VARAMIENTO DE CALDERONES DE ALETA LARGA EN LA PATAGONIA CHILENA: CONTRIBUCIÓN A LA ESTRUCTURA GENÉTICA, SOCIAL Y A LA FILOGEOGRAFÍA DE LA ESPECIE

Tesis

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TESIS DE MAGÍSTER

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Magíster presentada por el candidato:

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Mi flechazo por los océanos es tan remoto como mis primeros recuerdos. Gracias a mis padres mostré un gran interés por la fauna marina desde muy pequeño, particularmente peces, tiburones y rayas. Mis primeros libros, juguetes y mascotas siempre estuvieron relacionadas a esto.

Nací en Mannheim, Alemania, donde estuve hasta mis cinco años. Después llegamos a Chile, donde ingresé al Colegio Suizo de Santiago. Algunos de mis amigos me dicen que el primer recuerdo que tienen de mí es el de un niño sin muchas ganas de hablar, pero con una orca de juguete bajo el brazo. Durante esta etapa mi ramo favorito naturalmente fue biología y siempre tuve la certeza de que biología marina sería lo que estudiaría.

Así, entré a estudiar esta carrera en la Pontificia Universidad Católica de Chile el año 2009, donde, entre otras cosas, comenzaría a comprender lo amplio que es ese mundo (y lo equivocado que estaba respecto a mi relación con las matemáticas, que había dado por terminada luego de la PSU).

Luego de egresar, tuve la suerte de poder participar en muchos proyectos relacionados con tiburones, desde talleres de identificación y reuniones, hasta expediciones de toma de muestras y marcaje satelital. El año 2015 tuve la fortuna de ingresar al Laboratorio de Ecología Molecular, en el que, rodeado de excelentes personas, forjé un nuevo interés por otras áreas, como los cetáceos y la ciencia antártica. Aquí es donde más me he desarrollado como científico y he podido participar de diversos proyectos, entre ellos el Magister que comencé el año 2017.

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También quiero agradecer a mi familia, mi mamá, María José Gutiérrez, y mi papá, Bruno Kraft, que alimentaron mi amor por el océano desde pequeño, que ahora se ha transformado en eje central de mi vida. También me permitieron tener la fortuna de que nunca me faltara nada para poder perseguir este sueño. A mi hermana, Irene, con la que comparto el amor por la naturaleza.

A mis amigos, la familia que se elige mutuamente, que afortunadamente ha ido sumando integrantes con cada nueva etapa que comienzo. Aquí hay una cantidad de apoyo logístico, académico, emocional y humorístico inmensurable. A los amigos que hice durante mi época escolar, Francisca Garrido, Francisco de la Fuente, Ignacio Alzola, Joaquín Guevara, Josefine Bachmann, Karin Hofer, Manuel Hinrichsen, Pablo Moure, Sebastián Correa, Valentina Hasbún y Vicente Valenzuela. Durante pregrado, Arturo Greene, Bárbara Schultz, Carlos Abello, Benjamín Glasner, César Muñoz, Diego Sobera, Gonzalo Gomara, Hernán Peñaloza, Ítalo Fernández, Javier von Marées, Matías Barceló, Nicolás Cumplido, Nicolás Rodríguez, Rodrigo Muñoz, Tomás Ribba, Tomás Saratscheff. Y a aquellos ahora en mi tiempo en el LEM, Ana Guzmán-Castellanos, Camila Bravo, Claudia Maturana, Constanza Napolitano, Francisca Rodríguez, Francisco Concha, Franco Cianferoni, Guillaume Schwob, Javier Naretto, Nicolás Segovia, Sandra Brito, Sasha Millán, Simón Anguita, Valentina Bernal y Valentina Muñoz.

Pensar en todas las personas que me ha acompañado durante este camino me genera una sobrecogedora sensación de apoyo incondicional de la que estaré siempre agradecido.

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Sebastián

Índice

Introducción general

El estudio biológico y ecológico de cetáceos presenta dificultades inherentes en su desarrollo (Mann, 1999; Nowacek et al., 2016) debido principalmente a que habitan en un ambiente de difícil acceso y son altamente móviles. El levantamiento de información, y en particular la toma de muestras biológicas es sumamente difícil, sobre todo en especies que habitan mayormente en zonas alejadas de la costa, presentan un amplio rango de movilidad y conductualmente pueden llegar a ser muy elusivos (Mann, 1999; Moore et al., 2018; Pyenson, 2011). Adicionalmente, la mayoría de los cetáceos son de amplia distribución, siendo algunas virtualmente cosmopolitas, como el cachalote *Physeter macrocephalus* (Whitehead, 2018), la orca *Orcinus orca* (Ford, 2018) y algunas ballenas balaenoptéridas (LeDuc, 2018), lo que dificulta los estudios íntegros a lo largo de su área de ocurrencia.

En ocasiones, los cetáceos llegan a la costa en grandes números durante eventos conocidos como varamientos masivos. En este contexto, un varamiento está definido como el hallazgo de un cetáceo vivo o muerto en la costa o fuera de su hábitat usual (Moore et al., 2018) y es considerado masivo cuando dos o más individuos, exceptuando parejas de madre y cría, varan en el mismo lugar y momento aproximados (Geraci and Lounsbury, 2005). Las causas de estas mortalidades aún están siendo estudiadas ya que son eventos de carácter complejo y generalmente ocurren por un conjunto de razones de distinta naturaleza (Moore et al., 2018). A través del tiempo, nuestra percepción de estos eventos e interacción con ellos ha cambiado desde aproximaciones netamente descriptivas a evaluaciones más científicas del fenómeno (Heyning, 1991; Moore et al., 2018). Esto se ve reflejado, por ejemplo, en el

aumento del esfuerzo para documentar y del interés público respecto al tema (Evans et al., 2005), junto con el mejoramiento sostenido de la colección y ordenamiento de esta información en las últimas décadas (Heyning, 1991). De esta manera, en la actualidad se cuenta con registros detallados y de larga data sobre estos eventos en las costas del Reino Unido (Sheldrick, 1976), Estados Unidos (Geraci and St. Aubin, 1979), Nueva Zelanda (Brabyn, 1991) y Holanda (van der Meij and Camphuysen, 2006). La recolección de datos y muestras a partir estos sucesos representan una fuente muy importante de información (Geraci and St. Aubin, 1977). Mucha de la información disponible sobre varias especies es producto de la recolección de datos durante estos eventos (Geraci and St. Aubin, 1979; Wilkinson and Worthy, 1999).

Los calderones de aleta larga (*Globicephala melas*) son odontocetos que cumplen con muchas de las características que hacen a los cetáceos difíciles de estudiar: son de hábitos principalmente oceánicos y presentan una amplia distribución antitropical, con una subespecie presente en cada hemisferio: *G. m. edwardii* habita las aguas temperadas a subpolares del Hemisferio sur, mientras que *G. m. melas* está restringida a las aguas del Atlántico norte y Mediterráneo (Olson and Reilly, 2002). Además son capaces de recorrer grandes distancias en breves períodos, desplazándose a velocidades de hasta 14.5 km/h y recorriendo hasta 200 km en un mismo día (Bloch et al., 2003). Esta especie presenta una particular organización social entre cetáceos. Los grupos se conforman alrededor de asociaciones matrilineales fuertes entre hembras cercanamente emparentadas y su descendencia de ambos sexos (Amos et al., 1991; Jefferson et al., 1996; Olson and Reilly, 2002). Individuos de esta especie forman grupos temporalmente estables cuyo tamaño oscila alrededor de los diez individuos (Augusto et al., 2017a; de Stephanis et al., 2008; C. A.

Ottensmeyer and Whitehead, 2003), a su vez que puede incrementar a cientos de individuos por medio de agregaciones (Bernard and Reilly, 1994; Sergeant, 1982). Los varamientos masivos son particularmente comunes en esta especie, junto al cachalote *Physeter macrocephalus*, la falsa orca *Pseudorca crassidens* y el calderón de aleta corta *Globicephala macrorhynchus* (Moore et al., 2018; Olson and Reilly, 2002; Sergeant, 1982), todas especies consideradas de sociedades matrilineales. Estos varamientos masivos han sido previamente utilizados para realizar estudios genéticos intra e interpoblacionales en esta especie, mayormente en el Atlántico norte, el mar Mediterráneo y el Pacífico Sudoeste (Amos et al., 1991, 1993; de Stephanis et al., 2008; Miralles et al., 2016; Monteiro, 2013; Oremus et al., 2009; Oremus et al., 2013; Verborgh, 2015).

El registro de varamientos y mortalidades de cetáceos en Chile ha ido incrementando recientemente, principalmente impulsado por la ocurrencia del mayor evento de mortalidad masiva a nivel mundial de misticetos, registrada en el Golfo de Penas que involucró a al menos 343 ballenas sei *Balaenoptera borealis* (Häussermann et al., 2017). Junto a este registro, entre las especies que documentan varamientos masivos en mayores números en las costas de Chile se encuentran la falsa orca *Pseudorca crassidens* (Haro et al., 2015) y el calderón de aleta larga *Globicephala melas* (Alvarado-Rybak et al., 2019). Si bien los registros de varamientos de esta última especie están reportados a lo largo de toda la costa de Chile continental, la mayoría se concentra en la región austral, donde también esta especie aparentemente es más común (Aguayo-Lobo et al., 1998). Hasta la actualidad se han reportado cinco varamientos masivos para esta especie, todos en la Patagonia chilena, específicamente en Tierra del Fuego (Goodall, 1978), Estrecho de Magallanes y Canal Beagle (Mansilla et al., 2012), Isla Navarino (Venegas and Sielfeld, 1980) y más

recientemente en Isla Clemente (Alvarado-Rybak et al., 2019), los que pese a haber sido reportados, no habían sido aprovechados como oportunidad de levantamiento de información.

El año 2016, un varamiento masivo fue reportado en Isla Clemente, región de Aysén (45°35' 57.50" S, 74°34' 30.32" O). En esta oportunidad, se realizó una expedición multidisciplinaria en conjunto con varias organizaciones, entre ellas la Armada de Chile y el Servicio Nacional de Pesca y Acuicultura (SERNAPESCA). Durante la expedición se colectaron muestras de tejido y/o hueso de 124 individuos encontrados, que fueron derivadas al Laboratorio de Ecología Molecular de la Universidad de Chile. La colección de estas muestras a partir del varamiento masivo permitió el desarrollo de esta investigación que explora, a escala local, la estructura y diversidad genética de la especie en la zona del Pacífico suroriental y, en un contexto más global, al estudio filogeográfico de los calderones de aleta larga, contando con una información nueva de una zona que no ha sido incluida en los análisis biogeográficos globales.

Capítulo 1

Hipótesis:

Las poblaciones patagónicas de *Globicephala melas* siguen el modelo de estructura social matrilineal de odontocetos.

Predicciones:

- Los individuos presentes en el varamiento mostrarán baja diversidad genética mitocondrial.
- Los individuos presentes en el varamiento presentarán alto grado parentesco.
- Los diferentes varamientos mostrarán estructura genética poblacional.

Objetivo general: Establecer las relaciones genéticas inter- e intra-varamiento de calderones de aleta larga de dos varamientos ocurridos en la Patagonia chilena.

i. *Objetivo específico 1:* Estimar la diversidad genética dentro y entre los varamientos masivos de Isla Clemente e Isla Navarino, a nivel de ADN mitocondrial y microsatélites.
ii. *Objetivo específico 2:* Evaluar la presencia de estructuración poblacional entre ambos varamientos, utilizando ADN mitocondrial y microsatélites.
iii. *Objetivo específico 3:* Estimar el grado de parentesco genético dentro y entre los varamientos masivos de Isla Clemente e Isla Navarino mediante el uso de microsatélites.

Capítulo 2

Hipótesis: Las poblaciones de *Globicephala melas* del Hemisferio sur (HS) pertenecen a una misma Unidad Evolutiva Significativa (ESU), distinta de las poblaciones del Hemisferio norte (HN).

Predicciones:

-Baja o ausente estructura filogeográfica entre poblaciones del Hemisferio Sur.

-Marcada estructura genética poblacional a nivel inter-hemisferio.

Objetivo general: Proponer una hipótesis biogeográfica en base a la estructura filogeografía de la especie adicionando muestras del Pacífico suroriental.

i. *Objetivo específico 1:* Determinar la estructura filogeográfica de las poblaciones del Pacífico sureste con aquellas del resto de su distribución en el HS y HN.

ii. *Objetivo específico 2:* Poner a prueba escenarios biogeográficos históricos que habrían originado su distribución antitropical actual.

Capítulo 1

Genetic structure and relatedness of long-finned pilot whales *Globicephala melas* in the Chilean Patagonia

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Abstract

Among odontocetes, matrilineal species such as pilot whales are particularly kin-inclined and tend to form stable groups with long lasting bonds among their close relatives. These core units can aggregate to form larger groups for feeding, mating, predator avoidance and calf raising. Pilot whales are among the most frequently recorded species in mass strandings, also stranding in some of the largest numbers. In this study, socio-genetic, genetic population structure and genetic diversity studies were conducted from two mass strandings of long-finned pilot whales that occurred in the Chilean Patagonia (Isla Clemente, n=78; Isla Navarino, n=12), thanks to collective efforts to gather data and samples. As previously reported for this species, we observed low mitochondrial and microsatellite diversity. We also detected population structure between both strandings with the mitochondrial control region, but not with microsatellite dataset. This suggests male-mediated gene flow and lower dispersal rates for female pilot whales, concordantly with previous studies that suggest that males move among pods in order to reproduce and avoid inbreeding. The majority of individuals in Isla Clemente stranding, despite overall relatedness being lower than expected, have a first-degree relationship with another member of the group. Hence, the Isla Clemente mass stranding was likely composed of several matrilineal units that stranded together, as suggested by the mitochondrial and microsatellite data and supported by other genetic and behavioural studies.

Resumen

Entre los odontocetos, las especies matrilineales como los calderones de aleta larga están particularmente inclinados a formar grupos entre parientes cercanos, estableciendo lazos estables entre individuos. Estas unidades centrales se pueden agregar para formar grupos más grandes para alimentarse, reproducirse, evadir depredadores y criar su descendencia. Los calderones de aleta larga se encuentran entre las especies más documentadas en varamientos masivos, también varando en grandes números. En este trabajo, se realizaron estudios socio-genéticos, de estructura genética poblacional y diversidad genética a partir de dos varamientos masivos de esta especie que ocurrieron en la Patagonia chilena (Isla Clemente, n=78; Isla Navarino, n=12), gracias al esfuerzo colectivo de colectar datos y muestras a partir de estos eventos. Como ha sido reportado previamente para la especie, observamos baja diversidad mitocondrial y de loci microsatélites. Además, detectamos estructuración poblacional con los datos mitocondriales entre los dos varamientos, pero no con los datos de microsatélites. Esto sugiere un flujo genético mediado por machos y una tasa de dispersión menor para las hembras, lo que concuerda con estudios previos que sugieren que los machos se mueven entre grupos para reproducirse y evitar la endogamia. A pesar de que encontramos un bajo nivel de parentesco global, la mayoría de los individuos del varamiento de Isla Clemente presentaron una relación de primer grado con otro individuo del mismo varamiento. Así, este varamiento masivo muestra haber estado compuesto de varias unidades matrilineales que vararon juntas, como es sugerido por los datos mitocondriales y microsatélites y apoyado por otros estudios genéticos y conductuales.

Introduction

Some of the most complex forms of social organization can be found in mammals. For instance, a number of primate species present matrilocal organization that can last for several generations (Welker, 1985); lions are hierarchically organized in prides (Schaller, 1972); elephants engage in multileveled, fission–fusion social structures (Wittemyer et al., 2005) and even eusociality, among the most extreme deviations from panmixia, has been described in naked mole-rats (Jarvis, 1981). Cetaceans have themselves adopted a wide array of social strategies, which conspicuously differ among baleen and toothed whales (Tyack, 1986).

The social connections in mysticetes are straightforward and labile, as interactions among individuals are generally limited to particular events such as feeding, mating and calf-raising (Acevedo-Gutiérrez, 2002; 2018; Berta and Sumich, 1999; Trillmich and Cantor, 2018). Odontocetes, much like mysticetes, also incur in group formation for feeding, mating, predator avoidance and calf raising, which usually is more prolonged (Pomeroy et al., 2018). However, toothed whales hold intricate social behaviors in a much more elemental role when grouping (Acevedo-Gutiérrez, 2002; Tyack, 1986). They extensively favor kin selection (Acevedo-Gutiérrez, 2018; Trillmich and Cantor, 2018) and increment the net benefits of interactions with conspecifics when incurring in group formation (Trillmich and Cantor, 2018) by establishing bonds that are more stable in time (Tyack, 1986). Alloparental care, a form of kin-selection in which adults tend unrelated offspring, has also been described for odontocetes (Hamilton, 1964). These engagements have been reported in species with various social structures, like the fission-fusion groups of bottlenose dolphins *Tursiops truncatus* (Caldwell and Caldwell, 1966; Mann and Smuts, 1998), labile group formation of belugas *Delphinapterus leucas* (O'Corry-Crowe, 2018), but is particularly common in cetacean

species that present matrilineal societies (Augusto et al., 2017; Bigg et al., 1987; Whitehead, 1996). Matrilineal units are conceived around a central group of closely related, adult females and their descent, which can remain within their group of birth their entire lifetime (Jefferson et al., 1996; Olson and Reilly, 2002; Whitehead, 1998). Additional complexities to this system have been detailed in some species, like sperm whales and orcas, which show different levels of organization that also include close kin rather than strictly matrilineal members (Bigg et al., 1990; Konrad et al., 2018).

