A multilocus perspective on the worldwide population structure of common dolphins (genus *Delphinus*)

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ABSTRACT

Common dolphins (genus *Delphinus*) presently comprise two species and four subspecies: the short-beaked common dolphin, *D. delphis delphis* Linnaeus, 1758 distributed in temperate waters of the Atlantic and Pacific Oceans; *D. delphis ponticus* Barabash, 1935 restricted to the Black Sea; the long-beaked common dolphin, *D. capensis capensis* Gray, 1828 distributed in nearshore sub-tropical and temperate waters of the Southern Atlantic and Pacific Oceans; and *D. capensis tropicalis* van Bree, 1971 distributed in the Indian Ocean. These species were initially described based on morphological data, with mitochondrial DNA and AFLPs also supporting the distinction between short-beaked and long-beaked populations in the Northeast Pacific. However, more recent analysis on a global scale using mitochondrial DNA has revealed some level of discordance between morphology and genetics. In this report we will present preliminary results from a multilocus analysis (including mitochondrial DNA, microsatellites and nuclear loci) aimed at clarifying the population structure and taxonomy of common dolphins.

KEYWORDS: common dolphins; short-beaked; long-beaked; Atlantic Ocean; Pacific Ocean; Indian Ocean; taxonomy; gene flow

INTRODUCTION

Common dolphins of the genus *Delphinus* are widely distributed small cetaceans that present great morphological variability throughout their distribution. At least 30 nominal species were described in the past (Hershkovitz 1966), but most cetacean biologists accepted the existence of a single species (*Delphinus delphis* Linnaeus, 1758), until Heyning and Perrin (1994) found evidence for two species of common dolphins occurring in sympatry on the coast of California in the Northeast Pacific. These include a short-beaked form and a long-beaked form, which the previous authors referred to as *D. delphis* and the nominal species *D. bairdii* Dall, 1873 from California, respectively. These authors found differences in morphological and skeletal characters such as coloration, overall body size, relative length of the rostrum and tooth counts. They noted that based on relative size and proportions of the rostrum, *D. bairdii* appeared to be a junior synonym of *D. capensis* Gray, 1828 from South Africa and concluded that the short-beaked and long-beaked morphotypes occurred around the world as two species. They reviewed the descriptions and holotype specimens of a number of other nominal species and referred them to *D. delphis* or *D. capensis* on morphological grounds. A genetic study based on the mitochondrial DNA control region gave support for the separation of the two species on the coast of California (Rosel et al., 1994). The possible existence of a third nominal species in the Indian Ocean, *D. tropicalis* (van Bree, 1971), remained controversial until a study by Jefferson and Van Waerebeek (2002) suggested that this form is most likely a long-beaked subspecies of *D. capensis*. Another subspecies is recognized to exist in the Black Sea, *D. d. ponticus* (Perrin 2009).

Despite the new classification into two species and four subspecies, morphological studies of common dolphins inhabiting regions such as the North Atlantic and Southwest Pacific regions have shown populations with measures of relative rostrum length and tooth counts not matching those of the short- and long-beaked forms described from the Northeast Pacific (Bell et al., 2002; Murphy et al., 2006; Westgate, 2007). Furthermore, subsequent molecular studies using nuclear and mitochondrial DNA markers have failed to support reciprocal monophyly between the two putative species (Amaral et al., 2007; Kingston and Rosel, 2004; LeDuc et al., 1999). In a broader study, which included samples from the North Atlantic, Mauritania, Argentina, South Africa and Northeast Pacific, including two morphologically defined long-beaked populations, there was significant genetic differentiation among populations inhabiting different oceans and different basins within oceans, but little or no differentiation between populations inhabiting the same side of an ocean basin (Natoli et al., 2006). Additionally, these authors found high genetic differentiation among the populations described as long-beaked. That study, however, failed to include individuals from the Indo-Pacific region, namely the *tropicalis*-type. More recently, a worldwide study including populations described as short-beaked, long-beaked and the *tropicalis* form showed that the distribution of the different morphotypes in the different geographic regions was not seen in the distribution of mitochondrial lineages, putting into question the current morphology-based taxonomy (Amaral et al., 2009).

