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Phylogenetic identification and population differentiation of bottlenose dolphins (*Tursiops* spp.) in Melanesia, as revealed by mitochondrial DNA

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Abstract

The taxonomic status of many dolphin populations remains uncertain in poorly studied regions of the world's ocean. Here we attempt to clarify the phylogenetic identity of two distinct forms of bottlenose dolphins (*Tursiops* spp.) described in the Melanesian region of the Pacific Ocean. Mitochondrial DNA control region sequences from samples collected in New Caledonia (n = 88) and the Solomon Islands (n = 19) were compared to previously published sequences of *Tursiops* spp., representing four phylogenetic units currently recognized within the genus. Phylogenetic reconstructions confirm that the smaller coastal form in Melanesia belongs to the same phylogenetic unit as *T. aduncus* populations in the Pacific, but differs from *T. aduncus* in Africa, and that the larger more oceanic form belongs to the species *T. truncatus*. Analyses of population diversity reveal high levels of regional population structuring among the two forms, with contrasting levels of diversity. From a conservation perspective, genetic isolation of *T. aduncus* in the Solomon Islands raises further concern about recent impacts of the commercial, live-capture export industry. Furthermore, the low level of mtDNA diversity in *T. aduncus* of New Caledonia suggests a recent population bottleneck or founder effect and isolation. This raises concerns for the conservation status of these local populations.

Key words: *Tursiops*, Melanesia, phylogeny, genetic diversity, population structure, mtDNA.

The taxonomic status of many regional dolphin populations remains uncertain (*e.g.*, Mendez *et al.* 2013). This is due in part to the fact that Delphinidae is the most speciose family of cetaceans (at least 38 species currently recognized), and is undergoing frequent revisions, including proposals for new species (*e.g.*, Beasley *et al.* 2005, Caballero *et al.* 2007). The Delphinidae also exhibit a wide range of morphological and ecological diversity, among and within species (Perrin 1991, LeDuc 2002). This can obscure species identification, especially in understudied areas where information on morphology, genetics, and life history is usually sparse.

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Molecular systematics and taxonomy provides a powerful approach to resolving species identity (Ross *et al.* 2003). Such techniques have proven to be particularly valuable for cetaceans, including forensics used to detect illegal products sold on markets or in restaurants, and species identification of obscure stranded and museum specimens (Dalebout *et al.* 2007, Baker 2008, Baker *et al.* 2010, Thompson *et al.* 2012). Previous attempts to resolve species identity in cetaceans have usually relied on mitochondrial (mt) DNA sequences, either the control region (CR) or cytochrome *b*. However, interpretation of mtDNA is not always straightforward, as intraspecific diversity is high and interspecific divergence is low among some closely related species of dolphins (Reeves *et al.* 2004). To adequately characterize the certainty or uncertainty in taxonomic identification, it is often necessary to use a data set of reference sequences that covers a large spectrum of the intra- and interspecies diversity (Funk and Omland 2003).

The genus *Tursiops* has been particularly challenging when it comes to assigning taxonomic units (Reeves *et al.* 2004). Two species are currently accepted on the basis of genetics, osteology, and external morphology: the common bottlenose dolphin (*Tursiops truncatus*) and the Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) (Ross 1977, LeDuc *et al.* 1999, Wang *et al.* 1999, Hale *et al.* 2000, Shirakihara 2003, Kurihara and Oda 2007). However, the Indo-Pacific bottlenose dolphin apparently refers to two different phylogenetic units (*i.e.*, species or subspecies); one identified along the coast of Africa (Natoli *et al.* 2004, Särnblad *et al.* 2011), and one found in several locations of the western Pacific Ocean, including China, Japan, Korea, and Australia (Wang *et al.* 1999, Kakuda *et al.* 2002, Perrin *et al.* 2007, Kim *et al.* 2010, Kita *et al.* 2013), and the eastern Indian Ocean (in Indonesia, Wang *et al.* 1999). The taxonomic status of the Shark Bay bottlenose dolphin population, in western Australia, remains unclear with mtDNA control region haplotypes apparently clustering either with *T. aduncus* or *T. truncatus* sequences (Krützen 2002). Furthermore, evidence of past hybridizations was found within the insular populations of *T. truncatus* in Hawaii, including one individual carrying a *T. aduncus*-like mtDNA haplotype (Martien *et al.* 2011). However, since the origin of this hybrid ancestry is unknown, with no evidence for a population of *T. aduncus* in this region, the eastern limit for the current distribution of *T. aduncus* is thought to be located in the western Pacific (Wang and Yang 2009).