At the population level, sociality and kinship can influence the genetic structure of different mammal species (Gerkey, 2015; Storz, 1999) and cetaceans are an illustrative example of this. In mysticetes, population genetic structure generally reflects their migratory patterns (Palsbøll et al., 1995) as strong social drivers are generally absent. In odontocetes, particularly matrilineal species, socially acquired traits have shown to give way to genetic structure. For instance, a link between population structure and vocal clans –rather than geographic origin- has been suggested for sperm whales from the Pacific (Rendell et al., 2012). Similarly, North Pacific populations of orcas are classified in ecotypes, which emerged in sympatry as a result of prey specialization and social behavior (Hoelzel et al., 2007). Gene flow does occur among groups in matrilineal species and is thought to be undertaken by males, as commonly described among mammals (Greenwood, 1980) and consequently marine mammals, (e.g. Brown Gladden et al., 1999; Fabiani, 2003; Lyrholm et al., 1999; Pilot et al., 2010).

Pilot whales form groups that are also structurally based on strong matrilineal associations (Jefferson et al., 1996; Olson and Reilly, 2002) and, as highly social animals, form stable bonds in groups of a mean size around 10 individuals (Augusto et al., 2017; de Stephanis et al., 2008; Ottensmeyer and Whitehead, 2003) up to hundreds of individuals through aggregation of

these groups (Bernard and Reilly, 1994; Sergeant, 1982).

Long-finned pilot whales are considered the most common cetacean species involved in mass strandings, together with the matrilineal false killer whale *Pseudorca crassidens* and the short-finned pilot whale *Globicephala macrorhynchus,* (Moore et al., 2018; Olson and Reilly, 2002; Sergeant, 1982). Their closely-knitted relationships are thought to be one of the driving forces behind these events (Perrin and Geraci, 2002). Strandings of long-finned pilot whales occur along most of their distribution range (e.g. Geraci and St. Aubin, 1977; Venegas and Sielfeld, 1980; Oremus et al., 2013) and present unique opportunities to collect a large number of samples, compared to the alternative of sampling free-ranging individuals. In turn, studies based on samples collected from strandings have been carried out in areas of the North Atlantic and the southwestern Pacific (Miralles et al., 2016; Oremus et al., 2009; Siemann, 1994). Reports of stranding events exists for the Southeastern Pacific, yet the genetic diversity, genetic structure and relatedness of long-finned pilot whales in this area remain to be studied. Within the Chilean territory, single strandings of *G. melas* (Sanino and Yáñez, 2001) and at least five mass events have been reported: Bahía Flinders, Tierra del Fuego in 1971 (n=9) (Goodall, 1978); Bahía Windhood, Isla Navarino in 1979 (n=125), Bahía Poseidón, Estrecho de Magallanes in 1982 (n=61), Islotes de Holger, Canal Beagle, in 2006 (n=13) (Mansilla et al., 2012); and Isla Clemente in 2016 (n=124) (Alvarado-Rybak et al., 2019).

In order to study the genetic diversity, connectivity and relatedness of long-finned pilot whales in the southeastern Pacific, we analyzed samples collected from the two most recently reported mass strandings that took place in the Chilean Patagonia, using mitochondrial and microsatellite markers.

Materials and methods

Sample collection DNA extraction, sequencing and microsatellite genotyping

Tissue samples were collected in 2006 from twelve individuals in a stranding event that occurred in Isla Navarino (55°15'S; 67°30'W) (Mansilla et al., 2012) (Figure 1, a). In 2016, 124 *Globicephala melas* were sampled from the mass stranding event that took place in Isla Clemente (45°35' 57.50" S, 74°34' 30.32" W). All samples were preserved in 90-95% ethanol. DNA extractions were performed following a modified salt-extraction protocol (Aljanabi and Martinez, 1997).

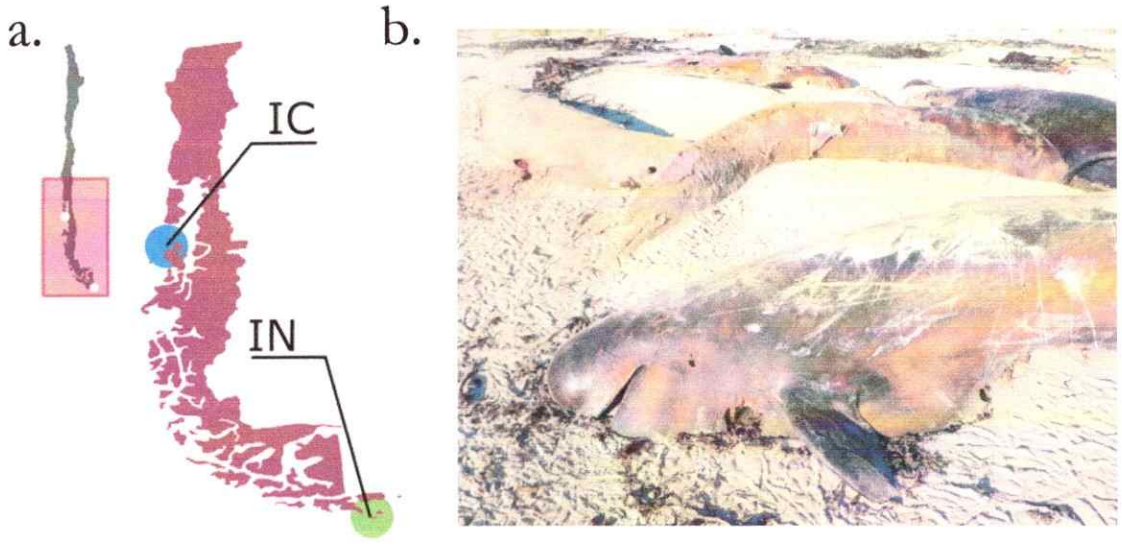

Figure 1: a. Sampling sites of the mass strandings that occurred in Isla Navarino in 2006 (green circle) and Isla Clemente in 2016 (blue circle); b. individuals found at the Isla Clemente event, in advanced stages of decomposition.

Sex identification

The sex of each individual was evaluated at two stages: first, visually in the field and later by amplification of the sex chromosome fragments ZFX and ZFY. The primers used for the X chromosome were P1-5EZ 5'-ATAATCACATGGAGAGCCACAAGCT-3' and P2-3EZ 5'-GCACTTCTTTGGTATCTGAGAAAGT-3', and the primers used for the Y chromosome were Y53-3D 5'-ATTTTAGCCTTCCGACGAGGTCGATA-3' and Y53-3C 5'-CCCATGAACGCATTCAATGTGTGG-3' (Aasen and Medrano, 1990; Gilson et al., 1998). For the amplification protocol, the total volume of PCR reactions was 21 μL: 7.16 μL water, 4 μL 10X Buffer (Invitrogen), 1.6μL 50 mM $MgCl_2$ (Invitrogen), 2 μL 10 pM dNTPs (Invitrogen), 1μL 10 pM of each primer (4 μL total), 0.24 μL Taq polymerase (Invitrogen) and 2 μL DNA at 50-200 ng/μL. PCR profile was as follows: a preliminary denaturation stage at 94 °C for 2 minutes; 35 cycles of: denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, polymerase extension at 72 °C for 60 s; and a final polymerase extension stage at 72 °C for 10 minutes and a final infinite hold temperature of 4 °C. Each PCR run included a positive control for each sex and a negative control. The sex of each individual was identified visually in a 2% agarose gel. Females present a single band of approximately 450 base pairs (bp) that corresponds to the X chromosome fragment, while males present an additional band of lower molecular weight (approximately 174 bp) that corresponds to the Y chromosome fragment (Aasen and Medrano, 1990; Gilson et al., 1998). To avoid errors, sex identification was done independently by two researchers.

Mitochondrial control region data

The mtDNA control region was amplified using the primers described by Dalebout et al., (2005) M13 Dlp1.5 5'-TGTAAAACGACAGCCAGTTC ACCCAAAGCTGRARTTCTA-3'(forward) and 8G 5'-GGAGTACTATGTCC TGTAACCA-3'(reverse). The amplification protocol was as follows: 25.6 μL reaction volume for each PCR reaction consisted of 12.7 μL water, 5 μL 10X Buffer (Invitrogen), 2 μL 50 mM $MgCl_2$ (Invitrogen), 2 μL 10 pM dNTPs (Invitrogen), 1 μL 10 pM of each primer (2 μL total), 0.5 μL Taq polymerase (Invitrogen) and 70-150 ng of DNA. A Thermo Hybaid PxE 0.5 thermal cycler was used for all amplifications, with the following profile: preliminary denaturation of 2 minutes at 94 °C, followed by 30 cycles of: denaturation for 30 s at 94 °C, annealing for 40 s at 56 °C, polymerase extension for 40 s at 72 °C and a final polymerase extension for 10 minutes at 72 °C and an infinite hold temperature of 4 °C. Each PCR run included positive and negative controls. Fragments were run in a 1% agarose gel, each well containing 3 μL of PCR product mixed with an equal volume of loading dye with 0.3% Gel Red and visualized in a gel documentation system (Maestrogen SMU-01). PCR product purification and sequencing were done at Macrogen Inc., Seoul, South Korea with a 3730XL DNA Analyzer (Applied Biosystems). All sequences obtained were aligned manually in *ProSeq* 3.5 (Filatov, 2009). Prior to molecular analyses, the species for each sample was corroborated with two platforms of comparative analysis of sequences: BLAST (Basic Local Alignment Search Tool, www.blast.ncbi.nlm.nih.gov and DNA Surveillance (Ross et al., 2003).

mtDNA genetic diversity and structure

The genetic diversity indices number of polymorphic sites (k), number of haplotypes. (k), haplotype diversity (Hd), pairwise differences between sequences ($\prod$) and nucleotide diversity (π) were estimated in *Arlequin* v3.5.2 (Excoffier and Lischer, 2010). The haplotype network was constructed in *PopART* v1.7 (Leigh and Bryant, 2015). Analyses of genetic structure (F_{ST}) and phylogeographic structure (Φ_{ST}) were conducted in *Arlequin* v3.5.2 with 1 000 permutations and a significance level of 0.05.

Sequence editing

With an exploratory examination of the global haplotype network, it was noted that site 156 of the alignment generated three loops in the network. This hypervariable site was considered to be interfering with the phylogeographic signal of the data and was consequently removed, in order to eliminate a potential homoplasy signal. Additionally, a repeated TA motif starting at position 90 was identified as a possible microsatellite. We modified all sequences at this position by deleting one of the nucleotides within each repeat, so each motif was considered as a single mutational step, instead of each nucleotide separately.

Microsatellites

A total of 19 loci were amplified: 464/465 (Amos et al., 1993), DlrFCB1, DlrFCB6 (Buchanan et al., 1996), Ev1, Ev14, EV37 (Valsecchi and Amos, 1996), GATA53, (Palsboll et al., 1997), GT6, GT51 (Caldwell et al., 2002), GT23, GT211, GT509, GT575 (Berube et al., 2000), MK5, MK9 (Krützen et al., 2001), PPHO131 (Rosel et al., 1999), Sgui03, Sgui06 and Sgui16 (Cunha and Watts, 2007). PCR reactions were done with a Multiplex PCR kit (Qiagen), each reaction containing: 12,5 µL water, 5 µL MM2x Buffer and 1 µL each primer at 10 pM. Between two and four loci with different fluorescent dyes were combined in each reaction. Allele scoring was done with the software *GeneMarker* v2.6.0 (www.softgenetics.com) with a 500liz standard. The loci were tested for scoring errors, allele dropout and null alleles in *Micro-Checker* v.2.2.3 (Van Oosterhout et al., 2004), their inbreeding coefficient (F_{IS}) calculated in *Genetix* to test whether there was more non-random mating than expected by chance, and checked for outliers in *Arlequin* v3.5.2. Detection of loci under selection is done by examining the distribution of loci under a hierarchical island model, using coalescent simulations and locus-specific F_{ST} and heterozygosity values. Fisher's paired comparisons were done among allelic richness and expected heterozygosity of both strandings with a two-tail exact test and 10 000 randomizations in *Rundom Pro* 1.1 (Jadwiszczak, 2003).

Genetic structure

Observed heterozygosity (H_o), expected heterozygosity (H_e), average number of alleles per locus (NA) were estimated in *Genetix* v4.05.2 (Belkhir et al., 2004). Allelic richness (AR) was calculated in *FSTAT* v2.9.4 (Goudet, 1995). Genetic structure was evaluated with the programs *Genetix*, *Structure* 2.3.4 (Pritchard et al., 2000) and the MS Excel add-in *GenAlEx*

6.51 (Peakall and Smouse, 2012, 2006).

Structure implements a Bayesian clustering method to evaluate population structure, using multilocus data to assign individuals to populations and to identify migrants or admixed individuals in the sample. The burn-in period was set to 100 000 repeats, followed by 1 000 000 MCMC repeats, with an admixture model and correlated allele frequencies in ten different runs. *CLUMPP* 1.1.2 (Jakobsson and Rosenberg, 2007) and *distruct* 1.1 (Rosenberg, 2003) were used to prepare the *Structure* output files and generate graphic outputs.

GenAlEx calculates G-statistics, a collection of five frequency-based population structure estimators for codominant data: G_{ST}, Nei's standardized G'_{ST}, Hedrick's G'_{ST} standardized for small numbers of populations, G''_{ST} and Jost's D_{EST} (F_{ST} is also calculated, in order to compare it with the other estimators).

- G_{ST} is based on a comparison of the expected heterozygosity within and among populations, but never reaches a maximum value of 1. This is because the values of genetic structure of natural populations depend on the within-population diversity, as higher variability lowers the maximum genetic structure value. This can derive in confusion when interpreting results, as a genetic structure value can be mistaken for a seemingly low value, when in fact it is the maximum that can be obtained given the data (Charlesworth, 1998; Hedrick, 1999). This issue has been addressed by several authors and is condensed in the work of Meirmans and Hedrick (2011).
- Hedrick's G'_{ST}: this estimator standardizes G_{ST} by dividing in the maximum value it can obtain given the observed within-population diversity, ensuring an upper limit of 1, *i.e.* not reduced by the within-population diversity (Hedrick, 2005; Meirmans and Hedrick, 2011).

Additionally, biases can arise when heterozygosities are estimated from allele frequencies calculated from a low number of individuals from the population (Meirmans and Hedrick, 2011). To tackle this issue, three estimators are used:

- Jost's D_{EST}: in addition to working on the issue with the upper limit imposed by the within-population diversity, is based on the effective number of alleles (NA) rather than expected heterozygosity (H_e). This is because NA scales linearly with increasing diversity, while H_e does not (Jost, 2008).
- Nei's standardized G'_{ST}: corrects G_{ST} by avoiding an included comparison of every population with itself, leading to an underestimation of genetic structure when the number of sampled populations is low (Nei, 1987).
- G''_{ST}: this estimator is an unbiased version of Hedrick's G'_{ST} that accounts for the same issue (Meirmans and Hedrick, 2011).

Relatedness and relationships

Estimations of relatedness and putative genetic relationships were performed with the R package *Related* 1.0 (Pew et al., 2015) and *ML-Relate* (Kalinowski et al., 2006). *Related* allows to calculate relatedness based on seven estimators and to compare their performance through Pearson's correlation coefficient, in order to select the one that suits the data better. One moments-based estimator and one likelihood-based estimator were selected, as their performance can vary depending on the data (Wang, 2014). Additionally, this package allows to test whether the group relatedness found within each stranding is significantly different from those found in a randomly assembled population. This is achieved by comparing the observed and expected values, which are generated via simulations. Individuals are randomly

shuffled between groups, which remain of constant size, and average relatedness is calculated after each randomization.

Relatedness of stranded individuals were also evaluated using *ML-Relate* software. Assuming a closed, outbreeding population, this program estimates the relatedness index (r) for each pair of individuals in the matrix, interpreted as the probability that an allele is identical by descent. The software also assigns each pair to one out of four putative genetic categories: unrelated (U), half-siblings (HS) or alternatively grandparent-grandchild (GG), full-siblings (FS) or parent-offspring (PO), calculated as the probability that two individuals share zero, one or two alleles identical by descent, per locus (Kalinowski et al., 2006).

Additionally, the software *SOCPROG* 2.9 (Whitehead, 2009) was used to associate individuals into groups, based on the first-order relationships found by *ML-Relate*.

Detection of first generation migrants

First generation migrants were detected with *GENECLASS 2* (Piry et al., 2004). Using multilocus genotype data, *GENECLASS* assigns to or excludes individuals from their reference populations, identifying potential first-generation migrants. A likelihood ratio is calculated with $L=L_{home}/L_{max}$ for each individual, where L_{home} is the likelihood calculated from the sampling population, whereas L_{max} is the highest likelihood value among all populations, including the corresponding sampling population. Individual probabilities of belonging to each population are calculated using MCMC resampling algorithms.

Results

Molecular sexing

Among both strandings, the sex of 35 individuals (27 females (F) and 8 males (M)) was identified, while 13 remained uncategorized. For IC, the sex of 23 individuals (17 F and 6 M) could be identified visually and/or genetically: four individuals (4 F) were assessed with both molecular and visual data, five individuals (3 F and 2 M) were molecularly assessed but had no visual determination and fourteen individuals (10 F and 4 M) were visually assessed with no successful amplification. All twelve individuals of the IN stranding were successfully amplified and had visual assessment as well (10 F and 2 M).

Amplification of mitochondrial DNA and microsatellites

A consensus fragment of 598 bp of the D-loop region was obtained for a total of 90 samples, 78 from IC and 12 from IN. One haplotype was previously unreported (hereon referred to as haplotype R2). Additionally, 16 of the 19 microsatellite loci were successfully genotyped across most individuals of the total 48. Particularly, 36 individuals from the IC stranding (5.6% of missing data) and all 12 from the IN event, with no missing data.