In this report, we revisit the mitochondrial DNA dataset of Amaral et al. (2009) by including individuals from the central eastern Atlantic, Brazil (Southwest Atlantic)

and New Zealand (Southwest Pacific). Additionally, we present preliminary results from 14 microsatellite loci and sequences from 5 nuclear loci in order to shed light on the worldwide population genetic structure of common dolphins.

MATERIAL AND METHODS

Sampling

In total, 345 common dolphin samples were analysed in this study. For *D. delphis,* the sampled regions were the Northeast (NE) Atlantic, *n* = 63, the Central Eastern (CE) Atlantic, *n* = 21, the Northwest (NW) Atlantic, *n* =27, the NE Pacific, *n* = 26, the Southwest (SW) Pacific, *n* = 42 (encompassing Eastern Australian waters) and *n* = 40 (encompassing New Zealand waters) and Southeast (SE) Indian Ocean (southern Australian waters), *n* = 27. For *D. capensis*, the sampled regions were the Northeast Pacific, *n* = 41, the SE Atlantic, off South Africa, *n* = 26 (These samples are here classified as *D. capensis* following Samaai et al. (2005) and P. Best (pers. comm.)), and the SW Atlantic, off Brazil, *n* =8. Finally, for the *tropicalis*-form, *n* = 25 were obtained from the Arabian Sea in the Western Indian Ocean.
For the microsatellite analyses we could not use individuals from the Western Indian Ocean and Southwest Atlantic due to poor DNA quality. Number of samples from the remaining regions also varied with a total of 340 common dolphin samples genotyped (Table 3).

mtDNA

All samples were preserved in pure ethanol. DNA was extracted from muscle or skin following standard proteinase K and two phenol-chloroform-isoamyl (24:1) extractions followed by ethanol precipitation (Rosel and Block, 1996). The cytochrome *b* gene was amplified (1121 bp) using primers on the transfer RNA (tRNA) genes on either side of the cytochrome *b*. The L-strand primer was on tRNA glutamine (L14724, 5'-TGACTTGAARAACCAYCG TTG 3') and the H-strand primer on tRNA threonine (5'CCTTTTCCGGTTTACAAGAC 3'). The thermocycle profile consisted of an initial denaturation step at 94°C for 3 min followed by 35 cycles of 45 s at 94°C, 45 s at 48°Cand 1 min at 72°C and a final extension step for 5 min at 72°C. The PCR products were cleaned by adding 0.5U of Shrimp Alkaline Phosphatase and 5U of Exonuclease I and incubating at 37ºC for 30 min and 80ºC for 15 min. Both strands were directly sequenced (BigDye Terminator CycleSequencing; Applied Biosystems) on an ABI 3730 automated sequencer (Applied Biosystems).

All sequences obtained were aligned using the software Sequencher, version 4.2 (Gene Codes Corporation). Diversity measures (nucleotide and haplotype diversities) and neutrality tests, Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997), were estimated in Arlequin v. 3.5 (Excoffier and Lischer 2010). Arlequin was also used to test for population differentiation, by calculating pairwise F_{ST} (using haplotype frequencies) and ϕ_{ST} (using genetic distance) between sampled regions and by performing an analysis of molecular variance (AMOVA).

Microsatellites

Samples were genotyped at 14 polymorphic microsatellite loci: 7 tetranucleotide (Tur4_80, Tur4_87, Tur4_92, Tur4_105, Tur4_141, Tur4_142 (Nater et al., 2009) and Dde59 (Coughlan et al., 2006) and 7 dinucleotide (Dde66, Dde70 (Coughlan et al. 2006), KW2, KW12 (Hoelzel et al., 1998), EV1 (Valsecchi and Amos, 1996), MK6 and MK8 (Krutzen et al., 2001)). Amplification reactions contained 50-100 ng DNA, 1x reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 μM of each primer and 1 unit of Taq DNA polymerase. The thermal cycler profile for the tetranucleotide loci and Dde66 and Dde70 consisted of initial denaturation at 94ºC for 3 min followed by a touchdown profile for 5 cycles with the annealing temperature starting at 63ºC and decreasing 2ºC per cycle, followed by 30 cycles with an annealing temperature of 53ºC, and a final extension step at 72ºC for 10 min. For the remaining dinucleotide loci conditions followed the original publications. All reactions included both positive and negative controls. Following amplification, samples were mixed with an internal size standard and run on an ABI 3130 Genetic Analyzer. The GeneMapper 4.1. software (Applied Biosystems, CA) was used for sizing of allele fragments.