The holotype of *T. aduncus* was collected in the Red Sea and analysis of mtDNA control region sequences from this specimen groups with the African taxon (Perrin *et al.* 2007). Therefore, the taxonomy of dolphins known as *T. aduncus* from the Pacific Ocean is likely to require revision. Since this formal taxonomy is still unresolved, we refer to these two taxa as the "African" *T. aduncus* and the "Pacific" *T. aduncus* (hereafter, when we refer to the two taxa without distinction, *T. aduncus* is mentioned alone). To add to the confusion, a fourth phylogenetic unit potentially representing a new distinct species of *Tursiops* was recently identified in the waters of southern Australia (Charlton-Robb *et al.* 2011). The name *T. australis* sp. nov. was proposed for it but since formal recognition has not been agreed (Committee on Taxonomy 2014), we chose to employ the terminology "putative *T. australis* sp. nov."

In Melanesia, two forms of *Tursiops* have been described based on observations made in New Caledonia and in the Solomon Islands (Kahn 2006, Garrigue 2007). Distinction between these two forms has relied primarily on morphological features but also on habitat preferences. The first of these two forms presents all the characteristics of *T. aduncus* with adult individuals reaching no more than 2.5 m and often

presenting spots on their side and ventrum (Wang and Yang 2009). Their habitat is strictly coastal and limited to shallow waters. In New Caledonia, they are widely distributed across the lagoon that surrounds the "Grande Terre" and the Isle of Pines, and are occasionally seen just along the external barrier reef (Garrigue and Poupon 2013). In the Solomon Islands, the *T. aduncus* form appears to be found exclusively close to shore, usually <1 km, and in <100 m water depth (Oremus *et al.* 2013*b*). The taxonomy of coastal bottlenose dolphins in the Solomon Islands is of particular interest given their recent history of live-capture. Since 2003, at least 108 dolphins have been removed from wild populations around the islands of Guadalcanal and Malaita and exported for commercial display (UNEP-WCMC 2012). Presumably more have been captured but died before export or are still held in captivity in the Solomon Islands (Oremus *et al.* 2013*b*). Strong concerns have been expressed about the consequences of such removals on local populations (Reeves and Brownell 2009). Note that *T. aduncus* in the Solomon Islands are not targeted by hunters although there is also a long-stand tradition of dolphin drive-hunting that is ongoing in this country (Oremus *et al.* 2013*a*).

The characteristics of the second form of bottlenose dolphins in Melanesia are more similar to *T. truncatus*, with adults presenting larger body size (total length > 3 m) and shorter beak relative to body length (Wang *et al.* 2000). In both New Caledonia and the Solomon Islands, they are primarily distributed offshore, in deeper waters (>100 m), although in New Caledonia occasional sightings have been made along the external barrier reef of the "Grande Terre" (Garrigue and Poupon 2013, Oremus *et al.* 2013*b*). However, there are no records of this species in the lagoon of this island (Garrigue and Poupon 2013). Such distribution is similar to other *T. truncatus* populations elsewhere, although this species is known to use a wide variety of habitats throughout its range (Wells and Scott 2009). On the basis of these morphological and ecological differences, the two *Tursiops* forms in Melanesia have been previously referred to as *T. truncatus* and *T. aduncus* (Kahn 2006, Garrigue 2007, Borsa *et al.* 2012). However, the taxonomic status of these populations has not been confirmed or clarified by molecular phylogenetics.