The loci DlrFCB6, GT51 and Sgui03 presented an excess of homozygotes in the IC samples, attributed to the possible presence of null alleles, and were removed from the dataset. No evidence for scoring error, large allele dropout or null alleles was found for IN. The loci were then checked for outliers and locus GT23 was found to be under selection and removed from further analyses (Figure 2). Inbreeding (F_{IS}) was found to be very low and not significant, for neither IC (F_{IS}=-0.014, p=0.300) nor IN (F_{IS}=0.015, p=0.400).

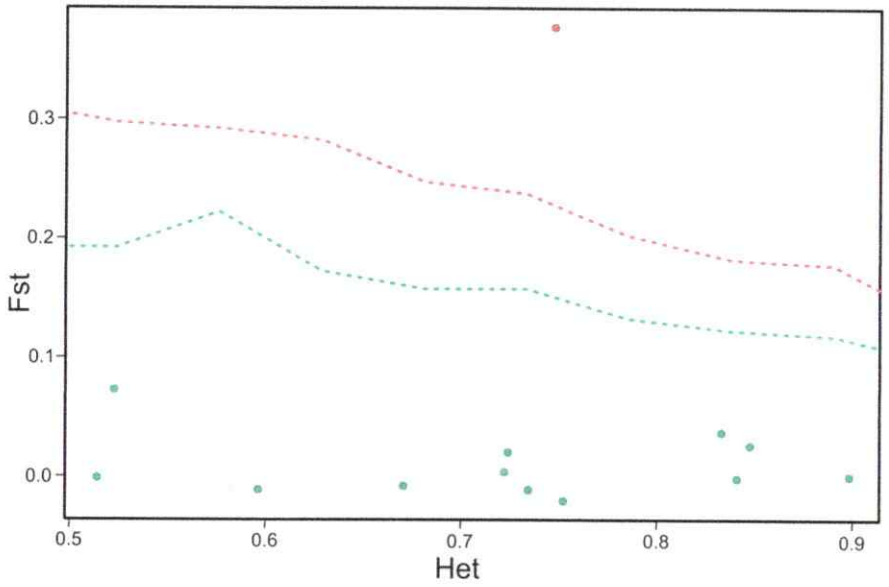

Figure 2: Detection of the outliers (red dot) with *Arlequin*, plotted as genetic structure (F_{ST}) versus heterozygosity (Het). The green and red lines represent distribution quantiles of 0.90 and 0.95 respectively. Dots represent each locus, with GT23 under positive selection in red.

Genetic diversity, genetic and phylogeographic structure

Genetic diversity - Mitochondrial DNA

A total of four haplotypes were identified among the 90 control region sequences (Table 1). Of these, two were present only in IC (haplotypes S+R, n=31, and Q+Y, n=12), one in IN (haplotype R2, n=2) and one was shared (haplotype P+U, n=35 in IC and n=10 in IN). The latter was also the most frequent haplotype, accounting for half of the sequences (Figure 3). The genetic diversity values obtained for IC (n=78) and IN (n=12) were, respectively: number of haplotypes (k): 3 and 2; segregating sites (S): 2 and 4; haplotype diversity (Hd): 0.625 and 0.303; and nucleotide diversity (π%): 0.125 and 0.203 (Table 1).

Table 1: Summary of the mtDNA control region genetic diversity of *Globicephala melas*, for each stranding and the complete dataset. The frequency of each haplotype (S+R, P+U, Q+Y, R2) is reported per stranding and in total. The genetic diversity indices number of polymorphic sites (k), number of haplotypes (k), haplotype diversity (Hd), nucleotide diversity (π) and pairwise differences between sequences ($\prod$) are also reported.

Locality	S+R	P+U	Q+Y	R2	Total	k	S	Hd	π (%)	$\prod$
Isla Clemente	31	35	12	-	78	3	2	0,625	0,125	0,749
Isla Navarino	-	10	-	2	12	2	4	0,303	0,203	1,212
Total	31	45	12	2	90	4	5	0,62	0,14	0,835

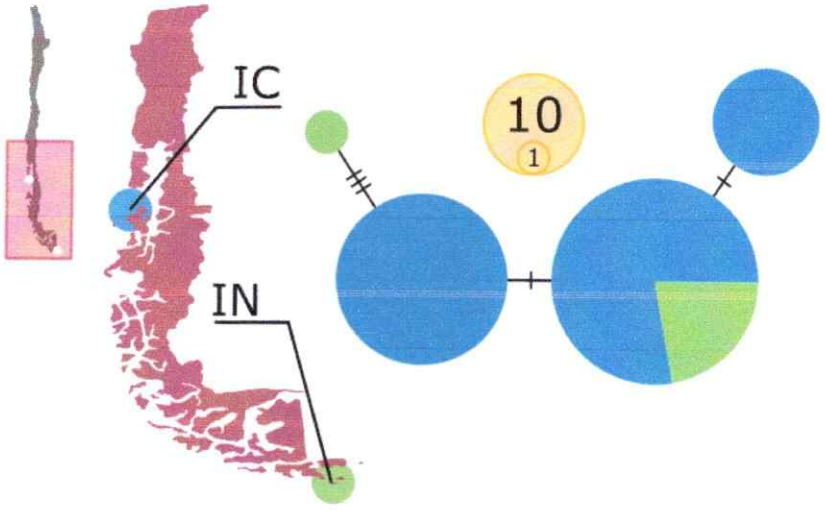

Figure 3: Haplotype network of the 90 sequences of *Globicephala melas* from the strandings at Isla Clemente (IC, n=78, blue) and Isla Navarino (IN, n=12, green) in southern Chile. Circle size is proportional to haplotype frequency. Each hash between haplotypes represents one mutational step.

Genetic diversity – Microsatellites

The genetic diversity indices obtained for the microsatellite data for IC and IN were, respectively: expected heterozygosity (H_e): 0.719 and 0.736; observed heterozygosity (H_o): 0.739 and 0.757; average number of alleles per locus (NA): 7.75 and 6.50; average allelic richness (AR) based on the lowest sample size, n=12: 5.95 and 6.50 (Table 2). Neither H_e nor AR were statistically different among both strandings (Fisher's paired comparisons, p=0.775 and p=0.062 respectively, after 10 000 randomizations). Alleles and allele frequencies for each locus are available in the supplementary table ST1.

Table 2: Microsatellite diversity and inbreeding coefficient F_{IS} per locality and of the complete dataset of 12 loci. Number of genotyped individuals (n), number of alleles (nA), allelic richness (AR), observed heterozygosity (H_o), expected heterozygosity (H_e).

	Locus	n	nA	AR	Range	Ho	He	Fis	% val. >	% val. <	% val. =	p-value (%)	Private alleles
Isla Clemente	464/465	27	9	7,24	139-157	0,741	0,772	0,059	17,3	67,8	14,9	32,2	2
	DlrFCB1	36	8	6,13	123-143	0,722	0,750	0,052	19,9	67,3	12,8	32,7	2
	Ev1	33	8	5,28	149-167	0,727	0,705	-0,017	47,9	36	16,1	52,1	2
	Ev14	30	6	5,31	131-141	0,867	0,739	-0,156	92	2,8	5,2	8	1
	GT39	36	5	3,23	140-156	0,611	0,506	-0,194	87,2	6,2	6,6	12,8	3
	GT48	31	16	10,81	195-233	0,807	0,868	0,087	4,7	86,6	8,7	13,4	6
	GT575	36	7	6,75	153-167	0,833	0,821	-0,001	37,8	43,5	18,7	62,2	-
	MK5	36	6	4,40	219-231	0,694	0,642	-0,068	65,4	21,7	12,9	34,6	2
	MK9	36	4	3,51	158-164	0,528	0,572	0,092	16,9	70	13,1	30	-
	PPHO131	34	5	4,50	191-199	0,677	0,664	-0,004	40,1	42,7	17,2	59,9	-
	Sgui06	30	10	7,67	203-229	0,867	0,814	-0,047	61,7	19,8	18,5	38,3	3
	Sgui16	35	9	6,58	161-177	0,800	0,769	-0,026	51,8	28,9	19,3	48,2	2
Isla Navarino	464/465	12	7	7	139-157	0,694	0,583	0,202	3,3	87,4	9,3	12,6	-
	DlrFCB1	12	6	6	125-135	0,667	0,754	0,158	7	79,7	13,3	20,3	-
	Ev1	12	7	7	149-163	0,750	0,788	0,092	12,2	62,8	25	37,2	1
	Ev14	12	5	5	131-139	0,667	0,597	-0,073	46,6	21,4	32	53,4	-
	GT39	12	2	2	140-152	0,750	0,497	-0,478	87,4	0,6	12	12,6	-
	GT48	12	12	12	195-235	0,917	0,906	0,032	13,4	51,6	35	48,4	2
	GT575	12	7	7	153-167	0,917	0,771	-0,147	72,9	6,3	20,8	27,1	-
	MK5	12	5	5	219-227	0,667	0,719	0,116	15,4	65,5	19,1	34,5	1
	MK9	12	4	4	158-164	0,667	0,642	0,006	32,1	41,6	26,3	58,4	-
	PPHO131	12	6	6	191-201	0,833	0,781	-0,023	37,5	31,9	30,6	62,5	1
	Sgui06	12	9	9	199-221	0,833	0,847	0,060	15,7	57,1	27,2	42,9	2
	Sgui16	12	8	8	163-185	0,833	0,833	0,043	19,5	51	29,5	49	1
Total	464/465	39	9	-	139-157	0,692	0,761	0,104	4,8	90,6	4,6	9,4	-
	DlrFCB1	48	8	-	123-143	0,708	0,754	0,070	14,1	76,1	9,8	23,9	-
	Ev1	45	9	-	149-167	0,733	0,732	0,010	38,9	45,6	15,5	54,4	-
	Ev14	42	6	-	131-141	0,810	0,708	-0,131	92,6	3	4,4	7,4	-
	GT39	48	5	-	140-156	0,646	0,509	-0,259	98,2	0,8	1	1,8	-
	GT48	43	18	-	195-235	0,837	0,889	0,070	4,1	88,4	7,5	11,6	-
	GT575	48	7	-	153-167	0,854	0,825	-0,024	58	26,4	15,7	42,1	-
	MK5	48	7	-	219-231	0,688	0,666	-0,021	51,4	36,4	12,2	48,6	-
	MK9	48	4	-	158-164	0,563	0,594	0,063	22,2	66,5	11,3	33,5	-
	PPHO131	46	6	-	191-201	0,717	0,708	-0,002	41	45,2	13,8	59	-
	Sgui06	42	12	-	199-229	0,857	0,833	-0,017	48,8	33	18,2	51,2	-
	Sgui16	47	10	-	161-185	0,809	0,805	0,007	38,7	45,8	15,5	54,2	-

Genetic structure

At the mtDNA level, evidence of genetic and phylogeographic structure was found with the complete mitochondrial dataset (F_{ST}=0.205, p=0.0039 and Φ_{ST}=0.131, p=0.00879). In contrast, the microsatellite dataset revealed an absence of genetic structure in *Genetix* (F_{ST}=0.0049, p=0.33), *Structure* (K=1, L(K) = -1981.8) and *GenAlEx* (Table 3, Figure 4). The estimators that correct the upper limit of the estimator effectively scored higher than G_{ST}, from two times as high (G'_{ST}(Nei)) to approximately eight times higher (G'_{ST} (Hed) and G''_{ST}). Corrected values were still an order of magnitude lower than the mitochondrial (F_{ST}=0.205).

Table 3: Genetic structure values of the six genetic structure estimators used by *GenAlEx* and their respective p-values.

Estimator	Value	p-value
F_{ST}	0.017	0.278
G_{ST}	0.002	0.226
G'_{ST} (Nei)	0.004	0.226
G'_{ST} (Hed)	0.015	0.222
G''_{ST}	0.017	0.222
D_{EST}	0.013	0.299

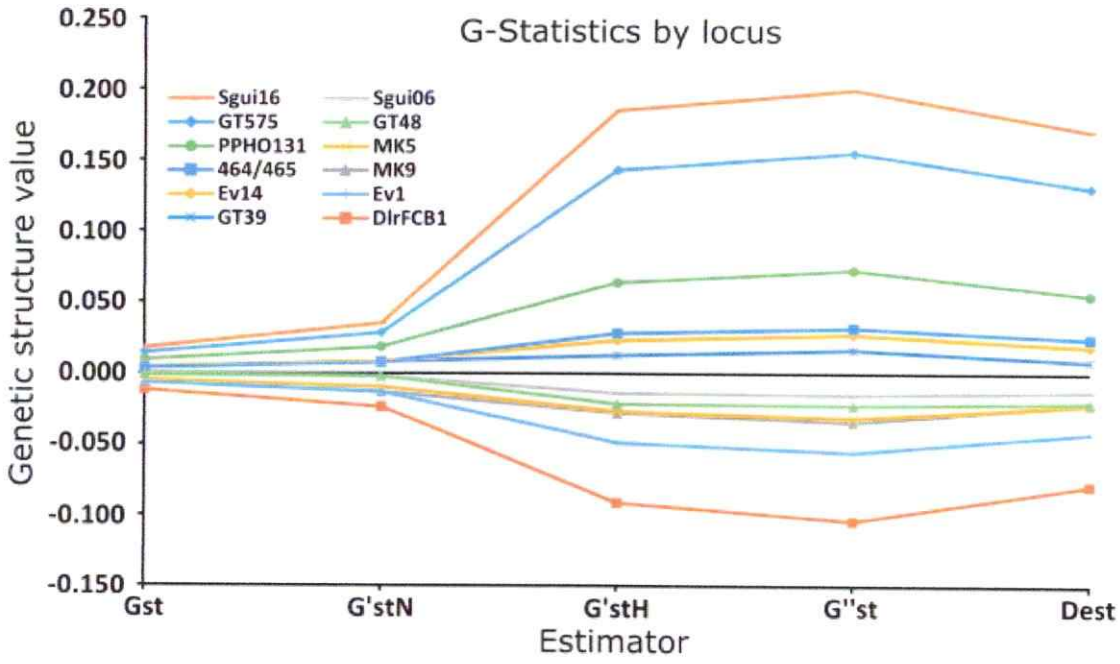

Figure 4: Locus-by-locus values of genetic structure of the 12 loci for each of the five estimators, G_{ST}, $G'_{ST}N$, $G'_{ST}H$, G''_{ST} and D_{EST}.

Estimation of gene flow

A total of four first generation migrants were detected with a set statistical significance of p=0.05. IC was proposed to have one migrant that was most likely from IN, while three individuals in IN were probably derived from IC (Table 4).

Table 4: The four first generation migrants detected by *GeneClass 2*, with a lower statistical significance than p=0,05.

Code of individual	Stranding of origin	-log (L home /L max)	IC –log (L)	IN –log (L)	p-value
G58	IC	2.353	20.606	**18.253**	0.0408
807	IN	3.564	**18.983**	22.547	0.0086
810	IN	2.254	**13.479**	15.733	0.0287
811	IN	2.259	**16.581**	18.840	0.0263

Relatedness and relationships

Relatedness

Based on estimator performance tested with Pearson's correlation coefficient (r), two estimators were selected: one of the five moments estimators (Wang, r=0.834) and one of the two likelihood estimators (TrioML, r=0.846). Average relatedness across all 48 samples was Wang = -0.013 and TrioML = 0.076. The average values obtained for IC were higher than the overall average (Wang= 0.019 and TrioML= 0.085), while those of IN were lower (Wang= -0.094 and TrioML=0.063). Estimates of relatedness among both strandings were Wang= -0.047 and TrioML=0.064. After estimating relatedness, the function *grouprel* was used to test if average relatedness was different from that of randomly grouped individuals. No significant differences were detected (*Wang*: $p < 0.895$; *TrioML*: $p < 0.58$). The function

performs separate tests for each group (i.e. stranding), which detected differences in IC (*Wang*: $p < 0.0025$; *TrioML*: $p < 0.02$), but showed no significant difference for IN (*Wang*: $p < 0975$; *TrioML*: $p < 0.745$). Relatedness estimations per sex showed similarly low values and neither male nor female dyads were not different from randomly grouped individuals (Table 5).

Table 5: Average relatedness values of Isla Clemente, Isla Navarino, overall data and per dyad (female, male, female-male), based on the TrioML estimator, Wang estimator and *ML-Relate*. Statistical significance of the obtained values is also reported, significant p-values in bold.

Estimator	Clemente	Navarino	Among	Overall	Female dyads	Male dyads	F-M dyads
TrioML	0.085	0.063	0.064	0.076	0.04 (0.076)	0.069 (0.070)	0.052 (0.112)
Wang	0.019	-0.094	-0.047	-0.013	-0.017 (0.172)	-0.010 (0.216)	-0.008 (0.189)
ML-Relate	0.067	0.049	0.045	0.057	0.049	0.069	0.068
Different from randomly grouped individuals							
TrioML	**p<0.02**	p<0.745	-	p<0.58	p<0.94	p<0.25	-
Wang	**p<0.0025**	p<0.975	-	p<0.895	p<0.568	p<0.505	-

Of the 20 highest relatedness values calculated with the TrioML estimator, half were pairs of females (FF), seven male-female (FM) and three were male pairs (MM). The highest MM value was in 10th place and the remaining two in 16th and 18th. Wang estimator showed a similar proportion, 8 HH, 10 HM and 2 MM pairs. The highest MM pair was ranked 6th and the second in 17th (Table 6).

Table 6: Highest 20 relatedness values obtained for each estimator among pairs of females (FF), males (MM) and female-male (FM).