The program Micro-checker v. 2.2.3. (Oosterhout et al. 2004) was used to check for the presence of genotyping errors such as scoring errors due to stuttering, large allele dropout or evidence for null alleles. Departures from Hardy-Weinberg Equilibrium were tested for each of the 14 microsatellite loci in each population using the Fisher exact test in Genepop v.4.0. (Rousset 2008). Genepop was also used to test for linkage disequilibrium among loci. Genetic diversity measures such as mean number of alleles per locus and observed and expected heterozygosities for each population were calculated in Arlequin v. 3.5.1. (Excoffier and Lischer 2010). The program FSTAT (Goudet 1995) was used to estimate another measure of genetic diversity, allelic richness, as well as to assess population differentiation between the putative populations by estimating the fixation index F_{ST}.

Nuclear Loci

Three anonymous nuclear loci [Del_12, Del_15 and Del_17 (Amaral et al. 2010)] and two introns [CHRNA1 (Roca et al., 2001) and PLP (Lyons et al., 1999)] were PCR amplified and sequenced for 92 common dolphin samples (*Delphinus delphis*: NE Atlantic, *n* = 10; CE Atlantic, *n* = 10; NW Atlantic, *n* = 10; SW Pacific Australia, *n* =10, SW Pacific New Zealand, *n* = 10; NE Pacific, *n* =10; *D. capensis*: SE Atlantic, *n* = 7; SW Atlantic, *n* = 9; NE Pacific, *n* = 10; *D. c. tropicalis*: W Indian, *n* = 6). The PCR reactions were performed in 25-μL reactions containing 10-100 ng DNA, 0.2 mM each dNTP, 0.3 μM each primer, 1 U Taq Polymerase and 1X Taq buffer. PCR products were separated on 1.0% agarose gels, stained with ethidium bromide and visualized with ultraviolet light. PCR products were cleaned with Exonuclease I and Shrimp Alkaline Phosphatase to remove free nucleotides and primers, and sequenced in both directions (BigDye Terminator CycleSequencing: Applied Biosystems) on an ABI 3730xl automated sequencer (Applied Biosystems).

All sequences obtained were aligned using the software Sequencher, version 4.2 (Gene Codes Corporation). jModeltest (Posada 2008) was used to infer the best-fitting evolutionary model for each locus. Models of evolution were chosen for subsequent analyses according to a second-order Akaike Information Criterion (AIC_c), with branch lengths included as additional parameters and a correction for small sample

sizes employed. A concatenated Bayesian phylogenetic tree was obtained in MrBayes v. 3.1.2. (Huelsenbeck and Ronquist, 2001) by running four simultaneous MCMC chains for 2 million generations, with trees sampled at intervals of 100 generations. The first 3000 trees were discarded as "burn-in". Sequences of *Globicephala melas* and *Phocoena phocoena* were used as outgroups.

RESULTS

mtDNA

A total of 1121 bp were sequenced for the cytochrome *b* gene, defining 186 haplotypes. Shared haplotypes (4) between all the three forms (-*delphis*, -*capensis* and –*tropicalis*) were found, as well as between several geographical regions sampled. Haplotypic and nucleotide diversities were high for most putative populations analysed, with *D. delphis* from the NE Pacific showing the highest nucleotide and haplotypic diversities and the *tropicalis* form showing the lowest haplotypic diversity (Table 1). The neutrality tests revealed negative and highly significant values of Tajima's *D* and Fu's *Fs* for NE and NW Atlantic and NE and SW Pacific Australia and New Zealand, suggesting that these populations are in expansion.

