

A preliminary note on population structure in eastern South Pacific common bottlenose dolphins, *Tursiops truncatus*

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ABSTRACT

Previous studies of eastern South Pacific common bottlenose dolphins, *Tursiops truncatus*, defined offshore and inshore ecotypes in Peru based on cranial and tooth morphology, documented the presence of a single resident inshore community ('pod-R') in central-north Chile, and confirmed the presence of offshore bottlenose dolphins off Chile. Here, mtDNA control region (331bp) was examined to evaluate genetic relationships between four geographic areas: inshore pod-R ($n=8$), Chilean offshore population ($n=8$), Peruvian inshore ($n=3$) and offshore ($n=12$) ecotypes. This is the first genetic analysis of *T. truncatus* in this ocean basin. Phylogenetic analysis grouped the three Peruvian specimens morphologically identified as inshore ecotype in an independent cluster, supported by 100% bootstrap value. The net genetic distance between Peruvian inshore and Peruvian offshore ecotypes was estimated at 2.9%, and even higher when compared with Chilean bottlenose dolphins. Morphological and mtDNA evidence combined argues for considering inshore and offshore ecotypes as evolutionary significant units, to be managed accordingly. Despite its inshore behavioural ecology, pod-R presented a high divergence from the Peruvian inshore ecotype and a relatively closer affinity with the Chilean offshore stock (3.41% and 0.87% net interpopulational distance, respectively). However, homogeneity tests showed significant genetic differences of pod-R with all other groups, including Chilean offshore. This, combined with a low nucleotide diversity (0.0069) and behavioural observations, suggest that pod-R may be reproductively isolated and active protection measures are recommended. Only one haplotype from a total of 21 was shared by Peruvian and Chilean offshore animals. Their net genetic distance was estimated at 0.024 and no significant differences were found in haplotype frequencies, suggesting a single, wide-ranging 'Peru-Chile offshore stock'.

KEYWORDS: GENETICS; STOCK IDENTITY; COMMON BOTTLENOSE DOLPHIN; PACIFIC OCEAN; SOUTH AMERICA; TAXONOMY; CONSERVATION; MANAGEMENT

INTRODUCTION

While the taxonomic status of the *Tursiops* genus (bottlenose dolphins) is still under discussion, most authors including ourselves presently recognise two species, one of cosmopolitan distribution, the common bottlenose dolphin, *T. truncatus*, Montagu, 1821 and the Indo-Pacific bottlenose dolphin, *T. aduncus*, Ehrenberg, 1833 (Ross, 1977; Curry, 1997; LeDuc, 1997; Hale *et al.*, 2000; Krützen *et al.*, 2001; Shirakihara *et al.*, 2003 and Wang *et al.*, 1999). However the existence of other species or subspecies cannot be ruled out.

Using general comparative morphology, cranial, diet and parasite load differences, as well as mtDNA analysis, two distinct common bottlenose dolphin ecotypes, offshore and inshore (syn. coastal), have been described for the eastern North Pacific (USA), western North Pacific (China and Japan), western North Atlantic (USA), eastern South Pacific (Peru), western South and Indo-Pacific (Australia and Solomon Islands), eastern South Atlantic (Namibia, South Africa), and the east coast of South Africa (Ross, 1977; 1984; Walker, 1981; Duffield *et al.*, 1983; Ross and Cockcroft, 1990; Van Waerebeek *et al.*, 1990; Findlay *et al.*, 1992; Mead and Potter, 1995; Hoelzel *et al.*, 1998 and Wang *et al.*, 1999).

In the Southeast Pacific Ocean, common bottlenose dolphins are known to occur from the Galápagos archipelago, continental Ecuador, the entire coast of Peru, northern and central Chile south to at least Quenu Island, off

Calbuco, X Region (41°45'S, 73°10'W)¹ and the offshore Chilean archipelagos of San Ambrosio and San Félix, Salas and Gómez Islands and the Juan Fernández Islands (e.g. Lévêque, 1963; Aguayo, 1975; Donovan, 1984; Guerra *et al.*, 1987; González *et al.*, 1989; Van Waerebeek *et al.*, 1990; Félix, 1994; Félix and Samaniego, 1994; Findlay *et al.*, 1998; Sanino and Yáñez, 2001).