Dyad	TrioML	Dyad	Wang
HM	0.653	HM	0.515
HH	0.561	HH	0.514
HM	0.552	HM	0.49
HM	0.543	HM	0.485
HH	0.500	HM	0.480
HM	0.495	MM	0.455
HH	0.493	HH	0.424
HM	0.467	HM	0.422
HH	0.457	HM	0.416
MM	0.439	HM	0.388
HH	0.406	HH	0.382
HH	0.395	HM	0.366
HM	0.382	HM	0.365
HH	0.381	HH	0.363
HH	0.376	HH	0.363
MM	0.370	HM	0.356
HH	0.370	MM	0.356
MM	0.370	HH	0.355
HH	0.358	HH	0.354
HM	0.352	HH	0.345

Relationships

A total of 166 relationships were estimated by *ML-Relate* (supplementary table ST1), particularly 17 first-degree (11 PO and 6 FS) and 138 second-degree (half-siblings) relationships, representing a 14.7% out of a possible 1128 dyad combinations, while almost 84% of dyads were unrelated.

Individuals from IC presented eight PO, all detected FS (n=6) and 87 HS relationships, while those from IN presented a single PO and 7 HS relationships. Related dyads were also detected among both strandings, particularly 2 PO and 44 HS relationships (Table 7). Of the analyzed 36 pilot whales of the IC stranding, eleven presented a PO relationship with another individual, while 10 showed FS relationships.

In contrast, average relatedness among pilot whales of IN did not differ from that of a randomly assembled group. The stranding contained a sole first-order relationship, corresponding to a mother-daughter pair. Nevertheless, despite the different sampling size, it accounted for a similar percentage of PO relationships as in IC (PO_{IN}=1.52%, PO_{IC}=1.27%) and a similar percentage of unrelated pairs was detected as well (U_{IN}=87.88%, U_{IC}=83.97%). The second-degree dyads showed some differences, representing a slightly lower proportion of dyads in IN (PO_{IN}=10.19%, PO_{IC}=13.81), while the most notorious difference was the absence of FS in the IN stranding.

Table 7: Number of relationships per category and within parentheses as a percentage of the total dyads in each category. Relationships were classified either as parent-offspring (PO), full-siblings (FS), half-siblings (HS) or unrelated (U).

	PO (%)	FS (%)	HS (%)	U (%)	Total
Isla Clemente	8 (1.27)	6 (0.95)	87 (13.81)	529 (83.97)	630
Isla Navarino	1 (1.52)	0 (0)	7 (10.61)	58 (87.88)	66
Among strandings	2 (0.46)	0 (0)	44 (10.19)	386 (89.35)	432
Total	11 (0.98)	6 (0.53)	138 (12.23)	973 (86.26)	1128

On the base of these first-degree genetic relationships, *Socprog* allowed the identification of 7 pairs and 4 kinship groups, of which three were composed of 3 individuals and one of 5. Among the individuals from IC, 23 (64%) presented a first-degree relationship with another pilot whale from the same stranding. Three IC individuals (one not included in the previous 23) formed a pair and a group of three with members of the IN stranding. Finally, IN only presented a single PO relationship (Figure 5).

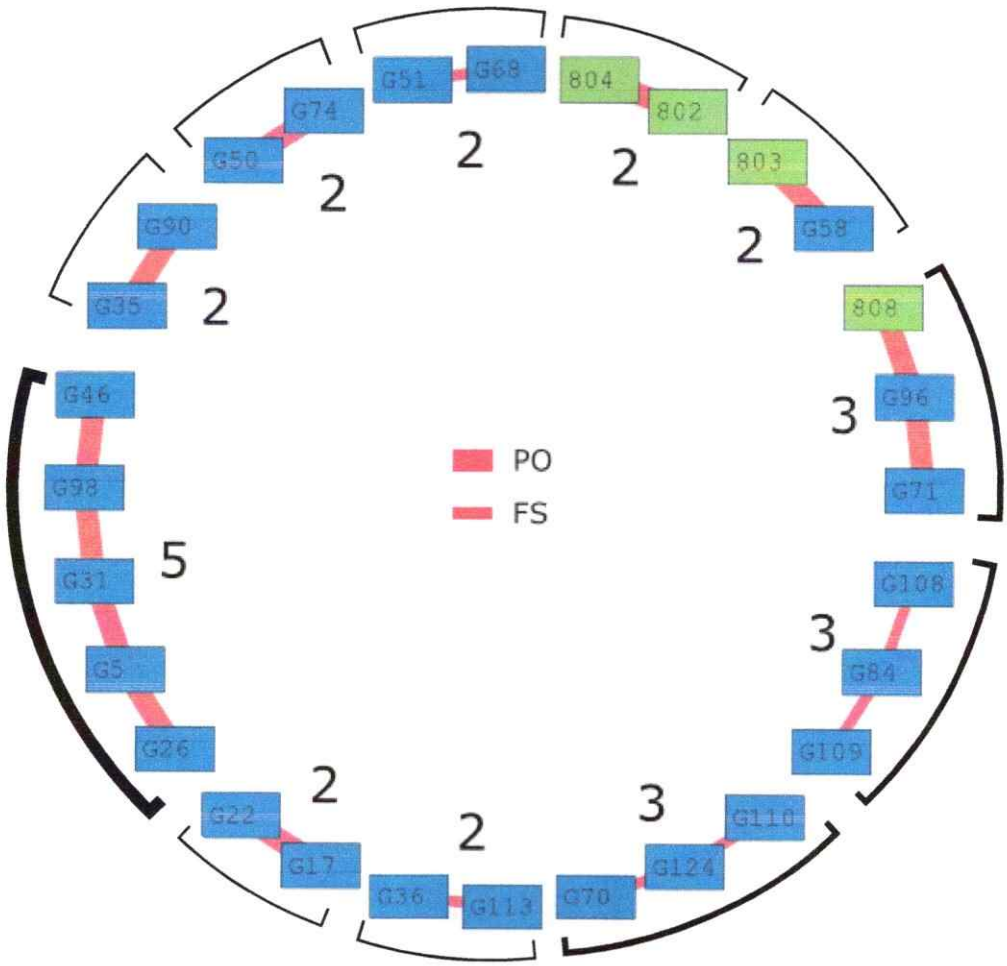

Figure 5: Genetic groups among the individuals that presented first-degree relationships of the strandings in Isla Clemente (blue) and Isla Navarino (green). Parent-offspring (PO) relationships are represented by the thick lines and full-siblings (FS) by the thin line. Numbers represent the size of each group and individuals within each are contained by brackets, with thickness proportional to group size.

Discussion

Before interpreting our findings, three aspects of the current study require some discussion, particularly the study of population structure based on stranded cetaceans and estimating relatedness with genetic markers and the nucleotide sequences. Pilot whales are highly social and groups tend to form around kin. Regarding this, previous evidence in brown trouts suggests that sampling close relatives instead of random individuals can induce bias in the estimation of allele frequencies (Hansen et al., 1997), and thus genetic structure. In this sense, results can potentially be influenced by using stranded instead of free-ranging animals particularly in cohesive and matrilineal species and at small geographic scales, but is not necessarily always the case (Bilgmann et al., 2011). Nevertheless, mass strandings have become a valuable source of information and samples(Geraci and St. Aubin, 1977) and much of the knowledge of many species of marine mammals has been obtained from such events (Geraci and St. Aubin, 1979; Wilkinson and Worthy, 1999). The elevated monetary, logistic and time costs that collecting a similar number of samples from free-ranging individuals signifies would have precluded the execution of this study.

Secondly, caution is advised when interpreting relatedness from molecular data, as the performance of estimators can vary greatly (Taylor, 2015) and depends on a reference population, which most commonly is the same studied population (Taylor, 2015; Wang, 2014). Effectively separating reference and study populations is highly challenging when researching wild populations. However, some estimators have included this factor (Wang, 2014) and we used two different estimators to consider this factor. In addition, especially when dealing with markers characterized by low diversity, identity by state of alleles is heightened and true genetic similarity can be masked (Taylor, 2015). This may turn into a

possible underestimation of relatedness and inbreeding level of closely related individuals, and vice versa for distantly or unrelated pairs (Taylor, 2015).

Finally, prior to conducting mtDNA analyses, we first decided to remove one polymorphic site that exhibited a strong homoplasy signal. This problem in mtDNA sequences of *G. melas* was already detected (Oremus et al., 2009), but not taken into account for the genetic analyses. Homoplasic sites have been described to hinder the resolution of mtDNA gene trees (McCracken and Sorenson, 2005) and have also been pointed out as potential confounders of evolutionary analysis in the mtDNA control region of humpback whales (Jackson et al., 2014) and human mtDNA coding regions (Herrnstadt et al., 2002), among others.

This study presents the first genetic data on population structure and relatedness of long-finned pilot whales in the southeastern Pacific region, using bi-parentally inherited nuclear microsatellite markers and maternally inherited mtDNA D-loop. The observed difference in amplification success among both strandings, principally for sex identification but also to some extent for the mtDNA and microsatellite markers, was attributed to the contrasting state in which the individuals of each event were. The IC stranding was sampled approximately two to three months after occurring (Alvarado-Rybak et al., 2019), while the IN individuals were sampled five days after stranding (Mansilla et al., 2012), precluding tissue deterioration, as seen in IC (Figure 1 b). Considering both sampling areas, the total number of identified females was almost three times higher relative to males. Although the sex of 27% of individuals could not be successfully identified, the higher number of females could be reflecting the matrilineal social structure of the species, as groups are composed of an excess of females relative to males (Olson, 2018; Sergeant, 1962).

Overall, long-finned pilot whales from the southeastern Pacific presented low mitochondrial

and microsatellite diversity -as displayed by matrilineal odontocetes in general-, another likely turnout of their social structure (Vachon et al., 2018; Whitehead et al., 2017). However, several differences were evident regarding diversity of the two strandings. Even if the number of mtDNA haplotypes in IC and IN are very similar (k_{IC}=3, k_{IN}=2), genetic diversity of IC doubled that of IN (Hd: 0.625 vs 0.303), mainly due to the presence of several medium-frequency haplotypes in IC. A possible explanation for this difference among both strandings may be that the pilot whales that stranded on IC belonged to multiple matriline units which were associated into a larger group at the time of the event, as previously proposed in Tasmania and New Zealand (Oremus et al., 2013). Social units are known to interact and form larger, more labile groups (Augusto et al., 2017; de Stephanis et al., 2008; Ottensmeyer and Whitehead, 2003). In contrast, the much smaller group stranded in IN would correspond mainly to a single matriline or at least part of one.

The presence of several matrilines in the IC stranding is further supported by the genetic diversity of microsatellites. Allelic richness and genetic diversity were roughly similar between both strandings (AR_{IC}=5.77 and AR_{IN}=6.50; H_{IC}=0.72 and H_{IN}=0.73), which are bi-parentally inherited. Yet, of the total 101 alleles, a quarter (n=25) were private to IC, approximately three times more than IN (7.9%). This might be indicative of several groups stranded together, each with their own private alleles. Nevertheless, we are aware that these differences in genetic diversity may also have been influenced by the unequal sample size (n_{IC}=36 vs n_{IN}=12).

Further observations regarding their social system can be made from the relationships that were found among all 48 individuals. Of the total dyads, 86.3% were unrelated and 13.7% (n=155) were categorized into one of three close relationships. The majority of these

corresponded to second-degree relationships (138 HS or GG), which were almost ten times more numerous than the first-degree relationships, where PO pairs almost doubled the FS dyads (11 vs 6). Pilot whales are polygynous and males are thought to move between groups in order to mate (Olson, 2018). They remain only a few months in the group they join, after which females give birth to a single calf in multiyear intervals (Amos et al., 1991; Balbuena and Raga, 1994). Thus, the expected relationship proportions to arise from such a dynamic are congruent with the pattern seen in these results. The absence of stable breeding pairs together with the conception of a single offspring per birth results in a higher proportion of half-siblings and parent-offspring pairs, relative to the cases of full siblings. Collectively in the IC stranding, 23 individuals presented a first-degree relationship, accounting for 64% of the analyzed pilot whales in this locality. Among these, we were able to identify distinct genetic groups, particularly 5 pairs and 3 distinct kinship groups, two of 3 individuals and one of 5. Similar results were obtained in a study of sperm whale social units in which 82.5% of individuals had a first-degree relationship with another individual in the same social unit (Konrad et al., 2018). The higher percentage of first-degree relationships could be a result of analyzing separate social units and not a stranding as a unit, as the latter would contain mixed individuals of different groups and increase the percentage of loosely related pairs. However, the finding of higher relatedness in the larger stranding contrasts with a previous report in the sister species *G. macrorhynchus*, where relatedness was shown to decrease with larger group size (Alves et al., 2013). Nevertheless, that study did not involve stranded individuals but alive, free-ranging animals of the sister species and differences can arise when conducting studies with either stranded or live individuals (Bilgmann et al., 2011). Average relatedness was lower than in other matrilineal odontocetes, like orcas (Queller & Goodnight estimator

= 0.102-0.305 vs. -0.002) and sperm whales (Wang=0.139 vs. 0.019) (Konrad et al., 2018). The difference among these studies and our own is once again probably caused by the nature of the IC stranding, which appears to be an assemblage of several units that stranded together, lowering overall relatedness. This is exemplified by the study on social units of sperm whales, in which comparatively lower relatedness was obtained when comparing among rather than within social units (Wang=0.004 vs 0.139 respectively) (Konrad et al., 2018). The existence of several matrilineal units in IC may also explain why our relatedness values fall closer to those registered in non-matrilineal species, as, for instance, stranded and accidentally entagled Franciscana *Pontoporia blainvillei* of northern Argentina. Relatedness values were higher for male pilot whales than male Franciscanas (*ML-Relate*: 0.069 vs. 0.030) and lower for female pilot whales compared to female Franciscanas (*ML-Relate*: 0.049 vs. 0.060) (Gariboldi et al., 2016). On the other hand, our results showed higher relatedness values that the overall estimations obtained for free-ranging short-beaked common dolphin *Delphinus delphis* from southern Australia, although slightly lower for females (*Delphinus delphis* TrioML estimator values: FF=0.043, MM=0.041 and MF=0.045) (Zanardo et al., 2016).

The differences in genetic diversity and in relatedness encountered between the more numerous IC stranding and the smaller IN stranding may be ascribe to the dynamic social structure of pilot whales. Core units are based on stable matrilineal associations among members of both sexes, generally of a size around 10 individuals (Augusto et al., 2017; de Stephanis et al., 2008; Ottensmeyer and Whitehead, 2003). Long-lasting bonds are usually formed among these smaller units, which can last for up to several years, hence members rarely leave the group (de Stephanis et al., 2008) unless a critical size is reached, when they are thought to split into smaller units (Augusto et al., 2017). In turn, these core groups

associate with others to form larger, potentially much shorter-lived groups of up to hundreds of individuals (Augusto et al., 2017; de Stephanis et al., 2008; Ottensmeyer and Whitehead, 2003) which, like in this case, can engage in mass stranding events.

Regarding the geographic distribution of genetic diversity, both strandings, separated by over 1 500 km apart, presented a significant mitochondrial DNA genetic structure. Geographic distance commonly underlies genetic structure, particularly in organisms of limited dispersal abilities. However, in this highly mobile species, this is an unlikely originator of the observed genetic structure, as adult female pilot whales have been documented to travel 1 577 km in as fast as 20 days (Bloch et al., 2003). Rather than geographic distance, prey distribution and abundance have been linked to population structure in some areas of their distribution (Fullard et al., 2000; Hátún and Gaard, 2010; Payne and Heinemann, 1993). In line with this, social and behavioral factors might be underlying the genetic structure (Ford et al., 1998). Accordingly, site fidelity has been widely described in matrilineal species. Resident pilot whale *Globicephala* spp. populations has been described in the Strait of Gibraltar, the Madeira Islands and the coast of California and Hawaii (Mahaffy, 2012; Olson, 2018), in sperm whales (Engelhaupt et al., 2009) and it has been suggested for a population of false killer whales (Martien et al., 2014).

Cetaceans also have extended nursing periods and high maternal energy investment (Trillmich and Cantor, 2018), which is more pronounced in matrilineal species. Consequently, the non-migrating sex usually relies more strongly on familiarity with local food resources, which in mammals most commonly is females (Pusey, 1987).

Contrarily, as suggested by the absence of genetic structure with the bi-parentally inherited microsatellite markers, gene flow appears to be male-mediated. The genetic relationships

among the two strandings are indicative of a possible connection of the two areas, particularly 44 second-degree relationships and two parent-offspring dyads. This could be a mostly southward gene flow, as there appears to be a greater number of first generation migrants from IC present in IN. This type of sex-biased gene flow is a common reproductive behavior among mammal species (Greenwood, 1980) and has been reported in several other marine mammals as well, such as southern elephant seals (Fabiani, 2003), belugas (Brown Gladden et al., 1999), bottlenose dolphins (Pérez-Alvarez et al., 2018), the matrilineal sperm whales and orcas (Lyrholm et al., 1999; Pilot et al., 2010). Previous studies have also suggested this for pilot whales, since males are thought to mate either during aggregations of pods or when moving between pods, as a reproductive strategy to avoid inbreeding (Amos et al., 1991; Andersen and Siegismund, 1994; Balbuena and Raga, 1994). This male-mediated gene flow is reflected in the fewer male-male pairs of high relatedness that were detected, with respect to female-female and female-male pairs (Table 6). The vagility of the species also coheres with these results, as a juvenile male long-finned pilot whale was recorded covering 3 003 km in just under a month and an adult male navigated 1 048 km in fifteen days (Bloch et al., 2003), while another study described two juvenile males travelling at least 984 km in sixteen days and at least 3 790 km within approximately four months (Nawojchik et al., 2003).

The results of this study present the utility of using data and samples collected from mass strandings to conduct genetic research in cetaceans. Secondly, exemplify the importance of integrating this with behavioural studies, in order to conduct ecological studies in wild populations.