Pairwise F_{ST} and ϕ_{ST} values show significant levels of genetic differentiation between most putative populations, with ϕ_{ST} values being generally higher than F_{ST} values (Table 2). This suggests that, at the population level, the differentiation observed is not recent. The *D. capensis* population from NE Pacific is highly differentiated from all other populations. The populations from SE Atlantic and the *–tropicalis* population from the Indian Ocean are also highly differentiated from all other populations. Lower levels of differentiation were obtained between *D. delphis* populations from NE and CE Atlantic, SE and SW Atlantic and NE Pacific and SW Pacific New Zealand, indicating that higher levels of gene flow occur between these populations. All possible patterns of population structure were tested with an AMOVA analysis. The hypothesis with which a higher percentage of variance was explained among groups was when populations were considered separately and when the statistics ϕ_{ST} was used (ϕ_{ST} = 0.25317).

Microsatellites

In total, 340 samples were genotyped at 14 microsatellite loci. Deviations from Hardy-Weinberg equilibrium (HWE) were found for 6 loci. 4 of these (Tur91, Tur142, Tur80 and KW2) only show deviations in one population each and were therefore included in subsequent analyses, whereas 2 (Tur141 and Dde66) show deviations in 5 and 3 populations, respectively. These deviations are due to a deficit of heterozygotes (significant F_{IS} values, Table 3). To test whether results would be affected by the inclusion of these two loci, all analyses were carried out with and without them. Since no differences in F_{ST} values were observed, all 14 loci were used in subsequent analyses.

Levels of genetic diversity, given by mean number of alleles, allelic richness and expected and observed heterozygosities were high for most populations (Table 3). Mean number of alleles varied from 8.21 to 11.64. Significant F_{IS} values were obtained for *D. delphis* from NE Pacific and SW Pacific Australia and New Zealand, which can be due to the presence of population sub-structure (i.e. Wahlund effect).

This is known to be the case for common dolphins around Australia and New Zealand (Bilgmann et al. 2008; Möller et al in review; Stockin et al in preparation).

Pairwise F_{ST} values obtained in FSTAT showed low but significant levels of differentiation between all putative populations (Table 4). The *D. capensis* population from NE Pacific is more differentiated from *D. capensis* and *D. delphis* populations from the Atlantic Ocean but less differentiated from *D. delphis* populations in the Pacific Ocean. Within the Atlantic Ocean, the *D. capensis* population is more differentiated from the *D. delphis* populations in the North Atlantic. There is also some level of differentiation between *D. delphis* populations from SW Pacific (including both Eastern Australian and New Zealand waters) and the population from the SE Indian Ocean. The AMOVA analyses resulted in significant values (F_{ST} = 0.0357) but accounted for only 3.57% of the variance among populations.

Nuclear Loci

A total of 3349 bp comprising five nuclear loci was sequenced for 92 common dolphin samples. Levels of polymorphism obtained were quite low, with only 39 variable sites found. The concatenated Bayesian tree is poorly resolved, with only a few clades supported by posterior probability values (Figure 1). These clades however do not show any geographical or taxonomic association. These dataset will be further explored in the near future using coalescent-based analyses.

DISCUSSION

Both mitochondrial DNA and microsatellite analyses support the existence of population differentiation between common dolphin populations inhabiting different oceans. The *D. capensis* population from NE Pacific showed high levels of divergence from all other populations including the other *D. capensis* populations from SE and SW Atlantic. This divergence had already been identified with the mtDNA (Natoli et al. 2006) but not with nuclear markers. Interestingly, no differentiation was detected between the *D. capensis* populations from SE and SW Atlantic for the mtDNA. Although sample size for the SW Atlantic population is small, this result suggests that long-beaked populations inhabiting the South Atlantic Ocean may share more genetic similarities amongst them than with the long-beaked population occurring in the NE Pacific. F_{ST} and ϕ_{ST} values obtained for the mitochondrial DNA do not support a strong differentiation between the *D. capensis* population from SE Atlantic and *D. delphis* populations from NE, CE and NW Atlantic. This result was also seen in the haplotypic network presented in Amaral et al. (2009). However, F_{ST} values obtained for the microsatellites seem to more strongly support this differentiation, which suggests that such differentiation is recent. This results further support the hypothesis that long-beaked populations in the Atlantic would be a result of local adaptation, occurring independently from the origin of long-beaked populations in the Pacific Ocean (Natoli et al. 2006). If this is the case, a taxonomic revision of the long-beaked populations is definitely needed. The *tropicalis* population from the Indian Ocean showed high levels of differentiation for the mtDNA, despite the existence of shared haplotypes with the *D. capensis* population from the NE Pacific and with *D. delphis* populations from the NW Atlantic. Unfortunately, it was not possible to obtain microsatellite data for this population to further explore levels of differentiation in the nuclear genome.