Van Waerebeek *et al.* (1990) found distinct cranial differences between offshore and inshore ecotypes of bottlenose dolphins in Peru, as well as clear differences in diets and helminth parasite loads, suggesting reproductively isolated populations. Santillán (2003), studying a larger sample, confirmed cranial differences. Recent management concerns, including direct takes (Sanino and Yáñez, 2000; 2001) led us to biopsy animals from an inshore dwelling community, named pod-R, at Choros Island (29°15'S, 71°26'W) in central-north Chile. These were originally thought to form part of a wider coastal population of bottlenose dolphins.

Video-identification studies revealed high site-fidelity of the 28 pod-R members, as well as morphological and behavioural differences in observed pods of offshore bottlenose dolphins (Sanino and Yáñez, 2001). The issue was raised as to whether pod-R bottlenose dolphins are

¹ Live-stranding of two adult bottlenose dolphins, trapped by fast receding tide, on 2 August 2004 (CMMR files, positive identification from photographic evidence published in *El Llanquihue* No. 36.642, 4 August 2004; Sociedad Periodística Araucanía, Puerto Montt, Chile).

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largely reproductively isolated or are part of, and mix with, other communities to form a wide-ranging Chilean coastal population.

In this study² mtDNA control region sequences were used to examine genetic diversity and phylogenetic relationships among common bottlenose dolphins from different localities in the southeast Pacific Ocean, with particular emphasis given to pod-R dolphins in central-north Chile and the Peruvian inshore ecotype.

MATERIALS AND METHODS

Samples and localities

Sampling localities and sample sizes for the bottlenose dolphins are as follows (Fig. 1): inshore Choros Island (Chile) (CL-I, $n=8$), offshore Chile (CL-O, $n=8$), Peruvian inshore (PE-I, $n=3$) and samples from Peru that included individuals from both confirmed offshore ecotype and indeterminate but like-offshore specimens for which no skulls were collected (PE-O, $n=12$). Morphological characteristics used to distinguish ecotypes in Peru include tooth diameter, the morphology of pterygoids, palatine bones, antorbital process and the separation of occipital condyles (Van Waerebeek *et al.*, 1990). Accompanying fisheries data were also taken into account, especially if the landing process was monitored by one of the authors. All tissue samples from Peru (PE-I and PE-O) were taken from either freshly landed specimens captured in a variety of fisheries, or from body remains found on beaches near fishing towns. Most were stored in dimethylsulphoxide (DMSO) solution with the remainder being stored in 70% ethanol.

CL-I skin samples were collected using a 'Golden Bear' long-bow with modified darts, as described in IWC (1991), mounted with a 6mm (diameter) tip. The samples were soaked in 70% ethanol for three weeks, after which the hypodermis was eliminated and the epidermis/dermis was transferred to a DMSO saturated saline solution. Examination of photographs and videotapes for individual identification precluded the possibility of more than one sample coming from the same animal. CL-O samples were collected with a Barnett crossbow and tethered bolts (IWC, 1991) from the bow of the R/V *Shonan Maru 2* during the third blue whale cruise of the IWC/SOWER programme (Findlay *et al.*, 1998). Sampling error was considered negligible as biopsies of bowriding specimens were taken near-randomly over a wide area (8° of latitude).

Extraction of DNA

Total cell DNA was extracted from samples of skin or other tissue. DNA extractions followed phenol/chloroform/isoamyl/alcohol protocols as described by Sambrook *et al.* (1989). Extracted DNA was resuspended in 500µl 0.1M Tris-HCl (pH 8.0), 0.05 mM EDTA.

Amplification of mtDNA control region

The first 500 nucleotides at the 5' end of the mitochondrial control region were amplified by the polymerase chain reaction. The oligonucleotides employed in the PCR amplification were MT4 (Arnason *et al.*, 1993%) and P2R (5'-GAA GAG GGA TCC CTG CCA AGC GG-3'). Reactions were carried out in 50 µL volumes containing 100 mM KCl, 20 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT; 0.5% Tween R20, 0.5% Nonidet RP-40, 200 µM dNTPs, 2.5