Approval

We confirm that all methods were carried out in accordance with relevant guidelines and regulations. Samples were taken from stranded, deceased animals with permission from the National Fisheries Service (SERNAPESCA, document ID 2016-11-13). All experimental protocols were approved by the Postgraduate Evaluation Committee at the Faculty of Science of the Universidad de Chile.

Capítulo 2

Global phylogeography and genetic diversity of the long-finned pilot whale *Globicephala melas*, with new data from the southeastern Pacific

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Abstract

The matrilineal long-finned pilot whale presents an antitropical distribution and is divided into two subspecies, one in the temperate seas of the Southern Hemisphere and the other restricted to the North Atlantic and Mediterranean. Until now, population genetic and phylogeographic studies have included localities of most of its Northern Hemisphere distribution, while only the southwestern Pacific has been sampled in the Southern Hemisphere. We add new genetic data from the southeastern Pacific to the published sequences. Low mitochondrial and nuclear diversity was encountered in this new area, as previously reported for other localities. Four haplotypes were found with only one new for the species. Fifteen haplotypes were detected in the global dataset, underlining the species' low diversity. As previously reported, the subspecies shared two haplotypes and presented a strong phylogeographic structure. The extant distribution of this species has been related to dispersal events during the Last Glacial Maximum. Using the genetic data and Approximate Bayesian Calculations, this study supports this historical biogeographic scenario. From a taxonomic perspective, even if genetic analyses do not support the subspecies category, this study endorses the incipient divergence process between hemispheres, thus maintaining their status and addressing them as Demographically Independent Populations is recommended.

Resumen

El calderón de aleta larga es una especie matrilineal que presenta una distribución antitropical y está dividida en dos subspecies, una en las aguas temperadas del Hemisferio sur y la otra restringida al Atlántico norte y Mediterráneo. Hasta ahora, los estudios filogeográficos y de genética de poblaciones han incluido localidades de la mayor parte de su distribución en el Hemisferio norte, mientras que solo el Pacífico sudoeste ha sido muestreado en el Hemisferio sur. En este trabajo agregamos datos genéticos nuevos del Pacífico sudeste a las secuencias publicadas. En esta nueva área se encontraron bajas diversidades mitocondrial y nuclear, como ha sido previamente reportado para otras localidades. Cuatro haplotipos fueron encontrados, siendo solo uno nuevo para la especie. Quince haplotipos fueron detectados en la base de datos global, resaltando la baja diversidad de la especie. Como ha sido reportado previamente, ambas subspecies comparten dos haplotipos y presentan una fuerte estructura filogeográfica. La distribución actual de esta especie ha sido asociada a eventos de colonización durante el Último Máximo Glacial. Usando datos genéticos y Computaciones Bayesianas Aproximadas, este estudio respalda este escenario biogeográfico histórico. Desde un punto de vista taxonómico, aunque los resultados genéticos no respalden las categorías de subspecies, este estudio avala el proceso de divergencia incipiente entre Hemisferios, manteniendo su estatus y se recomienda referirse a ellas como Poblaciones Demográficamente Independientes.

Introduction

Cetaceans have a diverse -and many times contrasting- array of geographic distributions. Some of the widest ranging animals species include the cosmopolitan sperm whale *Physeter macrocephalus* (Whitehead, 2018) and the orca *Orcinus orca* (Ford, 2018). Other species, especially small-sized odontocetes, generally present coastal and more restricted distributions, like the extreme case of the vaquita, *Phocoena sinus* (Rojas-Bracho et al., 2018). Some cetaceans have a particular distribution pattern known as disjunct or antitropical, in which the taxon is present at high latitudes in both hemispheres while being absent from lower latitudes (LeDuc, 2018). Examples include the mysticete genus *Eubalaena*, with *E. borealis* inhabiting the North Pacific, *E. japonica* the North Atlantic and their austral equivalent in the Southern Hemisphere, *E. australis* (Kenney, 2018). The phocoenid species pair *Phocoena phocoena* and *P. spinipinnis* is another example, found in the Northern Hemisphere and the coasts of South America, respectively (Jefferson et al., 1996). Among delphinids, the two *Lissodelphis* species also present this antitropical distribution pattern; *L. peronii* is present around the Southern Hemisphere while *L. borealis* is found in the North Pacific (Lipsky, 2002). A similar antitropical distribution is presented by the two subspecies of long-finned pilot whales *Globicephala melas*, where *G. m. edwardii* inhabits the temperate to subpolar waters of the Southern Hemisphere, while *G. m. melas* is restricted to the North Atlantic (Olson, 2018). Extinct populations of long-finned pilot whales have been reported in the North Pacific, from Japan (Kasuya, 1975) and Alaska (Frey et al., 2005), dating back 8 000-12 000 years and 2 500-3 500 years, respectively (Frey et al., 2005; Kasuya, 1975). Pilot whales are a highly social species of matrilineal odontocete, thought to be among the most gregarious cetaceans. The species is known to form groups with a mean size around tens of individuals

(Ottensmeyer and Whitehead, 2003) that can increase to hundreds through aggregations (Bernard and Reilly, 1994; Sergeant, 1982). These groups are structurally based on strong matrilineal associations (Jefferson et al., 1996; Olson, 2018) of closely related females and their descendants (Amos et al., 1991). Pilot whales are among the most common cetaceans involved in mass strandings (Jefferson et al., 1996; Olson, 2018; Sergeant, 1982). Southern Hemisphere long-finned pilot whales were originally described as a distinct species, *G. leucosagmaphora* (Rayner, 1939), but eventually ranked as one of the two subspecies of *G. melas* (Davies, 1960), based on observations of the coloration pattern and morphology (Davies, 1960; Sergeant, 1962). Sergeant (1962) stated that he found only minor differences in coloration and none in external morphology between specimens from the two hemispheres, albeit being in agreement with the subspecies classification proposed by Davies (1960). The author also expressed the need of including samples from additional localities in order to assess the variation of the colour pattern in the subspecies, as also recommended by other authors (Berta and Churchill, 2012). A more recent study found differences in skull morphometry of North and South Atlantic specimens (Marina et al., 2018), but no studies were found that account for geographic variation within each area. Little genetic evidence exists to support their classification status using mitochondrial DNA. Oremus et al., (2009) performed the first inter-hemisphere comparison of the taxa, between the southwestern Pacific and the North Atlantic (Oremus et al., 2009). The authors stated that the two subspecies do not qualify as Evolutionary Significant Units (ESU) according to the reciprocal monophyly criterion of Moritz (1994) (Moritz, 1994), only reporting restrictions to gene flow among the areas of distribution mainly based on differences in the frequency of shared haplotypes. In the study of Oremus et al., (2009), preliminary genetic data support a

biogeographic scenario previously proposed by Davies (1963). A colonization event from south to north would have taken place through a founder effect, followed by demographic population growth. Additional genetic research on pilot whales has been conducted mostly at the population level in the North Atlantic and Mediterranean Sea (Miralles et al., 2016; Monteiro, 2013; Verborgh, 2015), however, an integration of this available information is missing, together with the sampling of other areas such as the southeastern Pacific. In this study, we include new genetic data and results from samples collected in two mass standings in southern Chile, in order to improve the global phylogeographic overview of long-finned pilot whales. We also evaluate the historical biogeographic processes that originated the extant antitropical distribution of this species and discuss its taxonomic status.

Materials and methods

Southeastern Pacific sample collection

Tissue samples were collected in 2006 from twelve individuals in a stranding event that occurred in Isla Navarino, Chile (55° 15'S; 67° 30'W; Figure 1). In 2016, 124 *Globicephala melas* were sampled from the mass stranding event that occurred in Isla Clemente, Chile (45° 35' 57.50" S; 74° 34' 30.32" W). All samples were preserved in 90-95% ethanol.

DNA extraction

DNA extractions were performed following a modified salt-extraction protocol (Aljanabi and Martinez, 1997), adding a second step of digestion with proteinase K one hour after the first one.

Mitochondrial control region sequence data

The mtDNA control region was amplified using the primers M13 Dlp1.5 5'-TGTAAAACGACAGCCAGTTCACCCAAAGCTG RARTTCTA-3' (forward) and 8G 5'-GGAGTACTATGTCCTGTAACCA-3' (reverse) (Dalebout et al., 2005). The amplification protocol was as follows: 25,6 µL reaction volume for each PCR reaction consisted of 12,7µL water, 5µL 10X Buffer (Invitrogen), 2µL 50mM MgCl2 (Invitrogen), 2µL 10pM dNTPs (Invitrogen), 1µL 10pM of each primer (2 µL total), 0,5µL Taq polymerase (Invitrogen) and 70-150 ng of DNA. A Thermo Hybaid PxE 0.5 thermal cycler was used for all amplifications, with the following profile: Preliminary denaturation of 2 minutes at 94°C; followed by 30 cycles of: denaturation for 30 s at 94°C, annealing for 40 s at 56°C, polymerase extension for 40 s at 72°C; and a final polymerase extension for 10 minutes at 72°C and an infinite hold

temperature of 4°C. Each PCR run included positive and negative controls. Fragments were run in a 1% agarose gel, each well containing 3 μL of PCR product mixed with an equal volume of loading dye with 0,3% Gel Red and visualized in a gel documentation system (Maestrogen SMU-01).

PCR product purification and sequencing were done at Macrogen Inc., Seoul, South Korea with a 3730XL DNA Analyzer (Applied Biosystems). All sequences obtained were aligned manually in ProSeq 3.557 (Filatov, 2009). Prior to molecular analyses, the species for each sample was corroborated with two platforms of comparative analysis of sequences: BLAST (Basic Local Alignment Search Tool, www.blast.ncbi.nlm.nih.gov and DNA Surveillance (Ross et al., 2003).

An additional 922 control region sequences were obtained from five other sources: 1) Oremus et al., (2009) (n=573, Tasmania and New Zealand, GenBank access codes: FJ513342-54) (Oremus et al., 2009); 2) Siemann (1994) (n=59; western North Atlantic, Cape Cod, Newfoundland, Nova Scotia, Scotland and England; GenBank access codes: U20926-28) (Siemann, 1994); 3) Monteiro (2013) (n=116, eastern coast of the United States, Faroe Islands, United Kingdom and Iberian Peninsula, GenBank access codes: KC934932-34) (Monteiro, 2013); 4) Verborgh (2015) (n=117, eastern coast of the United States, Faroe Islands, United Kingdom, Euskadi, northeastern Atlantic, Iberian Peninsula, Strait of Gibraltar and the Mediterranean Sea; haplotypes reconstructed by hand) (Verborgh, 2015) and 5) Miralles et al., (2016) (n=57, Faroe Islands and Iberian Peninsula, GenBank access codes: KJ740360-71) (Miralles et al., 2016) (Table 1).

Table 1: Summary of number of haplotypes and genetic indices for each included locality and per subspecies (abbreviations are detailed in the methods section). The first row indicates the names of each haplotype according to the original authors. Numbers 60 and 62 represent the two last digits of the Genbank codes provided by Miralles et al., (2016) for identification.. Number of haplotypes (k), haplotype diversity (Hd), pairwise differences between sequences (Π) and nucleotide diversity (π) are also detailed.

Locality	S+R	P+U	Q+Y	R2	T	O	V	O2	W	Z	E+G	60	62	X	D	Total	k	S	Hd	π (%)	Π
TAS	51	32	72					14	15	31						215	6	4	0.783	0.394	1.356
NZ	6	333	3		1	14	1									358	6	5	0.133	0.040	0.136
CL	31	45	12	2												90	4	4	0.62	0.230	0.791
NWA	73	1												4		78	3	2	0.123	0.064	0.220
FI	55	17														72	2	1	0.365	0.100	0.365
UK	34	1									4					39	3	2	0.235	0.070	0.240
IB	39										1	1	1			42	4	3	0.139	0.055	0.188
NEA	20										1					21	2	1	0.095	0.027	0.095
GIB	44														20	64	2	1	0.436	0.126	0.436
MED	12														21	33	2	1	0.477	0.138	0.477
G.m.edwardii	88	410	87	2	1	14	1	14	15	31						663	10	9	0.580	0.233	0.802
G.m.melas	277	19									6	1	1	4	41	349	8	5	0.353	0.117	0.404
Total	365	429	87	2	1	14	1	14	15	31	6	1	1	4	41	1012	15	12	0.680	0.264	0.909

Sample grouping

Samples were grouped in two ways: (1) first in ten groups, according to their respective sampling locality: Tasmania (TAS), New Zealand (NZ), Chile (CL), northwestern Atlantic (NWA), Faroe Islands (FI), United Kingdom (UK), Iberian Peninsula (IB), northeastern Atlantic (NEA), Strait of Gibraltar (GIB) and the Mediterranean Sea (MED). NEA included samples ranging from UK to IB, and was combined with the locality Euskadi from the original study, since no significant F_{ST} structure was detected among them in that study (Verborgh, 2015) (Figure 1, Table 1). (2) The second way of grouping samples was according to the subspecies categories, i.e. *G. m. edwardii* from the South Pacific (SP) including the localities TAS, NZ and CL and *G. m. melas* from the North Atlantic (NA), including NWA, FI, UK, IB, NEA and the Mediterranean (GIB and MED).

Sequence editing

After alignment and trimming, a haplotype network was constructed in Network 5.0(Bandelt et al., 1999). With an exploratory examination of the global haplotype network, it was noted that site 156 of the alignment generated three loops in the network. This hypervariable site was considered to be interfering with the phylogeographic signal of the data and was consequently removed, in order to eliminate a potential homoplasy signal. Additionally, a repeated TA motif starting at position 90 was identified as a possible microsatellite. We modified the sequences by deleting one of the nucleotide positions within each repeat, so each motif was considered as a single mutational step, instead of each nucleotide separately. Thus the final fragment length was of 345 bp.

Genetic diversity and structure

The genetic diversity indices number of haplotypes (k), number of polymorphic sites (S), haplotype diversity (Hd), nucleotide diversity (π) and pairwise differences between sequences (Π) were estimated in Arlequin v3.5.2 (Excoffier and Lischer, 2010). Analyses of genetic structure (F_{ST}), phylogeographic structure (Φ_{ST}) and analysis of molecular variance (AMOVA) were conducted in Arlequin v3.5.2 with 1 000 permutations and a significance level of 0.05. Phylogeographic structure was also explored with Snn tests of genetic differentiation61, performed in DnaSP 5.10.01 (Librado and Rozas, 2009). For the AMOVA, the ten localities were grouped according to the distribution of each subspecies. A Correspondence Analysis (CA) was performed on all localities with the software Past 3.19 (Hammer et al., 2001), using the matrix of Table 1 in the form of presence/absence of haplotypes. In this kind of data representation, unique haplotypes (n=1 in the full dataset) can have a disproportionate weight

compared to better represented haplotypes. A second matrix without unique haplotypes was constructed to evaluate the effect these had on the results. Additionally, as suggested by the guidelines for the delimitation of cetacean subspecies using genetic data of Taylor et al., (2017), Nei's net nucleotide divergence d_A (Nei, 1987, equation 10.21) was calculated among the two putative subspecies in DnaSP. According to these guidelines, the net nucleotide divergence among two subspecies should be within the range of d_A=0.004-0.04.

Historical biogeography

The population history of the species was tested on the program DIYABC v2.1.0 (Cornuet et al., 2014). This software evaluates population histories using Approximate Bayesian Computation (ABC) with genetic data, by testing scenarios built through the combination of population divergence, admixture and population size changes. Two models were evaluated. The first model was defined based on a scenario previously proposed (Davies, 1963), together with evidence from the genetic results provided in the present study. The model considers a trans-equatorial, Last Glacial Maximum (LGM)-associated dispersal event, followed by a split in distribution and instantaneous population growth (Figure 1, a). The alternative model differs in that it considers a vicariance event, rather than a founder effect (Figure 1, b). The program was used to evaluate the accordance of these two scenarios with our genetic data. Priors were set as follows: Effective population size (N_e) of ancient population=1 000-100 000; Effective population size of founder effect (N_f) =10-1 000; time of dispersal event t2=10 000-35 000; time of instantaneous population growth t1=2 000-15 000 (with t2>t1) and mutation rate u=1.5 e^{-7} -1.5 e^{-8}. In accordance with the recommendations of the authors of

the software, we performed 6 000 000 simulations.

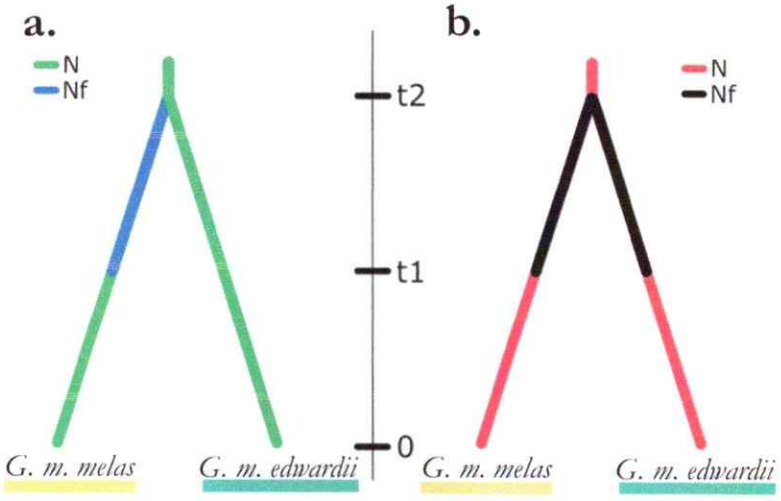

Figure 1: Proposed historical biogeographic scenarios tested in DIYABC: a. trans-equatorial colonization event followed by divergence and b. vicariance event. N=Previous effective population size, Nf = founder effect effective population size. Time scale: 0= present, t1= population size expansion, t2= a. colonization event or b. population split.