Levels of genetic differentiation for *D. delphis* populations are higher between different oceans than within oceans. For the mtDNA, F_{ST} and ϕ_{ST} values are lower across the Pacific (between NE and SW Pacific) than across the Atlantic (between NE and NW Atlantic). However, F_{ST} values for the microsatellites show the opposite pattern, suggesting that levels of recent gene flow may be higher across the North Atlantic (supporting results of Mirimin et al. 2009) than across the Pacific Ocean. Further analyses using coalescent-based approaches for estimating number of migrants will be conducted in order to elucidate levels and direction of gene flow.

Microsatellite markers are very informative for detecting population structure and assessing recent levels of gene flow. Nonetheless, having information from a different type of nuclear marker that can elucidate on older events can be useful to track the evolutionary history of a species or group of species. In the 3349 bp we have sequenced from the nuclear genome we found very low levels of polymorphism even between *D. capensis* from NE Pacific and *D. delphis* from the Atlantic and Pacific Oceans. This result illustrates the difficulty in obtaining informative nuclear markers and also reinforces the perception that the nuclear genome in cetaceans evolves slowly (e.g. Jackson et al. 2009). Nevertheless, this dataset will be further explored using newly developed coalescent-based approaches in an attempt to infer population and species trees.

ACKNOWLEDGEMENTS

The authors wish to thank all who kindly collected and provided tissue samples: Luca Mirimin (University College Cork), Jennifer Learmonth (SAC – Scottish Agricultural College and DEFRA), Ángela Llavona (CEMMA – Coordinadora para o Estudio dos Mamíferos Mariños), Marisa Ferreira (Sociedade Portuguesa de Vida Selvagem), Tim Collins and Robert Baldwin (Environment Society of Oman). Funding for this work was provided by Fundação para a Ciência e Tecnologia (Portugal) through a PhD grant to A. R. Amaral.

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Table 1. Descriptive statistics of genetic variability for the mtDNA cytochrome *b* gene in the sampled regions.

Species	**Region**	***N***	**Hd**	**π**	***D***	***Fs***
Delphinus	NE Atlantic	63	0.9145	0.015103	-1.36876	-9.85285**
delphis	CE Atlantic	21	0.8905	0.013859	-1.11923	-3.49843
	NW Atlantic	27	0.9630	0.026535	0.27464	-4.39754*
	NE Pacific	26	0.9908	0.034036	-1.73219*	-9.04236**
	SW Pacific_Australia	42	0.9837	0.021342	-1.74586**	-23.43580***
	SW Pacific_New Zealand	40	0.9885	0.027300	-1.72701*	-14.78562***
	SE Indian	27	0.9715	0.016671	-1.04795	-7.86971**
D. capensis	NE Pacific	41	0.7683	0.017975	-0.58044	1.05913
	SE Atlantic	26	0.9508	0.118306	-1.11851	2.49245
	SW Atlantic	8	0.7857	0.024587	-0.91562	5.82317
D. c. tropicalis	W Indian Ocean	25	0.6558	0.024829	-1.11029	3.80537
Total / Mean		345	0.8414	0.016539		

n – number of individuals sequenced; π - nucleotide diversity; Hd – haplotypic diversity; *D* – Tajima's *D*; *Fs* – Fu's *Fs*.

Table 2. Pairwise F_{ST} (below diagonal) and ϕ_{ST} (above diagonal) values obtained with the cytochrome *b* gene for the different geographical regions sampled.