Here, we first aim at clarifying the taxonomic status of the two forms of bottlenose dolphins identified in the waters of Melanesia. Based on morphological appearance, we referred to these as the *T. aduncus*-like form and the *T. truncatus*-like form of Melanesia. We analyzed skin samples collected in New Caledonia and the Solomon Islands and we reconstructed an extensive data set of reference mtDNA control region sequences from previous studies, representing the four phylogenetic groups of *Tursiops* described above: *T. truncatus* (represented by Pacific populations), "African" *T. aduncus*, "Pacific" *T. aduncus*, and putative *T. australis* sp. nov. No sequences from *T. truncatus* in other oceans of the world are included here because these are not relevant to our study of "bottlenose dolphins" in Melanesia. Phylogenetic reconstructions and evolutionary distances were used to assign the most likely species or phylogenetic units to which Melanesian *Tursiops* belongs. However, no attempt was made to resolve the phylogenetic relationships between the different *Tursiops* units since this complex issue would require additional genetic markers, (*e.g.*, Vilstrup *et al.* 2011, Amaral *et al.* 2012). After confirming that the *T. aduncus*-like form and the *T. truncatus*-like form of Melanesia belong to the "Pacific" *T. aduncus* and *T. truncatus* phylogenetic units, respectively, we conducted separate analyses of genetic diversity and population structure for each of the two phylogenetic units in the Pacific. These analyses provided preliminary information on the evolutionary history and degree of isolation of *Tursiops* populations in Melanesia.

Material and Methods

Study Sites and Sample Collection

The main island of New Caledonia, the "Grande Terre," is 400 km long and 50–80 km wide (Fig. 1). It is surrounded by over 1,600 km of barrier reef that delineates one of the largest lagoons on Earth (24,000 km²), with a mean depth of 24 m (range 0–80 m). The archipelago is also composed of several other islands, including the Isle of Pines and the Loyalty Islands (Maré, Lifou, Tiga, and Ouvea), as well as

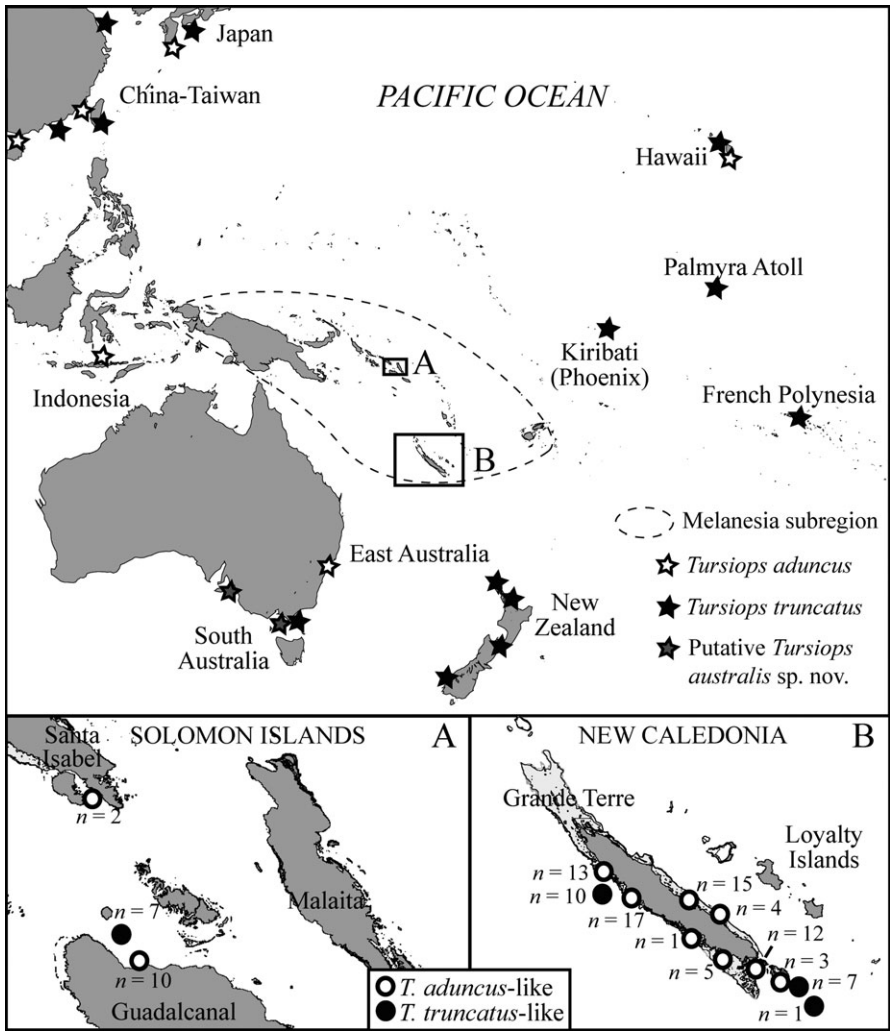

Figure 1. Regional location of origin for the samples from three phylogenetic units of *Tursiops* collected in the Pacific Ocean as represented in the reference data set. Enlargements of the study areas in the Solomon Islands (A) and New Caledonia (B) show sampling positions and number of sequences available (*n*) for the two forms of *Tursiops* observed in these regions.

many islets and reefs. The Solomon Islands consists of nearly 1,000 islands, representing over 5,000 km of coastline (Fig. 1). The continental shelf around these islands is usually narrow, and the ocean floor quickly falls to several hundred meters depth.