Microsatellite genotyping

A total of 19 loci were amplified in the samples from the Chilean Patagonia: 409/470, 464/465 (Amos et al., 1993), DlrFCB1, DlrFCB6 (Buchanan et al., 1996), Ev1, Ev14, EV37 (Valsecchi and Amos, 1996), GATA53 (Palsboll et al., 1997), GT6, GT51 (Caldwell et al., 2002), GT23, GT211, GT509, GT575 (Berube et al., 2000), MK5, MK9 (Krützen et al., 2001) and PPHO131 (Rosel et al., 1999). PCR reactions were done with a Multiplex PCR kit (Qiagen), each reaction containing: 12.5 µL of water, 5 µL of MM2x Buffer and 1 µL of each primer at 10 pM. Between two and four loci with different fluorescent dyes were combined in each reaction. Allele scoring was done with the software GeneMarker v2.6.0 (www.softgenetics.com) with a 500liz standard. The dataset was tested for scoring errors, allele dropout and null alleles in Micro-Checker v.2.2.3 (Van Oosterhout et al., 2004).

Microsatellite genetic diversity and inter-hemisphere comparison

Observed heterozygosity (H_o), expected heterozygosity (H_e), average number of alleles per locus (nA) and genetic structure (F_{ST}) were estimated in Genetix v4.05.2 (Belkhir et al., 2004). Published microsatellite data on this species was available from Tasmania (Oremus et al., 2013), NWA, UK (Fullard et al., 2000), Faroe Islands (Fullard et al., 2000; Miralles et al., 2016), Iberian Peninsula (Miralles et al., 2016), northeastern Atlantic, Strait of Gibraltar and the Mediterranean (Verborgh, 2015). However, because of the differences in the loci used in these studies, only a partial comparison of genetic diversity could be performed between populations of *G. m. melas* and *G. m. edwardii*. Only comparisons of allelic richness could be done, which were carried out in Rundom Pro 1.1 (Jadwiszczak, 2003) with 10 000 randomizations. Comparisons were intra-subspecies among southwestern and southeastern Pacific samples, and inter-subspecies among southwestern Pacific and North Atlantic samples.

Approval

We confirm that all methods were carried out in accordance with relevant guidelines and regulations. Samples were taken from stranded, deceased animals with permission from the National Fisheries Service (SERNAPESCA, document ID 2016-11-13). All experimental protocols were approved by the Postgraduate Evaluation Committee at the Faculty of Science of the Universidad de Chile.

Results

Southeastern Pacific sampling and genetic diversity

In total, 90 mtDNA sequences were obtained, defining four haplotypes. One haplotype was previously unreported (hereon referred to as haplotype R2). Haplotype and nucleotide diversities were low: Hd=0.620 and π=0.23% (Table 1). Microsatellites were successfully genotyped in n=44 samples (32 from Isla Clemente and 12 from Isla Navarino), across fourteen polymorphic loci. DlrFCB6, and GT51 presented an excess of homozygotes but were not used in the comparisons among Hemispheres. Regional diversity values of usable loci were H_o=0.655; H_e=0.700 and NA= 7.5 (Table 2).

Table 2: Comparisons among shared microsatellite loci from three studies including Tasmania (Oremus et al., 2013, Chile (this study) and the North Atlantic (Fullard et al., 2000). Dashes indicate either unreported information or no locus amplification.

	Tasmania				Chile				North Atlantic			
Locus	n	Alleles	Ho	He	n	Alleles	Ho	He	n	Alleles	Ho	He
409/470	262	10	0.844	0.825	-	-	-	-	529	9	-	0.567
415/416	242	9	0.798	0.801	-	-	-	-	529	5	-	0.567
464/465	122	9	0.648	0.681	44	9	0.676	529	6	-	0.670	-
DlrFCB1	264	15	0.777	0.774	44	8	0.682	-	-	-	-	
DlrFCB6	256	7	0.672	0.693	44	7	0.476	-	-	-	-	
EV1	262	14	0.756	0.773	44	10	0.756	-	-	-	-	
EV37	263	10	0.814	0.775	44	15	0.633	529	6	-	0.748	-
EV94	255	7	0.620	0.686	-	-	-	-	529	7	-	0.772
GT23	263	5	0.468	0.439	44	4	0.341	0.567	-	-	-	-
GT39	122	10	0.787	0.822	44	5	0.636	0.512	-	-	-	-
GT51	253	3	0.3	0.308	44	4	0.318	0.492	-	-	-	-
GT575	254	11	0.827	0.836	44	7	0.841	0.828	-	-	-	-
MK5	244	6	0.623	0.658	44	7	0.682	0.670	-	-	-	-
MK9	120	4	0.625	0.618	44	4	0.591	0.613	-	-	-	-
PPHO131	248	10	0.734	0.745	44	6	0.714	0.696	-	-	-	-

Global diversity analysis

After adding previously published sequences from other oceanic basins (Miralles et al., 2016; Monteiro, 2013; Oremus et al., 2009; Siemann, 1994; Verborgh, 2015), a total of 15

haplotypes were obtained after the elimination of the site that was generating phylogeographic noise in the 1012 sequences. This led to the combination of four previously described haplotypes in the following pairs: S with R, P with U, Q with Y, and E with G (Table 1). Nine of the twelve haplotypes reported by Miralles et al., (2016), originally of a consensus fragment size of 703 bp, were also merged into haplotype S+R.

Global and local haplotype networks

Adding previously published sequences from other oceanic basins, we detected that (1) two haplotypes were shared between *G. m. edwardii* (SP) and *G. m. melas* (NA and MED), and (2) one haplotype was shared by all SP localities but was absent from NA and MED (Table 1, Figure 2).

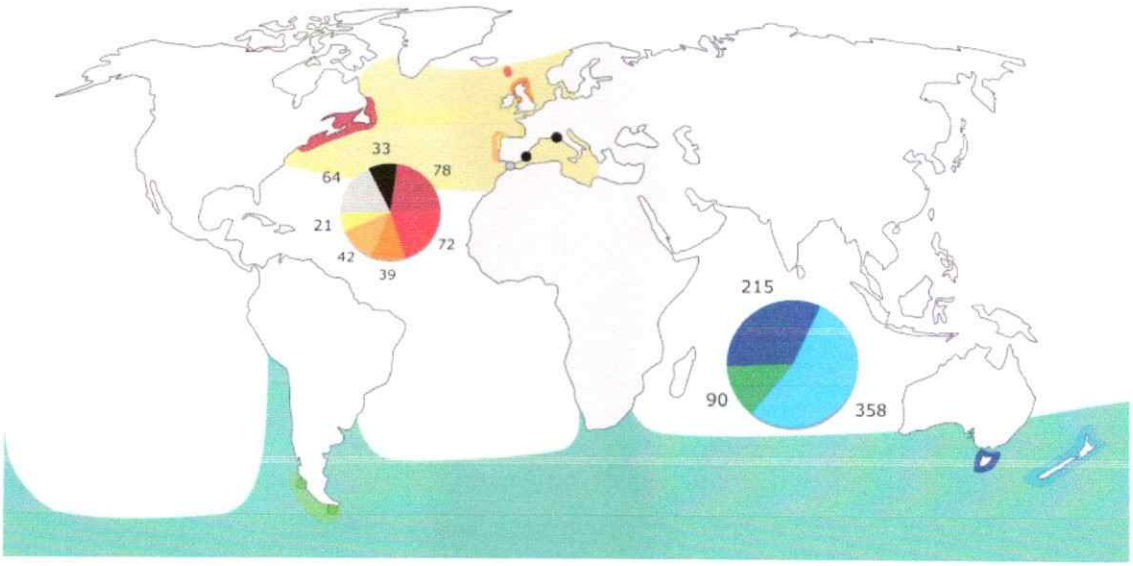

Figure 1: Origin of the 1012 sequences included in the study: Chile (CL, green), Tasmania (TAS, dark blue), New Zealand (NZ, light blue), northwestern Atlantic (NWA, dark red), Faroe Islands (FI, red), United Kingdom (UK, orange), Iberian Peninsula (IB, light orange), northeastern Atlantic (NEA, yellow), Strait of Gibraltar (GIB, grey), Mediterranean (MED, black). Pie charts indicate the number of sequences contained in each locality. Ocean areas in yellow represent the extant distribution of *G. m. melas* and in green of *G. m. edwardii*. Map adapted from www.svgsilh.com.

Haplotypes P+U and S+R were the only ones detected within the distribution range of both *G. melas* subspecies (Table 1, Figure 3, a): haplotype P+U is present in the SP and NA, while S+R was found in all three basins (SP, NA and MED), and was pivotal in the development of local diversity. Haplotype Q+Y was shared by all SP localities, but absent from NA and MED. The remaining 12 haplotypes represent in situ diversification within each corresponding basin: eight for SP, four for NA and one for MED. The haplotypes found in the Mediterranean area are represented either by S+R or by D, which derives directly from it. The same occurs with half (2) of haplotypes encountered in the NA and half (4) of those exclusive to the SP.

In the haplotype network by locality, haplotype S+R is present in all ten studied areas and originates much of the local diversity within them (Table 1, Figure 3, b). Haplotype P+U plays a similar, but downscaled role, in the South Pacific; it originates half (4) of the diversity that is exclusive to this area. This haplotype is also much more abundant in the localities corresponding to *G. m. edwardii* than in the range of *G. m. melas*, where it was detected in lower numbers in three localities (17 samples from FI, one from NWA and one from UK). One of the haplotypes that originates from P+U, haplotype Q+Y, is exclusive to the South Pacific and present in all localities. Ten of the fifteen haplotypes (67%) were exclusively present in one of the following five localities: TAS (3), NZ (3), CL (1), IB (2) and NWA (1). In the South Pacific 70% of haplotypes were private to one of the three localities; it was the only basin with at least one private haplotype in each. Three private haplotypes were encountered in the North Atlantic: one in the NWA and two in the IB.

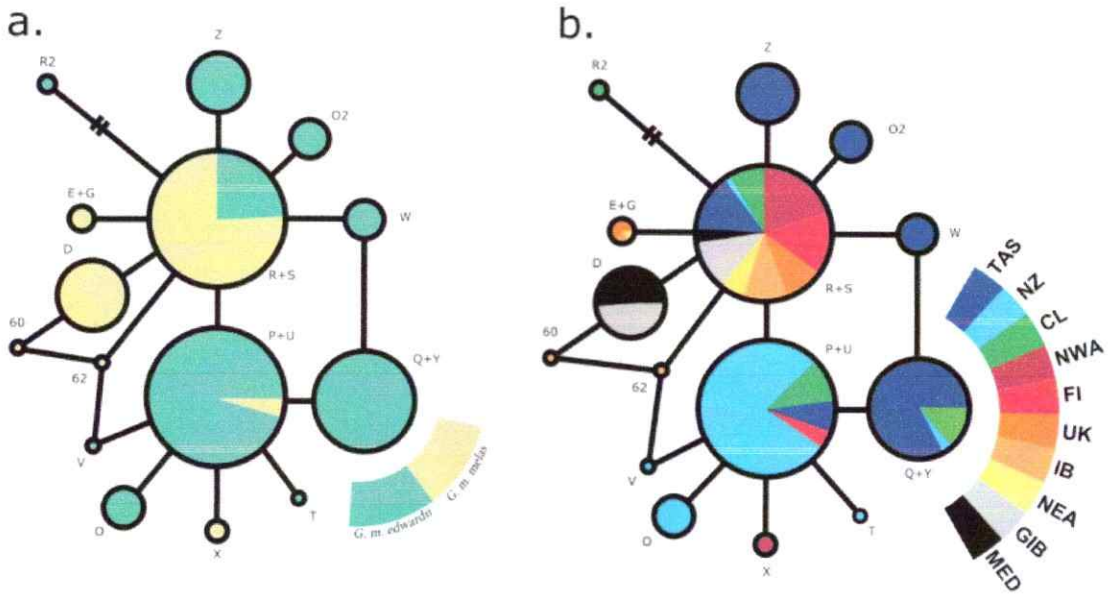

Figure 3: Haplotype networks of a) the global data coloured by subspecies: *G. m. melas* in yellow and *G. m. edwardii* in green; and b) coloured by locality (details in figure). Smallest circle size indicates a frequency of 1. Detailed haplotype frequencies can be found in Table 1.

Global and local genetic structure

Significant genetic and phylogeographic structure was also detected between the subspecies, i.e. South Pacific versus North Atlantic and Mediterranean (F_{ST} =0.439; Φ_{ST} =0.464, both p< 0.000004). The Snn test for genetic differentiation was statistically significant among the two disjoint distribution areas of the subspecies (i.e. *G. m. edwardii* and *G. m. melas*; Snn=0.830, P<0.001). Subsequently, samples were organized in this same way for the AMOVA. Similar structure results were obtained grouping the samples by the 10 worldwide localities. Average Φ_{ST} values were highest within the SP (avg. Φ_{ST} =0.283), lowest in the NA (avg. Φ_{ST} =0.060), and intermediate among the two Mediterranean localities (avg. Φ_{ST} =0.174) (Table 2).

Table 2: Genetic (F_{ST}) and phylogeographic structure (Φ_{ST}) values of the comparisons among the ten worldwide sampling localities. Structure values are beneath each diagonal and P-values are above them.

F_{ST}	NWA	FI	UK	IB	NEA	GIB	MED	TAS	NZ	CL
NWA	-	0	0.057	0.394	0.616	0	0	0	0	0
FI	0.136	-	0.019	0.001	0.034	0	0	0	0	0
UK	0.034	0.077	-	0.270	0.559	0.001	0	0	0	0
IB	-0.001	0.115	0.006	-	0.999	0	0	0	0	0
NEA	-0.007	0.109	-0.009	-0.030	-	0.006	0	0	0	0
GIB	0.226	0.156	0.15	0.184	0.172	-	0.003	0	0	0
MED	0.615	0.430	0.486	0.552	0.524	0.174	-	0	0	0
TAS	0.345	0.234	0.281	0.314	0.306	0.242	0.273	-	0	0
NZ	0.865	0.751	0.844	0.863	0.869	0.798	0.811	0.513	-	0
CL	0.435	0.199	0.337	0.388	0.368	0.301	0.358	0.116	0.399	-
Φ_{ST}										
NWA	-	0.002	0.03	0.058	0.145	0	0	0	0	0
FI	0.089	-	0.002	0.001	0.010	0	0	0	0	0
UK	0.043	0.131	-	0.091	0.693	0.001	0	0	0	0
IB	0.029	0.151	0.02	-	0.891	0.001	0	0	0	0
NEA	0.016	0.137	-0.018	-0.014	-	0.001	0	0	0	0
GIB	0.239	0.267	0.218	0.190	0.200	-	0.003	0	0	0
MED	0.578	0.530	0.538	0.540	0.544	0.174	-	0	0	0
TAS	0.253	0.178	0.248	0.260	0.239	0.307	0.394	-	0	0
NZ	0.849	0.762	0.864	0.873	0.878	0.855	0.893	0.421	-	0
CL	0.392	0.219	0.382	0.407	0.378	0.441	0.534	0.086	0.342	-

All Φ_{ST} comparisons were statistically significant within the South Pacific. In the North Atlantic, only the Faroe Islands showed a statistically significant phylogeographic structure with all other localities, while NWA did with FI and UK. All comparisons among localities from different basins showed statistically significant differences. The AMOVA showed significant differentiation among the two distribution ranges, with greater variation among groups than within them (Table 3).

Table 3: Results of the AMOVA among both subspecies of long-finned pilot whales in the South Pacific (i.e. *G. m. edwardii*) and North Atlantic with the Mediterranean (i.e. *G. m. melas*).

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups	1	124.096	0.23802 Va	39.19
Among populations within groups	8	78.725	0.11293 Vb	18.6
Within populations	1002	256.86	0.25635 Vc	48.33
Total	1011	459.681	0.6073	

In the Correspondence Analysis, the first axis separated the localities according to their respective hemisphere of origin. The three South Pacific localities (TAS, NZ and CL) were clustered separately from those of the Northern Hemisphere. The second axis divided the Strait of Gibraltar and the Mediterranean Sea from the remaining localities in the Northern Hemisphere (Figure 4, a). When eliminating unique haplotypes, the separation obtained by the first dimension was maintained, while the separation of GIB and MED from the rest of the North Hemisphere localities was not retrieved (Figure 4, b). The calculation of Nei's (1987) net nucleotide divergence (d_A) among the subspecies *G. m. edwardii* and *G. m. melas* resulted in d_A=0.00158 (SD=0.00019).

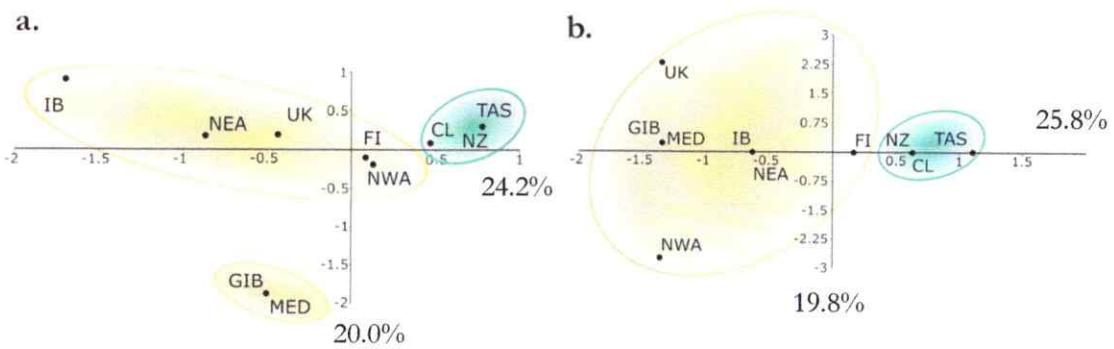

Figure 4: Correspondence Analysis scatter plot of presence/absence of haplotypes per locality, a. including and b. removing unique haplotypes. Localities within the distribution of *G. m. melas* are encircled in yellow, northwestern Atlantic (NWA), Faroe Islands (FI), United Kingdom (UK), Iberian Peninsula (IB), northeastern Atlantic (NEA), Strait of Gibraltar (GIB) and the Mediterranean Sea (MED), and the South Pacific localities of *G. m. edwardii* are in green: Tasmania (TAS), New Zealand (NZ), Chile (CL).