	Dc NEPAC	Dd NEATL	Dd SWPAC AUS	Dd EIND	Dct WIND	Dd NWATL	Dd NEPAC	Dc SEATL	Dd CEATL	Dd SWPAC NZ	Dc SWATL
Dc NEPAC		0.5413	0.4539	0.4995	0.5164	0.4641	0.3793	0.3336	0.5566	0.4140	0.5176
Dd NEATL	0.1371		0.0895	0.0416	0.5112	0.1784	0.1884	0.1843	0.0262	0.0794	0.2004
Dd SWPAC_AUS	0.1043	0.0461		0.0170	0.4348	0.1607	0.0582	0.1608	0.1131	*0.0048*	0.1992
Dd EIND	0.1135	0.0498	0.0162		0.4713	0.1813	0.1207	0.1191	0.0803	0.0358	0.1856
Dct WIND	0.2000	0.1849	0.1536	0.1663		0.2092	0.3023	0.2499	0.4994	0.3795	0.3957
Dd NWATL	0.1008	0.0407	0.0186	*0.0135*	0.1083		0.1172	0.1523	0.2404	0.1147	0.1834
Dd NEPAC	0.1043	0.0489	0.0128	0.0188	0.1573	0.0160		0.1427	0.1926	0.0297	0.1882
Dc SEATL	0.1239	0.0579	0.0324	0.0319	0.1773	0.0291	0.0292		0.1009	0.1494	*0.0617*
Dd CEATL	0.1401	*-0.0056*	0.0409	0.0543	0.1965	0.0396	0.0446	0.0614		0.1058	0.1982
Dd SWPAC_NZ	0.1016	0.0447	0.0115	*0.0110*	0.1512	0.0146	0.0069	0.0294	0.0443		0.1502
Dc SWATL	0.2011	0.1166	0.0991	0.0920	0.2703	0.0889	0.0979	*0.0300*	0.1386	0.0967	

Non-significant values are in italic. All other values were significant ($P < 0.001$).

Table 3. Descriptive statistics of genetic variability for 14 microsatellite loci in the sampled regions.

Species	**Region**	***N***	**Na**	A_R	H_E	H_O	F_{IS}
Delphinus	NE Atlantic	75	10.500	8.371	0.789	0.774	0.020
delphis	CE Atlantic	29	8.214	7.511	0.739	0.687	0.072
	NW Atlantic	38	9.286	8.184	0.785	0.745	0.051
	NE Pacific	40	11.643	9.424	0.784	0.730	0.069*
	SW Pacific_Australia	35	10.643	8.485	0.782	0.726	0.073*
	SW Pacific_New Zealand	39	10.500	9.130	0.792	0.697	0.121*
	SE Indian	25	7.571	7.163	0.700	0.696	0.006
D. capensis	NE Pacific	37	10.143	8.574	0.777	0.735	0.061
	SE Atlantic	22	6.643	6.512	0.735	0.702	0.046
Total / Mean		340	9.460	8.151	0.765	0.721	

n – number of samples genotyped; N_A – mean number of alleles; A_R – allelic richness; H_E – expected heterozygosity; H_O – observed heterozygosity; F_{IS} – inbreeding coefficient and respective significance level, $P < 0.05$.

Table 4. Pairwise F_{ST} values obtained for 14 microsatellite loci in the different geographical regions sampled.

	Dd NEATL	Dd SWPAC AUS	Dd NWATL	Dd NEPAC	Dc SEATL	Dd CEATL	Dd SWPAC NZ	Dc NEPAC
Dd NEATL								
Dd SWPAC AUS	0.0270							
Dd SEIND	0.0679	0.0497						
Dd NWATL	0.0044	0.0223	0.0726					
Dd NEPAC	0.0288	0.0096	0.0655	0.0276				
Dc SEATL	0.0472	0.0525	0.1081	0.0446	0.0485			
Dd CEATL	0.0111	0.0437	0.0878	0.0132	0.0430	0.0551		
Dd SWPAC NZ	0.0236	0.0107	0.0384	0.0240	0.0189	0.0558	0.0459	
Dc NEPAC	0.0619	0.0398	0.0941	0.0614	0.0340	0.0780	0.0776	0.0516

All values are significant ($P < 0.001$).