Each sample collected from these two locations was classified as coming from the *T. aduncus*-like form or the *T. truncatus*-like form based on a combination of several morphological and habitat characteristics as described above, some of which were supported by systematic collection of photo-identification data. Genetic samples were collected during systematic boat surveys conducted (1) between 2004 and 2009 around the "Grande Terre" and at the Isle of Pines in New Caledonia and (2) between 2009 and 2011 in the Solomon Islands. Skin samples were obtained using a small biopsy dart fired from a modified .22 caliber veterinary capture rifle equipped with a variable pressure valve (Krützen *et al.* 2002). This system was especially developed to assure minimal impact on small cetaceans (Noren and Mocklin 2012, Tezanos Pinto and Baker 2012). In New Caledonia, biopsy samples were collected from 18 *T. truncatus*-like and 70 *T. aduncus*-like. In the Solomon Islands, we collected seven biopsy samples of the *T. truncatus*-like form off the coast of Guadalcanal Island and two samples of the *T. aduncus*-like form off the coast of Santa Isabel.

In addition to biopsies, 33 skin samples of the *T. aduncus*-like form were obtained from captive dolphins captured in 2008 along the coast of Guadalcanal, Solomon Islands, and held in facilities at Honiara, Guadalcanal, and Gavutu, Florida Islands. Samples from captive dolphins were collected by local trainers using the skin-swabbing technique (Harlin *et al.* 1999). This technique consists of using a sterilized nylon scrub pad that is swabbed on the dorsal or lateral surface of the dolphin to remove and retain sloughed epidermal cells. It has the advantage of being almost noninvasive but has the disadvantage of providing poorer quality genetic material (Harlin *et al.* 1999). All samples were preserved in 70% ethanol and stored at −20°C for subsequent analyses.

DNA Extraction and Amplification

Total DNA was isolated from skin tissue by digestion with proteinase K followed by a standard phenol:chloroform extraction method (Sambrook *et al.* 1989) as modified for small samples by Baker *et al.* (1994). A 700 base pair (bp) fragment of the 5′ end of the mtDNA control region was amplified *via* PCR using the primers light-strand, tPro-whale M13-Dlp-1.5 (5′-TCACCCAAAGCTGRATTCTA-3′, Dalebout *et al.* 1998), and heavy strand, Dlp-8G (5′-GGAGTACTATGTCCTGTAACCA-3′, as reported in Dalebout *et al.* 2005). PCR reactions and sequencing were conducted as reported in Oremus *et al.* (2007).

Reference Data Set of mtDNA Sequences

In order to answer our research questions, we assembled 152 mtDNA control region haplotypes of *Tursiops* from previously published studies and/or from GenBank, representing a total of 939 individuals (Table 1). When possible, haplotype frequencies from putative regions were recorded in order to further investigate population structure at a regional scale. This reference data set represented the four phylogenetic units referred to in the Introduction, with the following sources:

(1) For "African" *T. aduncus*, sequences were obtained from South Africa (Natoli *et al.* 2008) and the associated holotype specimen from the Red Sea (Perrin *et al.* 2007). (2) For "Pacific" *T. aduncus*, sequences were obtained from China/Taiwan

Table 1. Summary of the reference sequences used to clarify the taxonomic status of *Tursiops* in Melanesia, stratified by phylogenetic unit and region of origin. It includes the number of samples analyzed in original studies, maximum length of haplotypes available, the number of unique haplotypes (before and after reduction to a homologous fragment of 364 bp), GenBank accession numbers and sources.