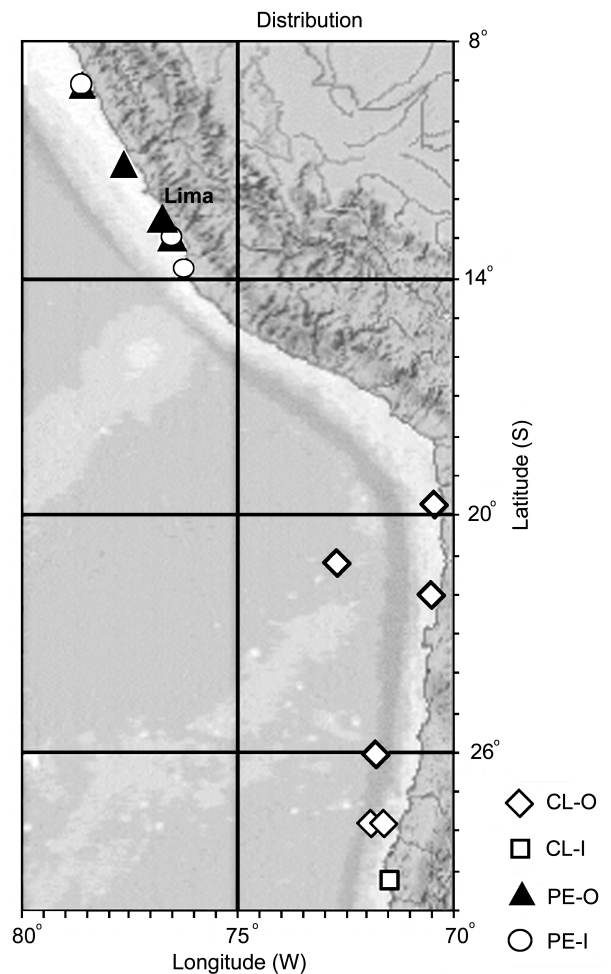


Fig. 1. Distributions of common bottlenose dolphins sampled in Peru and Chile.

pM each oligonucleotide and one unit of *Taq* DNA polymerase. After an initial denaturation step at 95°C for 5 minutes, a PCR amplification regime of 30 seconds at 94°C, followed by 30 seconds at 50°C and 30 seconds at 72°C was repeated 30 times. The amplification was completed with a final extension step of 10 minutes at 72°C. Subsequent cycle sequencing reactions were performed with 100ng of products generated in the above PCR amplifications using the *Prism*TM dRhodamine Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.). The oligonucleotides used to prime the cycle sequencing reaction were the same as employed in the initial PCR amplification listed above. A total of 25 cycles with 10 seconds at 96°C, 20 seconds at 56°C and four minutes at 60°C were performed. The nucleotide sequence of each cycle sequencing reaction was determined by electrophoresis through a 5% *Long Ranger*TM (FMC, Inc.) denaturing polyacrylamide matrix on a DNA *Prism*TM 377 DNA Sequencer (Applied Biosystems, Inc.) under standard conditions. Both strands were sequenced in their entirety for all samples.

Sequence analysis

Sequencing was performed with 100ng PCR products using the *PRISM*TM Ready Reaction Dye Deoxy Terminator Kit (Applied Biosystems) (ABI). Primers used for sequencing were the same as indicated above. The reaction was performed through 25 cycles of 96°C for 10sec, 56°C for 20sec and 60°C for 4min. The nucleotide sequence for each amplification was determined by electrophoresis through a 5% *Long Ranger*TM polyacrylamide matrix on an ABI DNA

² An earlier version of this paper (SC/55/SM22) was presented to the IWC Scientific Committee in 2003.

*Prism*TM 377, following the manufacturer protocols. For each sample both forward and reverse strands were sequenced. Sequences were aligned using the DNA sequence comparison software ‘*Sequence Navigator*’ developed by ABI.

Levels of polymorphism

Genetic distances among different haplotypes were estimated using Kimura’s two parameters method based on genetic distance among haplotypes (Kimura, 1980). Nucleotide diversity (π) was estimated following equation 10.5 of Nei (1987). The net genetic distance between populations (d_A) was estimated by subtracting the average level of variation within each population, following equation 10.21 of Nei (1987).

MtDNA genealogy

Phylogenetic reconstruction of haplotypes was made using the neighbour-joining method (Saitou and Nei, 1987). To evaluate the confidence limits of phylogenies, 1000 bootstrap simulations were conducted (Felsenstein, 1985). The phylogenies were rooted using the homologous sequence from a common dolphin (*Delphinus* sp.; GenBank accession number: U02652).

Homogeneity test

Homogeneity tests were conducted using the sequence (K_{st}^*) and haplotype (Hst) statistics proposed by Hudson *et al.* (1992). The degree of divergence was inferred as being larger than zero, if an equal or more extreme value of the K_{st}^* or Hst was observed in less than 5% of 10,000 Monte Carlo simulations.

