillas, Chile (33°S)	1	1
C. callorynchus	CCM655	Higuerillas, Chile (33°S)	1	2
C. callorynchus	CCM1054	Higuerillas, Chile (33°S)	7	NA
C. callorynchus	CCM1051	Higuerillas, Chile (33°S)	1	1
C. callorynchus	CCM1052	Higuerillas, Chile (33°S)	1	1
C. callorynchus	CCM1056	Higuerillas, Chile (33°S)	1	1
C. callorynchus	CCM1060	Higuerillas, Chile (33°S)	1	1
C. callorynchus	CCM1061	Higuerillas, Chile (33°S)	1	1
C. callorynchus	CCM1062	Higuerillas, Chile (33°S)	1	1
C. callorynchus	CCM1063	Higuerillas, Chile (33°S)	1	7
C. callorynchus	CCM1064	Higuerillas, Chile (33°S)	1	1
C. callorynchus	CCM1067	Higuerillas, Chile (33°S)	1	1
C. callorynchus	FCHIL268-07	Valparaiso, Chile (33°S)	NA	1
C. callorynchus	FCHIL269-07	Valparaiso, Chile (33°S)	NA	1
C. callorynchus	FCHIL270-07	Valparaiso, Chile (33°S)	NA	1
C. callorynchus	FCHIL271-07	Valparaiso, Chile (33°S)	NA	1
C. callorynchus	FCHIL272-07	Valparaiso, Chile (33°S)	NA	1
C. callorynchus	FCHIL273-07	Valparaiso, Chile (33°S)	NA	1
C. callorynchus	FCHIL274-07	Valparaiso, Chile (33°S)	NA	2

C. callorynchus	FCHIL157-06	Concepcion, Chile (36°S)	NA	1
C. callorynchus	FCHIL158-06	Concepcion, Chile (36°S)	NA	12
C. callorynchus	FCHIL159-06	Concepcion, Chile (36°S)	NA	1
C. callorynchus	FCHIL018-06	Golfo de Arauco, Chile (36°S)	NA	14
C. callorynchus	FCHIL019-06	Golfo de Arauco, Chile (36°S)	NA	1
C. callorynchus	FCHIL020-06	Golfo de Arauco, Chile (36°S)	NA	1
C. callorynchus	FCHIL021-06	Golfo de Arauco, Chile (36°S)	NA	12
C. callorynchus	FCHIL145-06	Talcahuano, Chile (36°S)	NA	1
C. callorynchus	FCHIL146-06	Talcahuano, Chile (36°S)	NA	1
C. callorynchus	CCM1083	Queule, Chile (39°S)	1	1
C. callorynchus	CCM1084	Queule, Chile (39°S)	1	1
C. callorynchus	CCM1086	Queule, Chile (39°S)	1	1
C. callorynchus	CCM1091	Queule, Chile (39°S)	1	1
C. callorynchus	CCM1092	Queule, Chile (39°S)	1	1
C. callorynchus	CCM1093	Queule, Chile (39°S)	NA	8
C. callorynchus	CCM1094	Queule, Chile (39°S)	1	1
C. callorynchus	CCM1096	Queule, Chile (39°S)	1	NA
C. callorynchus	CCM1097	Queule, Chile (39°S)	2	NA
C. callorynchus	CCM1098	Queule, Chile (39°S)	1	1
C. callorynchus	CCM1103	Queule, Chile (39°S)	1	1
C. callorynchus	CCM1104	Queule, Chile (39°S)	1	NA
C. callorynchus	CCM1105	Queule, Chile (39°S)	1	NA
C. callorynchus	CCM1106	Queule, Chile (39°S)	1	1
C. callorynchus	CCM1107	Queule, Chile (39°S)	1	NA
C. callorynchus	FARG330-07	Buenos Aires, Argentina (37°S)	NA	16
C. callorynchus	FARGB259-11	Buenos Aires, Argentina (37°S)	NA	10
C. callorynchus	CCB032-12	Buenos Aires, Argentina (37°S)	NA	1
C. callorynchus	A7	Golfo Nuevo, Argentina (43°S)	1	NA
C. callorynchus	A8	Golfo Nuevo, Argentina (43°S)	1	1
C. callorynchus	A9	Golfo Nuevo, Argentina (43°S)	1	4
C. callorynchus	A10	Golfo Nuevo, Argentina (43°S)	1	1
C. callorynchus	A14	Golfo Nuevo, Argentina (43°S)	1	9
C. callorynchus	A15	Golfo Nuevo, Argentina (43°S)	2	1