Species	Location of origin	No. samples	Original length (bp)	No. haplo.	No. haplo. reduced (364 bp)	GenBank	References
"Pacific" *Tursiops aduncus*	China/Taiwan	31	385/422	16	16	AF049100/AF056234-AF056243/ AF355576-AF355581	Wang *et al.* (1999), Yang *et al.* (2005)
	East Australia	185	403	8	7	AF287951-AF287954/EF581128/ GQ420670	Moller & Beheregaray (2001), Moller *et al.* (2007), Wiszniewski *et al.* (2010)
	Hawaii	1	399	1	1	EF672725	Martien *et al.* (2011)
	Indonesia	2	385	2	2	AF056237-AF056238	Wang *et al.* (1999)
	Japan	2	385	1	1	-	Kakuda *et al.* (2002)
"African" *Tursiops aduncus*	South Africa	50	599	6	4	EF636207-EF636212	Natoli *et al.* (2008)
	Red Sea (holotype)	1	399	1	1	DQ517442	Perrin *et al.* (2007)
Tursiops truncatus (Pacific)	East Australia	6	417	5	5	JN571470-JN571474	Charlton-Robb *et al.* (2011)
	French Polynesia	2	388	2	2	-	Tezanos-Pinto *et al.* (2009)
	Hawaii	118	400	19	19	EF672700-EF672718	Martien *et al.* (2011)
	Japan	165	427	21	21	AB303154-AB303174	Kita *et al.* (2013)
	Kiribati (Phoenix)	23	388	8	8	-	Tezanos-Pinto *et al.* (2009)
	China/Taiwan	33	386/424	18	18	AF056220-AF056232/ AF355582-AF355586	Wang *et al.* (1999), Yang *et al.* (2005)
	New Zealand	206	645	21	20	EU276389-EU276412	Tezanos-Pinto *et al.* (2009)
	Palmyra Atoll	11	401	7	7	EF672708-EF672723	Martien *et al.* (2011)
Putative *Tursiops australis* sp.nov.	South Australia	103	418/446	16	15	EF192140-EF192149/ JN571464-JN571469	Bilgman *et al.* (2007), Charlton-Robb *et al.* (2011)

(Wang *et al.* 1999, Yang *et al.* 2005), Japan (Kakuda *et al.* 2002), southeast Australia (Wiszniewski *et al.* 2010) and Hawaii (Martien *et al.* 2011). Although not from the Pacific Ocean, we also included two Indonesian sequences from Wang *et al.* (1999) in this group. (3) For the southern Australian bottlenose dolphin (*T. australis* sp. nov.), sequences were from Bilgmann *et al.* (2007) and Charlton-Robb *et al.* (2011). (4) For *T. truncatus*, we compiled sequences from samples collected in Australia (Charlton-Robb *et al.* 2011), China/Taiwan (Wang *et al.* 1999, Yang *et al.* 2005), Hawaii (Martien *et al.* 2011), Japan (Kita *et al.* 2013), New Zealand (Tezanos-Pinto *et al.* 2009), Palmyra Atoll (Martien *et al.* 2011), Kiribati and French Polynesia (Tezanos-Pinto *et al.* 2009) (Fig. 1, Table 1).

Sequences Alignment and Unique Haplotypes

Newly generated sequences from New Caledonia and the Solomon Islands were aligned using the MUSCLE alignment method with the default setting (Edgar 2004), as implemented in the software GENEIOUS v.6 (Drummond *et al.* 2009). Variable sites were identified and confirmed by visual inspection of peak heights. The new sequences were aligned with sequences from the reference data set using the same alignment method. The maximum length of the sequences available varied according to the different sources (ranging from 385 to 645 bp) and for the purpose of our analyses, sequences were truncated so that they all represent the same portion of the gene. Unique haplotypes were identified using the program DNAsp (Librado and Rozas 2009).