RESULTS

Level of polymorphism

The first 331 nucleotides were determined in the mtDNA control region for each of the 31 samples. A total of 32 polymorphic sites were detected (30 transitions, one transversion and one deletion), which defined 21 unique haplotypes (Table 1).

Nucleotide diversity for the whole sample population was estimated to be 0.02193, and the nucleotide diversity within a single sample ranged from 0.00201 in the Peruvian inshore (PE-I), to 0.02007 in the Chilean offshore group (CL-O) (Table 2).

Geographic distribution of haplotypes

The frequency of haplotypes in the bottlenose dolphin samples is shown in Fig. 2. Apart from haplotype ‘9’, which was shared by CL-O and PE-O, no shared haplotype occurred among CL-I, CL-O, PE-I and PE-O. All individuals in the PE-O group showed a different haplotype. Six haplotypes were defined in eight CL-O individuals, while only two haplotypes were defined in the eight CL-I animals. Two individuals of the Peruvian inshore ecotype shared the same haplotype (‘14’), although they were landed in ports 556km apart (Chimbote and San Andrés).

MtDNA haplotype genealogy

A neighbour-joining-based phylogenetic tree of the haplotypes (Fig. 2) shows haplotypes ‘4’ and ‘14’ as highly divergent from all others. Haplotype ‘14’ includes two individuals from the Peruvian inshore ecotype. Haplotype ‘4’ was represented by a single inshore individual (MFB-465), as determined by cranial and capture data. This was supported by sequencing results since MFB-465 clustered

very near to haplotype ‘14’ (with 100% bootstrap support). Two other clusters with a branch supported by a high bootstrap value (70%) were identified, however these clusters included individuals from different localities, but no Peruvian inshore specimens.

Net inter-populational distances

Table 2 shows the net inter-populational distances between areas. Individuals with haplotypes ‘4’ or ‘14’ were identified as the Peruvian inshore type based on morphological features (Van Waerebeek *et al.*, 1990) and capture circumstances. All pairwise comparisons involving this ecotype (PE-I, $n=3$) showed large genetic distances ranging from 0.02900 to 0.03412. The other pairwise comparisons resulted in genetic distances between 0.00024 and 0.00870. The smallest genetic distance was found between CL-O and PE-O.

Homogeneity tests

Pairwise comparisons between areas predominately resulted in significant genetic differences. However, the comparison between CL-O and PE-O, showed no significant difference and the comparison between CL-I and PE-I was only near-to-significant for Hst. The latter is presumably due to the small PE-I sample size since the corresponding K_{st}^* was highly significant (Table 3).

DISCUSSION

The three bottlenose dolphin specimens from Peru identified as the Peruvian Inshore ecotype through the evaluation of cranial characteristics, tooth diameters (Van Waerebeek *et al.*, 1990) and fisheries data (e.g. Van Waerebeek *et al.*, 1997; 2002), were found to be phylogenetically distinct from all other bottlenose dolphins studied in the eastern South Pacific. Furthermore we found a high net inter-populational distance of 2.9% between Peruvian inshore and offshore ecotypes, and even higher values when compared with another area (Chile). These results suggest that the Peruvian inshore ecotype should be considered an evolutionarily significant unit, and should be managed separately from the offshore ecotype, coinciding with morphological and ecological evidence (Van Waerebeek *et al.*, 1990; Santillán, 2003).

Inshore Chilean bottlenose dolphins (pod-R) were highly divergent from the Peruvian inshore ecotype based on phylogenetic analysis and the net inter-population distance. This is surprising since pod-R dolphins reside inshore with high site fidelity, which are characteristic of all inshore bottlenose dolphins. In fact, pod-R appeared more closely related to the Chilean offshore stock. However, homogeneity tests for pod-R in pairwise comparison with the Chilean offshore and the Peruvian offshore groups revealed significant genetic differences. Furthermore, the eight CL-I individuals showed only two haplotypes (Fig. 2) and a concomitant low nucleotide diversity. Strictly speaking, mtDNA divergence does not necessarily signify reproductive isolation since mtDNA is maternally inherited. However, these mtDNA analysis results are consistent with intensive field observations on pod-R, which suggest that the group may be reproductively isolated (Sanino and Yáñez, 2001), and a study to ascertain whether this is the case using nuclear DNA markers is planned. If reproductive isolation is indeed confirmed, the long-term survival of this community of some 30 individuals looks uncertain (Sanino and Yáñez, 2001). Pod-R might actually constitute the only remnant pod of a population. To date there is no evidence for

Table 1

Variable sites defining 21 haplotypes in bottlenose dolphins from the eastern South Pacific. The numbers above (reading vertically) list the nucleotide positions of the polymorphic sites starting from the 5' end of the mtDNA control region. Haplotypes '2' through '21' are listed with reference to haplotype '1'. A dot indicates an identical nucleotide at the position relative to haplotype '1'. A hyphen indicates a deletion.