C. callorynchus	A17	Golfo Nuevo, Argentina (43°S)	1	1
C. callorynchus	A18	Golfo Nuevo, Argentina (43°S)	2	1
C. callorynchus	A23	Golfo Nuevo, Argentina (43°S)	1	1
C. callorynchus	A42	Golfo Nuevo, Argentina (43°S)	4	1
C. callorynchus	A43	Golfo Nuevo, Argentina (43°S)	1	4
C. callorynchus	A44	Golfo Nuevo, Argentina (43°S)	NA	1
C. callorynchus	A45	Golfo Nuevo, Argentina (43°S)	4	1
C. callorynchus	A46	Golfo Nuevo, Argentina (43°S)	1	1
C. callorynchus	A47	Golfo Nuevo, Argentina (43°S)	1	1
C. callorynchus	A48	Golfo Nuevo, Argentina (43°S)	5	5

C. callorynchus	A49	Golfo Nuevo, Argentina (43°S)	1	1
C. callorynchus	A50	Golfo Nuevo, Argentina (43°S)	1	1
C. callorynchus	A61	Golfo Nuevo, Argentina (43°S)	1	6
C. callorynchus	A62	Golfo Nuevo, Argentina (43°S)	4	1
C. callorynchus	A21	Golfo San Jose, Argentina (43°S)	1	NA
C. callorynchus	A52	Golfo San Jose, Argentina (43°S)	1	1
C. callorynchus	A53	Golfo San Jose, Argentina (43°S)	1	1
C. callorynchus	A54	Golfo San Jose, Argentina (43°S)	1	5
C. callorynchus	A56	Golfo San Jose, Argentina (43°S)	1	1
C. callorynchus	A57	Golfo San Jose, Argentina (43°S)	1	6
C. callorynchus	A58	Golfo San Jose, Argentina (43°S)	1	1
C. callorynchus	A59	Golfo San Jose, Argentina (43°S)	1	1
C. callorynchus	A63	Golfo San Matías, Argentina (43°S)	1	10
C. callorynchus	A64	Golfo San Matías, Argentina (43°S)	4	1
C. callorynchus	A65	Golfo San Matías, Argentina (43°S)	1	1
C. callorynchus	A66	Golfo San Matías, Argentina (43°S)	1	1
C. callorynchus	A72	Golfo San Matías, Argentina (43°S)	1	11
C. callorynchus	FARG223-06	Patagonia, Argentina (43°S)	NA	1
C. callorynchus	FARG003-06	Patagonia, Argentina (46°S)	NA	13
C. callorynchus	FARG004-06	Patagonia, Argentina (46°S)	NA	1
C. callorynchus	FARG006-06	Patagonia, Argentina (46°S)	NA	1
C. callorynchus	FARG007-06	Patagonia, Argentina (46°S)	NA	1
C. callorynchus	FARG380-08	Patagonia, Argentina (46°S)	NA	1
C. callorynchus	FARG381-08	Patagonia, Argentina (46°S)	NA	1
C. callorynchus	FARG382-08	Patagonia, Argentina (46°S)	NA	1
C. callorynchus	ANGBF13022-18	Chile	NA	1
C. callorynchus	ANGBF13023-18	Chile	NA	1
C. callorynchus	ANGBF13025-18	Chile	NA	1
C. callorynchus	ANGBF13027-18	Chile	NA	1
C. callorynchus	ANGBF13029-18	Chile	NA	1
C. callorynchus	ANGBF13030-18	Chile	NA	1
C. callorynchus	ANGBF13031-18	Chile	NA	1
C. callorynchus	ANGBF13033-18	Chile	NA	1
C. callorynchus	ANGBF13034-18	Chile	NA	1
C. callorynchus	ANGBF13035-18	Chile	NA	1
C. callorynchus	ANGBF13036-18	Chile	NA	1
C. callorynchus	ANGBF13038-18	Chile	NA	1
C. callorynchus	ANGBF13039-18	Chile	NA	1
C. callorynchus	ANGBF13040-18	Chile	NA	1
C. callorynchus	ANGBF13041-18	Chile	NA	1
C. callorynchus	ANGBF13042-18	Chile	NA	1
C. callorynchus	ANGBF13043-18	Chile	NA	1
C. callorynchus	ANGBF13044-18	Chile	NA	1

C. callorynchus	ANGBF13045-18	Chile	NA	1