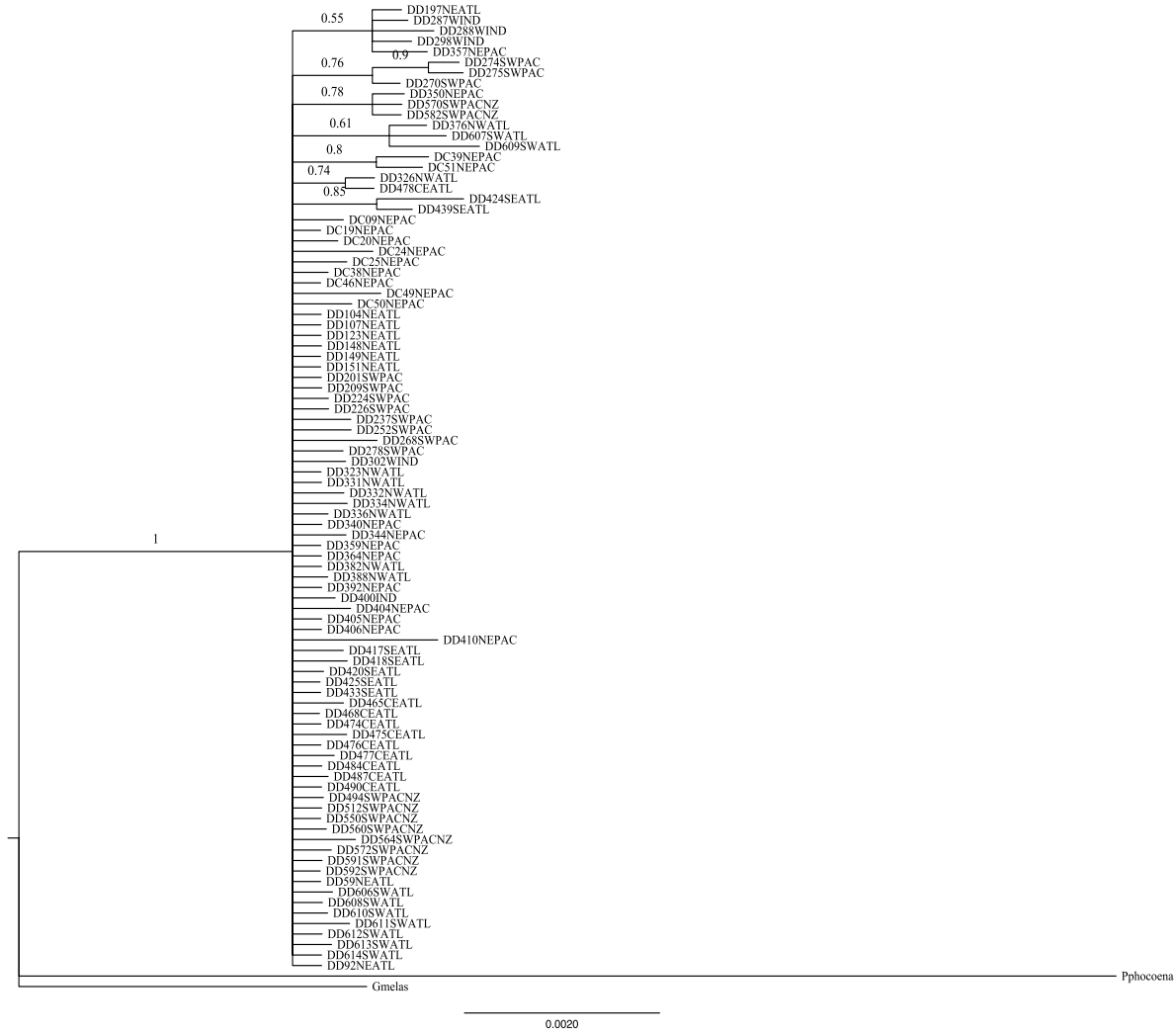

Figure 1. Bayesian phylogenetic tree generated in MrBayes from five concatenated nuclear loci. Posterior probability values are above nodes. Branch lengths are in substitutions/site. Taxa labels correspond to the acronym used in the text to define geographical origin of individuals.