		11	1111222222	2222222222	33
		233349901	1146034456	6777778899	00
		7013461270	1597545815	9237892334	35
1	MFB185/7	AGCTCTTTAT	GGCTCTCGCT	CATTTTTCCT	AT
2	MFB186	-.....T....TC	..
3	MFB441	-.....CC.T.	..
4	MFB465	-AT.T..C..	A..C..TATAC	..
5	MFB701	-..C.....C	..T...T..CCCC.TC	G.
6	MFB702C...	.AT...T..CC...TC	G.
7	KVW2393	-.....CC...	..
8	KVW2412	-.....	..T...T..CC.C.TC	G.
9	KVW2417C...	.AT...T..CC.C.TC	G.
10	KVW2439	-.....CC	T.....C...	..
11	KVW2440	-.....C	..
12	RBC54	-.....C..C..C	.C
13	JAS12C
14	JAS47	-AT.T..C..	A.....TATAC	..
15	GPSV-BP001	-.....	A...T.....	..C...C.T.	..
16	GPSV-BP002	-.....C...	..
17	TS97121301C...	.AT.....CC.C.TC	G.
18	TS97121303	-....C....C...	..
19	TS97121802	-.....	A.....C..C	.C
20	TS97122602	-.....C..C	..
21	TS97122604G.	..T..C...C	.G...C.TTC	..

Table 2

Nucleotide diversity (π , diagonal in bold) and net interpopulational distances (d_A , upper right), with respective sample sizes, for bottlenose dolphins in four geographic areas of the eastern South Pacific. Confidence intervals of the estimates are in parentheses.

Area	PE-I (n=3)	PE-O (n=12)	CL-I (n=8)	CL-O (n=8)
PE-I (n=3)	0.00201 (±0.00190)	0.02900 (±0.00820)	0.03412 (±0.01122)	0.03349 (±0.01004)
PE-O (n=12)		0.01794 (±0.00446)	0.00564 (±0.00160)	0.00024 (±0.00008)
CL-I (n=8)			0.00691 (±0.00228)	0.00870 (±0.00290)
CL-O (n=8)				0.02007 (±0.00766)

Table 3

Results of homogeneity tests for bottlenose dolphins in four geographic areas of the eastern South Pacific. Hst values are shown above the diagonal; Kst* values below diagonal. *=significant at the $P<0.05$ level; **=significant at the $P<0.01$ level.

Area	PE-I (n=3)	PE-O (n=12)	CL-I (n=8)	CL-O (n=8)
PE-I (n=3)	-	0.0577*	0.2177	0.1036*
PE-O (n=12)	0.2438**	-	0.1156**	0.0223
CL-I (n=8)	0.5100**	0.1284**	-	0.1633**
CL-O (n=8)	0.3271**	0.0207	0.1991**	-

the existence of a widely distributed inshore bottlenose dolphin population in Chile south of Punta Coloso (23°43'S), near Antofagasta (see Aguayo, 1975; Sielfeld, 1980; 1983; Guerra *et al.*, 1987; Van Waerebeek *et al.*, 1990). North of Punta Coloso, several undetermined ecotype specimens are curated at the University of

Antofagasta (Guerra *et al.*, 1987), and unconfirmed reports of dolphins in the surfzone off beaches around Iquique require further investigation.

No significant differences were found between Peruvian and Chilean offshore bottlenose dolphins, but only one haplotype was shared between them (No. 9, Fig. 2). These dolphins often travel at great speeds with steady bearing, performing high, energetic jumps (personal observations) and are thought to cover great distances with ease. Chilean and Peruvian offshore bottlenose dolphins probably form a single wide-ranging population, which we have provisionally named the 'Peru-Chile offshore bottlenose dolphin stock'. Affinities with other nominal bottlenose dolphin species described from the eastern Pacific Ocean³ should be established, including insular animals found around archipelagos.