C. callorynchus	ANGBF13046-18	Chile	NA	1
C. callorynchus	ANGBF13047-18	Chile	NA	1
C. callorynchus	ANGBF13048-18	Chile	NA	1
C. callorynchus	ANGBF13049-18	Chile	NA	1
C. callorynchus	ANGBF13050-18	Chile	NA	1
C. callorynchus	ANGBF13051-18	Chile	NA	1
C. callorynchus	ANGBF13052-18	Chile	NA	1
C. callorynchus	ANGBF13054-18	Chile	NA	1
C. callorynchus	ANGBF13056-18	Chile	NA	1
C. callorynchus	ANGBF13057-18	Chile	NA	1
C. callorynchus	ANGBF13058-18	Chile	NA	1
C. callorynchus	ANGBF13060-18	Chile	NA	1
C. callorynchus	ANGBF13061-18	Chile	NA	1
C. callorynchus	ANGBF13062-18	Chile	NA	1
C. callorynchus	ANGBF49312-19	Chile	NA	1
C. callorynchus	ANGBF49313-19	Chile	NA	1
C. callorynchus	ANGBF49316-19	Chile	NA	1
C. callorynchus	ANGBF49317-19	Chile	NA	1
C. callorynchus	ANGBF49318-19	Chile	NA	1
C. callorynchus	ANGBF49319-19	Chile	NA	1
C. callorynchus	ANGBF49320-19	Chile	NA	1
C. callorynchus	ANGBF49315-19	Chile	NA	4
C. callorynchus	ANGBF13026-18	Chile	NA	4
C. callorynchus	ANGBF13055-18	Chile	NA	6
C. callorynchus	ANGBF13028-18	Chile	NA	10
C. callorynchus	ANGBF13037-18	Chile	NA	10
C. callorynchus	ANGBF49314-19	Chile	NA	11
C. callorynchus	ANGBF13053-18	Chile	NA	11
C. callorynchus	ANGBF13032-18	Chile	NA	11
C. callorynchus	ANGBF13024-18	Chile	NA	18
C. callorynchus	ANGBF13059-18	Chile	NA	19
C. milii	KAH1402/68-2	South Island, New Zealand	9	20
C. milii	KAH1402/68-4	South Island, New Zealand	NA	23
C. milii	KAH1402/68-6	South Island, New Zealand	9	25
C. milii	KAH2302/60-1	South Island, New Zealand	11	21
C. milii	KAH2302/60-2	South Island, New Zealand	9	22
C. milii	KAH2302/61-1	South Island, New Zealand	12	24
C. milii	KAH2302/65-1	South Island, New Zealand	10	NA
C. milii	WLG12	North Island, New Zealand	NA	26
C. milii	FNZA544-08	New Zealand	NA	21
C. milii	FNZA545-08	New Zealand	NA	26
C. milii	FNZA953-10	New Zealand	NA	29

C. milii	FMVIC475-08	Western Australia, Australia	NA	27
C. milii	FMVIC477-08	South Australia, Australia	NA	27
C. milii	FMVIC476-08	Victoria, Australia	NA	27
C. milii	FOA261-04	Victoria, Australia	NA	27
C. milii	FOA263-04	Victoria, Australia	NA	27
C. milii	FOA264-04	New South Wales, Australia	NA	27
C. milii	FOA265-04	Tasmania, Australia	NA	27
C. milii	FOAN178-11	Tasmania, Australia	NA	27
C. milii	FOAN179-11	Tasmania, Australia	NA	27
C. milii	FOA262-04	Australia	NA	28
C. milii	IRREK410-08	Australia	NA	27
C. milii	IRREK411-08	Australia	NA	27
C. capensis	CO1_SJ1	St Joseph, South Africa	13	30
C. capensis	CO1_SJ2	St Joseph, South Africa	14	31
C. capensis	CO1_SJ3	St Joseph, South Africa	15	30
C. capensis	DSFSE128-07	South Africa	NA	32
C. capensis	DSFSE129-07	South Africa	NA	30
C. capensis	DSFSE130-07	South Africa	NA	30
C. capensis	DSFSE131-07	South Africa	NA	33
C. capensis	DSFSE132-07	South Africa	NA	30
C. capensis	HVDBF427-11	Namibia	NA	30
C. capensis	HVDBF428-11	Namibia	NA	30