Historical biogeography

Based on the genetic data provided, both scenarios were realistic (Figure 5, a), even though the simulations performed were more supportive of the founder effect population history scenario (Figure 5, b). Parameter estimations fell within the proposed ranges and the prior and posterior values from our dataset were not statistically different from simulations. A range expansion would have occurred around 12 900 years ago (t2), followed by a distribution split and population growth, 9 380 years ago (t1) (Figure 5, c). The mutation rate was estimated to be $u=4.44e^{-8}$.

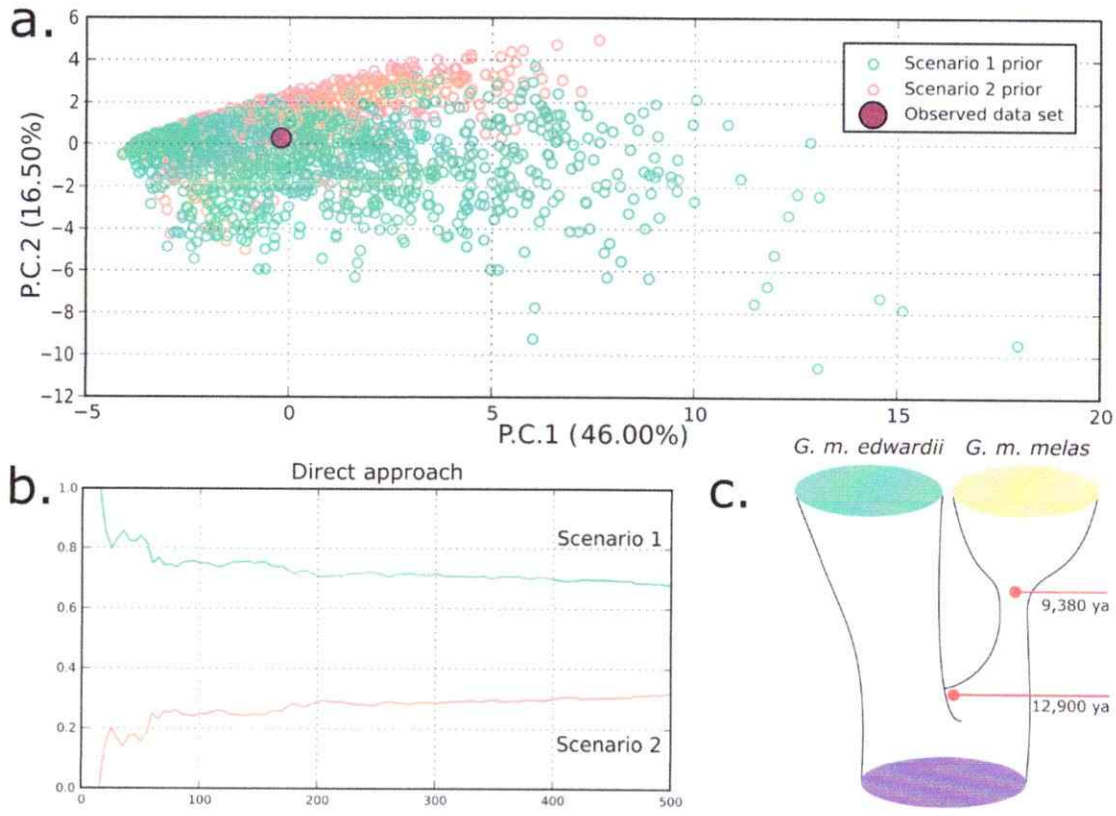

Figure 5: a. PCA showing the scenario and prior combinations of scenario 1 (green) and scenario 2 (orange); b. Estimates of posterior probability of the two scenarios, via direct approach over the 500 scenarios closest to the observed dataset; c. The supported historical biogeographic scenario, with approximate times of colonization and population expansion.

Microsatellite diversity

Previous data on microsatellite diversity was found for the southwestern Pacific (Oremus et al., 2013) and the North Atlantic (Fullard et al., 2000; Miralles et al., 2016; Verborgh, 2015). The diversity by locus reported by (Oremus et al., 2013) and (Fullard et al., 2000) is shown in (Table 4), with five loci in common to both. The number of alleles for each shared locus were, respectively: 409/470 (10 vs. 9), 415/416 (9 vs. 5), 464/465 (9 vs. 6), EV37 (10 vs. 6) and EV94 (7 vs. 7). Two of these loci were also present in our database of 44 analyzed individuals from the southeastern Pacific (464/465 and EV37), with 9 and 15 alleles in total. The upper-tail exact test among the five shared loci of *G. m. edwardii* (Oremus et al., 2013) and *G. m. melas* (Fullard et al., 2000) showed that South Pacific samples had significantly more alleles per locus ($p=0.028$). By contrast, the two-tail exact test performed with the 12 loci shared between *G. m. edwardii.* from the southwestern (Oremus et al., 2013) and southeastern Pacific (this study) did not detect a statistically significant difference ($p=0.166$).

Discussion

Sampling cetacean species for population genetic studies has proven to be logistically challenging. A large number of individuals need to be sampled for this kind of studies, but they are generally widespread geographically and low in density. Mass strandings of cetaceans represent unique opportunities to collect large numbers of samples, as in the case of the franciscana *Pontoporia blainvillei* (Cunha et al., 2014), bottlenose dolphin *Tursiops truncatus* (Parsons et al., 2002), Cuvier's beaked whale *Ziphius cavirostris* (Dalebout et al., 2005) and the short-beaked common dolphin *Delphinus delphis* (Viricel et al., 2008). The long-finned pilot whale is a species for which almost all genetic data has been obtained from sampling mass strandings. A large part of its distribution range has been already sampled in the northwestern and northeastern Atlantic (Miralles et al., 2016; Monteiro, 2013; Siemann, 1994; Verborgh, 2015), but within their broader distribution in the Southern Hemisphere, only the southwestern Pacific had been covered (Oremus et al., 2009). This study presents new data from two recent mass standings in southern Chile, in order to produce the first genetic information for *Globicephala melas* in the southeastern Pacific and to integrate this new information into the global phylogeography of the species.

Contrasting tissue quality was found between both strandings, which we attribute to the time of sampling of each event. Our sampling of the Isla Clemente stranding took place an estimated two to three months after occurring (Alvarado-Rybak et al., 2019), while the Isla Navarino individuals were sampled five days after stranding (Mansilla et al., 2012), precluding tissue the deterioration seen in the former event. This highlights the importance of swift responses to stranding events, in order to secure the best tissue quality possible.

Prior to conducting mtDNA analyses, we first decided to remove one polymorphic site that

exhibited a strong homoplasy signal, as described in the previous chapter. Despite the addition of 90 new sequences from the southeastern Pacific to the set of available sequences in the literature -over 9 000 km from New Zealand, the nearest sampled locality- the global haplotype network was expanded by just one private low-frequency haplotype, further confirming the worldwide low mitochondrial diversity of *G. melas*. Such low mtDNA diversity has been reported for other matrilineal odontocetes, including orcas (π=0.52%) (Hoelzel et al., 2002), false killer whales (π=0.01-0.3%) (Martien et al., 2014) and sperm whales (π=0.131-0.407%) (Alexander et al., 2016). In contrast, cetaceans with more labile social cohesion such as mysticetes and non-matrilineal odontocetes, generally present much higher nucleotide diversity, a feature that appears to be common among these species (Whitehead et al., 2017). Cultural hitchhiking has been regarded as a driver of their low mitochondrial diversity (Whitehead, 2005; Whitehead et al., 2017). An analogous effect of behaviour on mitochondrial diversity was described in a resident coastal bottlenose dolphin population in central Chile (Pérez-Alvarez et al., 2018). This particular population operates akin to pilot whales as it is also composed of adult females and their descendants, both male and female. That study found that the genetic diversity of this matrilineal population (Hd=0.63; π=0.8%) was lower than that of the non-matrilineal transient-pelagic group adjacent to them (Hd=0.95; π=1.4%), suggesting the importance of social structure in shaping the pattern of genetic diversity in cetaceans (at least odontocetes). As previously reported for other areas (Miralles et al., 2016; Monteiro, 2013; Oremus et al., 2009; Siemann, 1994), our results show that southeastern Pacific long-finned pilot whales (n=90) have low genetic diversity, in particular for haplotype richness (k=4) and nucleotide diversity (π=0.23%, Table 1). Within the South Pacific, the diversity indices of southeastern Pacific (*i.e.* Chilean) samples were

similar to the values obtained for Tasmania. Nevertheless, genetic diversity in these two localities was very different from that of New Zealand. Despite accounting for 54% of the samples in this basin, the genetic and nucleotide diversity of New Zealand dolphins were the lowest (Table 1), particularly because of the high predominance of one haplotype (93%, haplotype P+U). Such overrepresentation of a single haplotype in a matrilineal species may derive from sampling bias, for example, by sampling a single, large mass stranding event. However, in that study, the sampling period spanned from 1993 to 2007 and included numerous single and mass strandings that took place in various localities (Oremus et al., 2009). Therefore, we can assume that this diversity accurately reflects what is present in this area. The distinctiveness of New Zealand from Tasmania and Chile is further supported by high Φ_{ST} values (Φ_{ST}=0.421 and 0.342 respectively) (Table 2), which are four and five times higher than in Chile and Tasmania (Φ_{ST} =0.086). The absence of obvious geographic or oceanographic barriers between NZ and the other localities in the South Pacific does not allow a simple interpretation of the pattern of genetic structure found here. This genetic differentiation could also have been attained through ecological specialization, as previously pointed out (Oremus et al., 2009). Sea surface temperature and its influence on prey distribution has been regarded as a possible ecological factor underlying genetic differentiation in extant *G. melas* in North Atlantic waters (Fullard et al., 2000) and similar trends have been observed on the Scotian shelf (Gowans and Whitehead, 1995).

Differentiation over relatively short distances without any conspicuous geographical barriers has also been detected in other odontocetes. For example, genetic differentiation was detected in the Chilean dolphin *Cephalorhynchus eutropia* between two differing coastal habitats along the uninterrupted Chilean coastline, attributed to habitat adaptation and specific

hunting strategies(Pérez-Alvarez et al., 2015). Also, the Eastern Pacific Barrier has been proposed as a driver behind the differentiation of the short-finned pilot whale *Globicephala macrorhynchus* into two subspecies(Van Cise et al., 2019).

The haplotype network of NZ specimens presents a typical star-like shape (Figure 6, a.), suggesting that long-finned pilot whales around New Zealand represent a young population, perhaps tracing back to the Last Glacial Maximum. During this period, a 6-10 °C cooling occurred in superficial waters of southeast New Zealand, the strongest temperature drop reported in this area of the southwestern Pacific (Barrows et al., 2000). Such changes in environmental conditions, probably associated to a shift in the distribution of marine biota, may have provoked a typical population contraction-expansion in the long-finned pilot whale population in this area, as described for cold-temperate and polar marine species (Marko et al., 2010), including cetaceans (O'Corry-Crowe, 2008; Pérez-Alvarez et al., 2016). A similar case of strong genetic differentiation among populations of long-finned pilot whales is observed between the North Atlantic and Mediterranean populations, where the latter exhibits high phylogeographic and genetic differences with the North Atlantic localities (Verborgh, 2015). In this case, geographic and oceanographic discontinuities between the Mediterranean Sea and the Atlantic Ocean provide a robust explanation for the observed genetic structure. The separation of Mediterranean populations from North Atlantic ones has been previously reported in various marine species such as shallow water crustaceans (García-Merchán et al., 2012), sea stars (Baus et al., 2005), white sharks (O'Leary et al., 2015) and other odontocetes, like sperm whales (Drouot et al., 2004), striped dolphins *Stenella coeruleoalba* (Garcia-martinez et al., 1999) as well as Cuvier's beaked whales *Ziphius cavirostris* (Dalebout et al., 2005) and Risso's dolphins *Grampus griseus* (Gaspari et al., 2007).

Widespread cetacean taxa occurring in both the Northern and Southern Hemispheres generally exhibit strong phylogeographic structure and genetic divergence between regions. Such genetic differentiation has been generally exemplified by fixed substitutions in mtDNA control region sequences. This is the case of the fin whale *Balaenoptera physalus*, with one fixed difference between South and North Atlantic samples, thus presenting no shared haplotypes (Archer et al., 2013), the harbour porpoise *Phocoena phocoena*, with high divergence among ocean basins (Rosel et al., 1999) and the more closely related false killer whale *Pseudorca crassidens* (Martien et al., 2014). In the latter species, although the study had a low sample size in the North Atlantic, no shared haplotypes were found and at least 10 substitutions separated the Atlantic from the Indo-Pacific. In the case of *G. melas*, despite its antitropical distribution and the large geographic discontinuity between northern and southern distribution areas, the subspecies shared their two most abundant haplotypes. This genetic pattern may reflect (1) contemporary gene flow between hemispheres, or alternatively (2) an ancestral polymorphism resulting from an incipient divergence process. The strong phylogeographic structure detected among the subspecies supports the second hypothesis.

The South Pacific network holds much of the species' genetic diversity and is therefore very similar to the global network in overall shape (Figure 6, d). In contrast, the star-like haplotype network of North Atlantic and Mediterranean samples is typically presented by recently expanding populations. A biogeographic scenario of a dispersal event over the equator during previous glacial period has been proposed (Davies, 1963), with a split in distribution occurred after the Last Glacial Maximum, 10 000-15 000 years ago. This hypothesis was further expanded (Oremus et al., 2009), mentioning that a trans-equatorial dispersal event rather than vicariance might have taken place, on account of the lower diversity in the Northern

Hemisphere subspecies. Thus the hypothesis is here supported by (1) the contrasting haplotype networks (Figure 6, c and d), (2) the higher mitochondrial and microsatellite diversity indices of South Pacific long-finned pilot whales compared to their counterparts in the North Atlantic and Mediterranean (Tables 1 and 4) and (3) the clear separation of areas by subspecies in the haplotype presence/absence PCA (Figure 4). The validity of the proposed scenario of population history was further explored here with the DIYABC population history simulations, comparing a founder effect scenario with a vicariance scenario. The patterns of population diversity and structure observed in *G. melas* were consistent with the data simulated under the scenario of a trans-equatorial dispersal event followed by divergence (Figure 5). Posterior estimation of scenario parameters allowed estimating the time at which the different events occurred, setting the dispersal from the Southern Hemisphere to the North Atlantic at around 12 900 years ago, followed by a population demographic expansion around 9 380 years ago. However, even considering this scenario of divergence, the absence of reciprocal monophyly does not qualify northern and southern *G. melas* as different Evolutionary Significant Units (ESU) following the criterion of Moritz (1994) (Moritz, 1994). It is likely that not enough time has passed to sort lineages, or some level of gene flow still occurs (Morin and Dizon, 2018), as they still share haplotypes, but in differing frequencies (Oremus et al., 2009). Additionally, net nucleotide divergence d_A and Percent Diagnosability (PD) had been previously calculated for the long-finned pilot whale subspecies (Rosel et al., 2017), obtaining values of d_A=0.00128 and PD=0.84286. Therefore, addressing them as Demographically Independent Populations (DIP), a transitional state between a panmictic population and separate ESUs (Morin and Dizon, 2018) might be more accurate. Finally, from a conservation perspective, even if genetic

analyses do not support the subspecies category, we recommend maintaining their current taxonomic status, since these DIP might be undergoing a recent divergence process which has yet to mature into fully sorted lineages.

Concluding remarks

The results here presented should be considered as preliminary evidence, as the use of a single mtDNA marker for phylogeographic and demographic inferences has been deemed problematic before (Morin et al., 2010). As previously stated, such molecular studies should include nuclear markers together with mitochondrial DNA, even more when delimitating species and subspecies (Berta and Churchill, 2012). However, to date, the mtDNA control region is the only molecular marker for which sequences are available with sufficient sample size from different ocean basins to perform a worldwide phylogeographic study in *G. melas* (Siemann, 1994). To incorporate new genetic markers to a global phylogeographic study would require tremendous sampling effort and expenses or the establishment of strong collaborative work, and may also strongly depend on the occurrence of mass strandings in all these regions of the world. Finally, we believe that collaborative studies surveying uncharted areas, are fundamental to obtain complete data on the worldwide phylogeography and taxonomy of *G. melas* and to lay the groundwork for future research on these topics. This is especially true within the greater distribution range of long-finned pilot whales in the Southern Hemisphere, where areas like the Falkland Islands/Islas Malvinas, the Atlantic coast of South America and South Africa present no published work. Modern genetic tools, such as complete mitogenome and SNPs, has been already used to revise the taxonomic status of short-finned pilot whales(Van Cise et al., 2019), which could be replicated in long-finned pilot

whales.

Finally, we believe that surveying uncharted areas, especially within the greater distribution range of long-finned pilot whales in the Southern Hemisphere, in areas like the Falkland Islands/Malvinas, the Atlantic coast of South America and South Africa, is fundamental to obtain complete data on the worldwide phylogeography of *G. melas* and to lay the groundwork for future research on this topic.