The mtDNA results presented here, together with morphological data and parasite load differences (Van Waerebeek *et al.*, 1990) show that the Chilean and Peruvian inshore stocks of bottlenose dolphins should each be managed as distinct reproductive units.

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³ In particular *Tursiops nuuanu* Andrews, 1911 of the eastern tropical Pacific and *T. truncatus gillii* Dall, 1873 of southern and Baja California (see comparative discussion by Andrews, 1911).

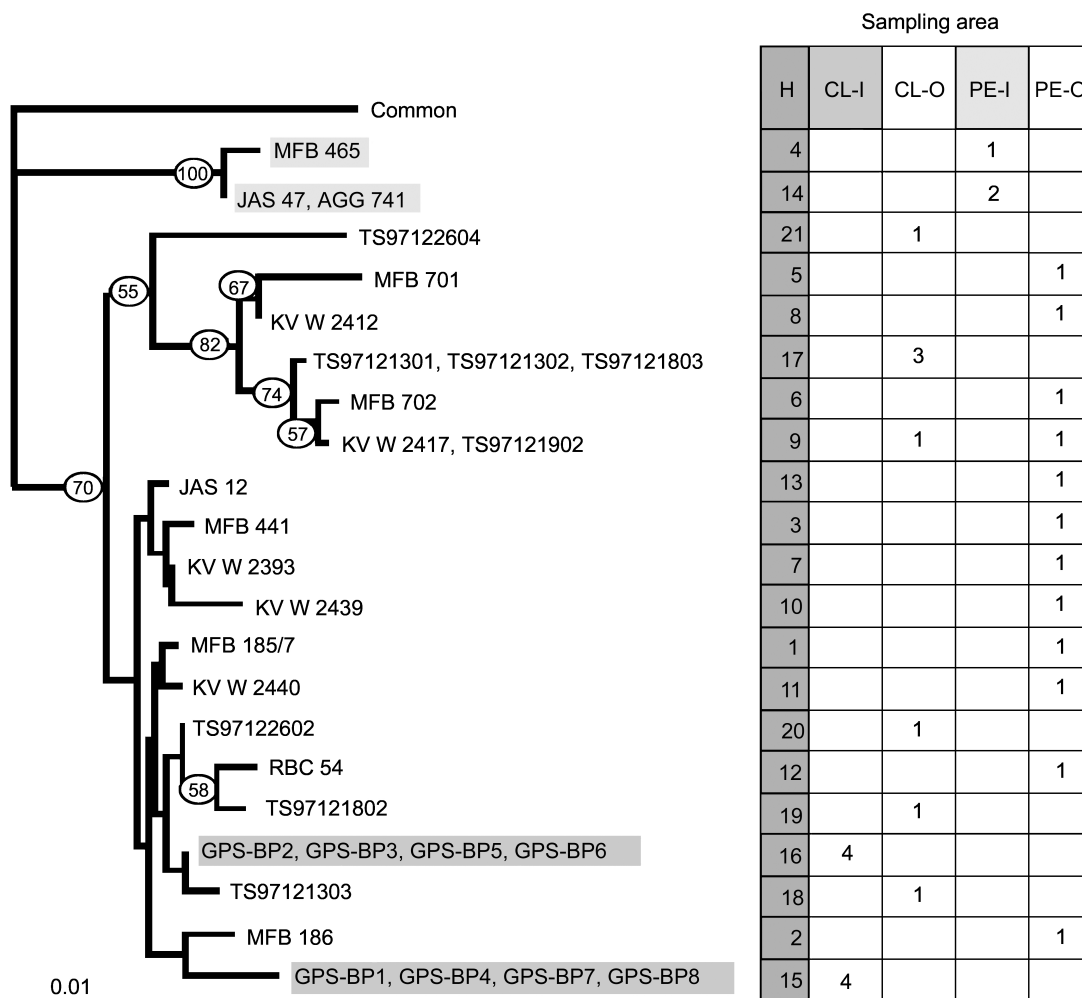


Fig. 2. Phylogenetic tree and frequencies of haplotypes (H) for common bottlenose dolphins from the four study areas (CL-I: Inshore Chile, CL-O: offshore Chile, PE-I: inshore Peru, PE-O: offshore Peru). In circles are the bootstrap values over 50% –in 1,000 Monte Carlo simulations.

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