Phylogenetic Analyses and Haplotype Divergence

The phylogenetic relationships of the mtDNA haplotypes were reconstructed using Maximum Likelihood (ML) and Bayesian (BA) analyses, as implemented in programs MEGA v.5 (Tamura *et al.* 2011) and MrBAYES v.3.2 (Ronquist *et al.* 2011), respectively. The best model of nucleotide substitution for our data was determined with jMODELTEST v.2 (Guindon and Gascuel 2003, Darriba *et al.* 2012), using the Akaike Information Criterion (AIC). It was the Tamura-Nei model (Tamura and Nei 1993) with a proportion of invariable sites and a gamma-shaped distribution of rates across sites (G = 0.4). For ML, we implemented this model and we used the Nearest-Neighbor-Interchange as heuristic method, neighbor-joining to create the initial tree and a moderate Branch Swap Filter. For the BA, we used the HKY + G nucleotide model (Hasegawa *et al*. 1985) as it is the best substitution model selected for our data by jMODELTEST (second best model overall) and which can be implemented in MrBAYES. Two independent analyses were run simultaneously and four chains were used for the phylogeny estimation. Analyses were started with a random tree, and run for 1,500,000 generations (every 1,000th tree was sampled). The initial 5,000 trees were discarded as burn-in. We checked that the standard deviation of split frequencies approach zero at the end of the runs, as a convergence diagnostic. The BA consensus tree was visualized using the program FigTree v.1.4 (http://tree.bio.ed.ac.uk/software/figtree/). The robustness of phylogenetic groupings was assessed by bootstrap resampling for the ML (1,000 replicates) and posterior probabilities for the BA. Rough-toothed dolphin (*Steno bredanensis*) and the short-finned pilot whale (*Globicephala macrorhynchus*) sequences were used as outgroups.

To further evaluate the relationship of Melanesian *Tursiops*, we used MEGA to calculate the mean gross (d_{xy}) and net (d_A) sequence divergence among the four

phylogenetic groups of *Tursiops*, as represented within the reference data set. We used the same model of substitution than noted above and the standard errors (SE) were calculated with 5,000 bootstrap replicates.

mtDNA Diversity and Population Structure

The analyses below were conducted separately for the "Pacific" *T. aduncus* and *T. truncatus*. The levels of mtDNA diversity and population structure were assessed within and between the regions for which haplotype frequencies were available. For "Pacific" *T. aduncus*, these were: China/Taiwan, east Australia, the Solomon Islands, and New Caledonia; and for *T. truncatus*: China/Taiwan, Hawaii, Kiribati, New Zealand, Palmyra Atoll, the Solomon Islands, and New Caledonia. First, we estimated standard indices of genetic variation, *i.e.*, nucleotide diversity, π, and haplotype diversity, h (Nei 1987). We then tested for departure from mutation-drift equilibrium within each group with Tajima's D test (Tajima 1989) and Fu's F_s test (Fu 1997). Significance of both statistics was inferred by randomization (10,000 steps), using a coalescent simulation algorithm (Hudson 1990).

Global genetic differentiation was estimated using an analysis of molecular variance (AMOVA) with conventional F_{ST} (based on haplotype frequencies) and its nucleotide equivalent, Φ_{ST} (using the Tamura-Nei model), which incorporates information on the genetic distance between haplotypes. Pairwise F_{ST} and Φ_{ST} were also estimated among regions. The significance of these differences was tested with a permutation procedure (10,000 steps, with significance set at $\alpha = 0.05$). Global and pairwise comparisons were also carried out using exact tests of sample differentiation (1,000,000 Markov chain steps; 1,000 000 dememorization steps, with significance set at $\alpha = 0.05$; Raymond and Rousset 1995). The AMOVA analyses were conducted using the software ARLEQUIN v.3.5 (Excoffier and Lischer 2010). Given the interest in describing units to conserve and the known conservative bias of the Bonferroni adjustment (Nakagawa 2004, Narum 2006), we reported unadjusted pairwise P-values as well as level of significance after applying a sequential Bonferroni correction for multiple comparisons (Holm 1979, Rice 1989).

Results

Tursiops *Sequences from Melanesia*

High quality sequences of the mtDNA control region were obtained from all of the biopsy samples from Melanesian *Tursiops* ($n = 97$). Unfortunately, little or no skin could be retrieved for most of the scrub pad samples of the Solomon Islands captive *T. aduncus*-like form and, therefore, we were able to amplify mtDNA from only 10 of the 33 original scrubby samples. Overall, we obtained mtDNA control region sequences of a homologous fragment of 640 bp from 107 Melanesian *Tursiops* representing a total of 27 unique haplotypes. Among these, 82 sequences were classified as *T. aduncus*-like, representing 9 unique haplotypes, and 25 were classified as *T. truncatus*-like, representing 18 unique haplotypes. Sequences representing each of the Melanesian haplotypes have been submitted to GenBank (KF555565 to KF555591; see Table 2 for details). The new sequences from Melanesia were then reduced to a fragment of 364 bp in order to match the longest fragment available for all sequences of the reference data set (see next paragraph). Doing so, we lost one haplotype among

T. aduncus-like form from the Solomon Islands and two haplotypes among *T. truncatus*-like form from New Caledonia, giving a total number of 24 unique haplotypes for *Tursiops* in Melanesia based on a fragment of 364 bp (Table 2).