Conclusiones generales

Los dos capítulos de este estudio sobre calderones de aleta larga de la Patagonia chilena conforman la primera investigación a nivel genético para esta especie dentro del Pacífico sureste. Presentaron patrones previamente descritos para la especie y otros odontocetos de estructura social matrilineal, al mostrar baja diversidad genética en comparación a otros cetáceos, estructuración genética a nivel de ADN mitocondrial, lo que no se detectó utilizando marcadores biparentales, sugiriendo un flujo genético mediado por machos. En relación a la estructura genética poblacional, los resultados muestran que existe estructuración poblacional a nivel de ADN mitocondrial entre los calderones de los dos varamientos analizados, sin embargo, el análisis con loci microsatélites indica que existe una conexión genética entre ambas localidades, que estaría mediada por el movimiento de machos.

El nivel de parentesco a nivel global no fue tan alto como el esperado para una especie de estrategia social matrilineal, pero sí se encontraron relaciones de primer grado para un alto número de integrantes del varamiento ocurrido en Isla Clemente. Estas dos líneas de evidencia, el bajo parentesco global y la gran cantidad de relaciones de primer grado, indican que el varamiento masivo en Isla Clemente estaba compuesto por varias unidades sociales, que previamente se habrían asociado en un grupo más grande y eventualmente protagonizaron el varamiento masivo.

Si bien ambos capítulos tratan a escalas contrastantes, están articulados por un factor común, que responde a la estrategia social de esta especie: la presencia de estructura genética mitocondrial y, en el caso de Patagonia, ausencia de esta utilizando marcadores biparentales. En este sentido, el uso de estos mismos marcadores o de SNPs presenta una oportunidad para evaluar si este patrón se repite a escala global.

La evidencia genética no respalda completamente la actual división taxonómica en dos subespecies, debido a que las poblaciones de ambos hemisferios aún comparten los dos haplotipos de mayor frecuencia y las métricas de separación para subespecies recientemente propuestas no logran discriminar entre ambas. Aun así, se reconoce el proceso de divergencia incipiente en el que se encuentran.

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Supplementary table ST1: Allele size and frequency for each of the 12 loci microsatellite used. Frequencies were calculated in *Genetix*.

Locus	Allele 1	Allele 2	Allele 3	Allele 4	Allele 5	Allele 6	Allele 7	Allele 8	Allele 9	Allele 10	Allele 11	Allele 12	Allele 13	Allele 14	Allele 15	Allele 16	Allele 17	Allele 18
464/465	139	141	143	145	149	151	153	155	157									
464/465	0,0256	0,1667	0,0385	0,0641	0,0128	0,0641	0,4231	0,0897	0,1154									
DlrFCB1	123	125	127	129	131	133	135	143										
DlrFCB1	0,0521	0,0729	0,0521	0,2083	0,3958	0,1875	0,0208	0,0104										
Ev1	149	151	153	155	157	159	161	163	167									
Ev1	0,0444	0,3222	0,3667	0,0333	0,1556	0,0111	0,0444	0,0111	0,0111									
Ev14	131	133	135	137	139	141												
Ev14	0,0714	0,1786	0,4643	0,1667	0,1071	0,0119												
GT39	140	150	152	154	156													
GT39	0,3542	0,0104	0,6042	0,0104	0,0208													
GT48	195	199	201	203	205	207	209	211	213	215	217	219	225	229	231	233	235	237
GT48	0,0349	0,0233	0,0116	0,1279	0,0581	0,2326	0,0233	0,1047	0,093	0,0349	0,093	0,0349	0,0116	0,0465	0,0116	0,0349	0,0116	0,0116
GT575	153	155	157	159	161	163	167											
GT575	0,1354	0,1563	0,0625	0,1042	0,2917	0,1042	0,1458											
MK5	219	221	223	225	227	229	231											
MK5	0,0625	0,4063	0,3958	0,0833	0,0313	0,0104	0,0104											
MK9	158	160	162	164														
MK9	0,0313	0,2917	0,5521	0,125														
PPHO131	191	193	195	197	199	201												
PPHO131	0,0435	0,4239	0,2935	0,1196	0,0978	0,0217												
Sgui06	199	203	205	207	209	211	213	215	217	219	221	229						
Sgui06	0,0119	0,0119	0,0357	0,0952	0,0238	0,2738	0,1548	0,2143	0,0476	0,0833	0,0357	0,0119						
Sgui16	161	163	165	167	169	171	173	175	177	185								
Sgui16	0,0106	0,3404	0,1064	0,2021	0,0213	0,0851	0,117	0,0532	0,0426	0,0213								

Supplementary table ST2: Relatedness (below diagonal) and relationships (above diagonal) estimated among all 48 pilot whales of both strandings, calculated with the software *ML-Relate*.

	G5	G7	G17	G22	G26	G29	G31	G33	G35	G36	G43	G45	G46	G50	G51	G55	G58	G62	G63	G68	G69	G70	G71	G74	G76	G84	G86	G90	G96	G98	G105	G108	G109	G110	G113	G124	801	802	803	804	805	806	807	808	809	810	811	812
G5		HS	U	U	PO	U	PO	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	U	U	U	U	HS	HS	U	U	U	U	U	U	U	U	U	U	U
G7	0,22		U	HS	HS	U	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	HS	HS	U	U	U	HS	U	HS	U	U	U	U	U	U	U	U	U	U	U	HS
G17	0,02	0		PO	U	HS	U	U	U	U	U	U	HS	U	U	U	U	HS	U	U	U	HS	HS	U	U	U	U	HS	U	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	U	U	U
G22	0	0,16	0,53		U	U	U	U	U	U	HS	U	HS	U	HS	U	U	HS	U	HS	U	HS	U	U	U	U	U	U	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
G26	0,54	0,2	0,06	0		HS	U	U	U	U	HS	U	U	U	U	U	U	U	U	HS	U	U	U	U	U	U	U	U	U	HS	U	U	HS	U	U	U	HS	U	U	U	U	U	U	U	U	HS	U	HS
G29	0,01	0,16	0,16	0	0,2		HS	U	HS	U	U	HS	U	U	U	U	U	U	HS	U	U	U	U	HS	U	U	U	HS	U	U	U	HS	U	HS	U	HS	U	U	U	U	U	U	U	U	U	U	U	HS
G31	0,5	0,3	0	0	0,13	0,16		U	U	U	U	HS	U	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	PO	U	U	U	U	U	HS	HS	HS	U	U	U	HS	U	U	U	U	U	U
G33	0	0	0	0	0	0	0		HS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
G35	0	0	0	0	0	0,24	0,15	0,28		U	U	HS	U	U	U	U	U	U	HS	U	U	U	U	HS	U	U	HS	PO	U	U	HS	U	U	HS	HS	U	U	U	U	U	HS	HS	U	U	U	U	U	U
G36	0	0,02	0,12	0	0,09	0	0	0,08	0		U	U	U	U	U	U	U	U	U	U	U	U	HS	U	HS	U	U	U	U	U	HS	U	U	U	FS	U	U	U	U	U	U	U	U	HS	U	HS	U	U
G43	0	0	0,15	0,15	0,27	0	0	0	0	0,03		U	U	U	U	U	U	U	U	U	U	U	U	HS	U	U	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	U	U	U
G45	0	0,12	0	0,04	0	0,24	0,12	0,06	0,27	0	0		U	HS	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
G46	0	0	0,19	0,13	0	0,11	0,15	0	0	0	0	0		U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	PO	U	HS	U	U	U	HS	U	U	U	U	U	HS	U	U	U	U	U	U
G50	0,07	0	0	0	0,03	0	0,18	0,24	0,07	0	0	0,33	0		U	U	U	U	U	U	U	U	U	PO	U	HS	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
G51	0	0	0,08	0,27	0	0	0	0	0	0	0,01	0	0	0		U	U	HS	U	U	U	U	HS	U	U	U	U	U	U	U	HS	U	U	U	HS	U	U	U	U	U	U	U	U	HS	U	U	U	U
G55	0	0	0	0,1	0	0	0,06	0	0,13	0	0	0,11	0,01	0	0,07		U	U	U	U	HS	U	U	U	U	U	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
G58	0	0	0,02	0,11	0	0	0	0	0	0,06	0,08	0	0	0,01	0,06	0,05		U	U	U	U	U	HS	U	U	U	HS	U	U	U	U	U	U	U	U	U	U	U	PO	U	U	U	U	U	U	U	U	U
G62	0,1	0	0,36	0,33	0	0	0	0	0	0,13	0	0	0	0,02	0,15	0	0,12		U	U	U	HS	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	U	U	U	U	U	U	U
G63	0	0	0	0	0,12	0,29	0	0	0,13	0,04	0,06	0	0,01	0,07	0	0	0	0		U	U	U	U	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U	HS
G68	0	0	0	0,2	0,19	0	0	0	0	0	0,11	0	0,04	0,05	0,47	0,05	0	0	0,07		U	U	U	U	U	U	U	U	U	U	U	U	HS	U	U	U	U	U	U	U	U	U	U	U	U	U	HS	U
G69	0,01	0,07	0	0	0,17	0	0	0,07	0	0,06	0,08	0,21	0	0	0	0,17	0	0	0,04	0		U	U	U	U	U	U	U	U	U	HS	U	U	U	U	U	U	U	HS	U	U	U	U	U	U	U	U	U
G70	0	0,04	0,2	0,33	0	0,06	0	0	0	0,05	0,15	0	0,04	0,09	0	0	0	0,19	0	0,13	0		U	U	U	U	U	U	U	U	U	U	U	U	U	FS	HS	U	U	U	U	U	U	U	U	U	U	U
G71	0	0	0,14	0,08	0	0	0	0,02	0,09	0,18	0,04	0	0,04	0	0,26	0,01	0,35	0,36	0	0	0	0		U	U	U	U	U	PO	U	U	U	U	U	U	U	U	HS	HS	HS	U	U	U	U	HS	U	U	U
G74	0	0,1	0	0	0,08	0,3	0	0,13	0,14	0,22	0,13	0	0,02	0,5	0	0	0	0	0,16	0	0	0	0		U	HS	HS	U	U	HS	U	U	U	U	HS	U	U	U	U	U	U	U	U	U	U	U	HS	U
G76	0	0,07	0	0	0	0	0,02	0	0,04	0,33	0	0,04	0	0	0,01	0,07	0	0	0,06	0	0	0	0,11	0		U	U	U	U	U	U	U	U	U	HS	U	U	U	U	U	U	U	U	U	U	HS	U	U
G84	0	0,36	0	0,11	0	0,2	0	0,06	0	0,08	0,11	0,04	0,12	0,31	0	0	0	0	0	0	0	0,14	0,09	0,35	0		U	U	HS	U	U	FS	FS	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
G86	0	0	0	0,16	0,02	0	0	0,14	0,34	0	0,17	0,21	0	0,34	0	0,14	0,15	0,01	0	0,2	0	0,04	0	0,19	0	0,11		U	U	U	U	U	U	U	HS	U	U	U	U	HS	U	U	U	U	U	U	U	U
G90	0,08	0,09	0,21	0	0	0,26	0	0	0,5	0,01	0	0,01	0,05	0	0	0,05	0	0,01	0,16	0	0,02	0	0	0	0	0	0		U	U	U	U	U	HS	U	U	U	U	U	HS	U	U	U	U	U	U	HS	U
G96	0	0,13	0,03	0,35	0	0	0	0,25	0,09	0,06	0	0,18	0,03	0	0	0,05	0,14	0,1	0	0	0	0	0,5	0	0	0,28	0,27	0		HS	U	HS	HS	U	U	U	U	HS	U	U	U	U	U	PO	HS	U	HS	U
G98	0,26	0,3	0,15	0,07	0,15	0,13	0,5	0	0	0,12	0,06	0,12	0,5	0,1	0	0	0	0	0	0	0	0	0	0,32	0	0	0	0,19	0,22		U	U	U	U	U	U	HS	U	U	U	U	U	U	U	U	U	HS	U
G105	0	0	0	0,06	0,12	0	0	0,18	0,25	0,19	0,09	0,02	0	0,25	0,19	0,09	0	0	0	0,1	0,31	0	0	0	0,16	0	0,1	0,05	0	0		U	U	HS	HS	U	U	U	U	U	U	U	U	U	U	U	U	U
G108	0	0,08	0	0,16	0	0,27	0	0	0	0	0	0	0,27	0	0	0	0,01	0	0,12	0	0	0	0	0	0,05	0,28	0	0,19	0,24	0	0		U	U	U	U	U	U	U	U	U	U	U	U	U	HS	U	U
G109	0,12	0,39	0	0,09	0,17	0,3	0	0	0	0	0	0	0	0,1	0	0	0	0	0,07	0,23	0	0	0	0	0	0,41	0	0,03	0,16	0,09	0	0,08		U	U	U	U	U	U	U	HS	U	U	U	U	U	U	HS
G110	0,06	0,17	0	0,04	0,11	0,29	0,04	0	0,27	0	0	0	0	0,03	0	0,03	0	0,02	0	0,08	0,03	0,3	0,02	0	0	0	0	0,25	0	0,12	0,23	0,03	0,04		U	FS	U	HS	U	U	U	U	U	U	U	U	HS	U
G113	0	0	0	0	0	0	0	0,27	0,17	0,36	0,05	0	0	0,16	0,15	0,09	0	0,03	0	0,03	0	0	0	0,13	0,35	0	0,13	0	0,03	0,12	0,28	0	0	0		U	U	U	U	U	U	HS	U	U	U	U	U	U
G124	0,2	0,25	0,07	0,03	0	0,26	0,22	0	0	0	0	0	0,22	0	0	0,01	0	0	0	0,09	0	0,33	0	0	0	0,15	0	0,03	0	0	0	0	0	0,34	0		U	U	U	U	U	HS	HS	U	U	U	U	U
801	0,22	0	0	0,06	0,23	0	0,27	0	0,09	0	0	0,08	0,2	0	0	0,04	0	0	0	0	0	0,18	0	0,01	0,03	0,11	0	0	0	0,23	0	0	0,05	0	0	0		U	U	U	U	U	HS	U	U	U	HS	HS
802	0	0	0	0	0	0	0,21	0	0	0	0,07	0	0	0,21	0	0	0,02	0,06	0	0	0	0	0,14	0,01	0	0,01	0	0,02	0,24	0	0	0,1	0,09	0,13	0	0,12	0		U	PO	U	U	HS	U	U	U	U	U
803	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0,5	0	0,06	0	0,18	0	0,18	0	0,05	0	0	0,02	0,07	0	0,08	0	0	0	0	0	0	0,12		U	HS	U	U	U	U	U	U	U
804	0,03	0	0	0,12	0	0,11	0,1	0	0,22	0,2	0	0,13	0,1	0	0	0	0	0,21	0	0	0	0	0,15	0	0	0	0,26	0,22	0,03	0	0	0,03	0	0,05	0,05	0,08	0	0,52	0,13		U	U	U	U	U	U	U	U
805	0	0	0	0,01	0	0,12	0	0	0,17	0	0,01	0	0	0,01	0,01	0	0,13	0	0,03	0,01	0	0	0	0	0	0	0,05	0,19	0	0	0	0	0,34	0,07	0	0	0	0	0,2	0,1		U	U	U	U	U	U	U
806	0,05	0	0,01	0	0	0,05	0,17	0,03	0,15	0	0	0	0,2	0	0,1	0	0,02	0,05	0	0	0,02	0	0	0	0	0	0	0	0	0,02	0,08	0,05	0	0,03	0,24	0,17	0,04	0	0,06	0	0,07		U	U	U	HS	U	U
807	0,09	0	0,06	0,07	0,08	0,12	0	0	0	0	0	0,02	0,09	0,07	0	0,02	0,08	0,03	0,01	0,07	0	0,02	0,01	0,01	0	0,04	0	0	0	0	0	0,03	0	0,02	0	0,1	0,15	0,13	0	0	0	0		U	U	U	U	U
808	0	0,05	0,39	0,13	0	0	0	0	0	0,23	0,13	0,01	0	0	0,29	0,05	0,03	0,16	0	0,12	0,02	0	0,1	0,03	0	0	0	0,05	0,52	0,11	0,1	0	0	0	0	0	0	0	0	0,01	0,06	0	0		HS	U	U	U
809	0,06	0	0	0	0	0	0	0,1	0,02	0	0	0,05	0,02	0	0	0	0	0,04	0	0	0	0	0,18	0	0	0,04	0	0	0,21	0	0	0,02	0	0	0	0	0,02	0,05	0,07	0,06	0,13	0	0	0,16		U	U	U
810	0	0,05	0,06	0,01	0,19	0,06	0	0	0,1	0,4	0,08	0	0,04	0,04	0,21	0,1	0	0	0	0,07	0	0,06	0,16	0,01	0,2	0,02	0,11	0,02	0	0	0,06	0,09	0,04	0	0,08	0	0	0	0	0	0	0,22	0	0,19	0		U	U
811	0,18	0	0	0	0	0,13	0	0,04	0,19	0	0	0,05	0	0	0	0	0	0,09	0	0,27	0	0,01	0,02	0,16	0	0	0,02	0,18	0,14	0,27	0	0	0	0,23	0	0,01	0,14	0	0	0,1	0	0,04	0	0,08	0	0		U
812	0,13	0,31	0,08	0	0,2	0,14	0	0	0,07	0,11	0,12	0	0	0	0	0	0	0,01	0,18	0	0,01	0,07	0,09	0	0	0	0	0	0	0,02	0	0	0,38	0	0	0,09	0,16	0	0	0,14	0,01	0	0	0	0	0	0,05	
	G5	G7	G17	G22	G26	G29	G31	G33	G35	G36	G43	G45	G46	G50	G51	G55	G58	G62	G63	G68	G69	G70	G71	G74	G76	G84	G86	G90	G96	G98	G105	G108	G109	G110	G113	G124	801	802	803	804	805	806	807	808	809	810	811	812