Reference Data Set and Reduction to Homologous Fragment

After truncating the 152 sequences of the reference data set to a 364 bp consensus fragment, a comparison revealed 20 matching haplotypes for a total of 132 unique haplotypes defined by 96 polymorphic sites of which 81 were transitions, 16 transversions, and four indels. Among these haplotypes: 27 were from "Pacific" *T. aduncus*, six were from "African" *T. aduncus*, 84 were from *T. truncatus*, and 15 were from the putative *T. australis* sp. nov. There were no matching haplotypes among the four phylogenetic units (Table 1).

Phylogenetic Identification of Melanesian Tursiops *spp.*

Bayesian and maximum likelihood tree reconstructions failed to resolved sister-taxa relationships among the different forms of *Tursiops*. However, they both resolved distinct monophyletic clades for the "Pacific" *T. aduncus*, the "African" *T. aduncus*, and the South Australia bottlenose dolphin. There was strong support for each of these clades with posterior probabilities above 0.95 for BA and bootstrap values of 96, 89, and 93, respectively, for ML (Fig. 2). On the other hand, there was no support for a monophyletic clade of *T. truncatus*, perhaps because of the high diversity and low phylogenetic signal of the short consensus sequences. Fixed nucleotide differences were found between each of the four putative phylogenetic groups as represented in the reference data set, ranging from 1 to 10 differences in pairwise comparisons (Table 3).

None of the haplotypes from *T. truncatus*-like in Melanesia were included within one of the three supported clades. Instead, they were found to be closely related to haplotypes of *T. truncatus* from other parts of the Pacific Ocean, as shown by calculations of mean gross and net divergences between groups, as well as the number of fixed nucleotide differences (Table 3). Furthermore, haplotype Ttr-NCal05 was an exact match with haplotypes from *T. truncatus* in China, Hawaii, Kiribati, and Palmyra Atoll, while haplotype Ttr-NCal10 matched with haplotypes from *T. truncatus* in Hawaii, Kiribati, and New Zealand.

All haplotypes of *T. aduncus*-like form from Melanesia fell within the clade of the "Pacific" *T. aduncus* (Fig. 2). One haplotype identified in New Caledonia (Tad-NCal01) was an exact match to most the common haplotype of *T. aduncus* from southeast Australia (GenBank accession AF287952, Wiszniewski *et al.* 2010). The mean gross and net divergences and the number of fixed nucleotide differences among the four *Tursiops* groups further indicated that the Melanesian *T. aduncus*-like are closely related to the "Pacific" *T. aduncus* (Table 3).

Regional Diversity and Differentiation of T. truncatus *in the Pacific Ocean*

Levels of mtDNA diversity in regional populations of *T. truncatus*-like form of the Pacific Ocean were found to be fairly similar among regions (Table 4). Overall, the indices of haplotype and nucleotide diversity were high, including within the two Melanesian regions. The lowest level of nucleotide diversity was found in Kiribati.

Table 2. Number and origin of the *Tursiops* sequences and haplotypes from samples collected in Melanesia. GenBank accession numbers refer to the haplotypes identified from 640 bp.

Putative form	Location of origin	No. sequences	No. haplotypes (640 bp)	No. haplotypes reduced (364 bp)	GenBank#
T. aduncus-like	Solomon Islands	12	7	6	KF555565-KF555571
	New Caledonia	70	2	2	KF555572-KF555573
T. truncatus-like	Solomon Islands	7	6	6	KF555574-KF555579
	New Caledonia	18	12	10	KF555580-KF555591

In Melanesia, *T. truncatus* presents a higher level of mtDNA diversity than "Pacific" *T. aduncus* in both New Caledonia and the Solomon Islands (Table 4). The difference is particularly striking in New Caledonia. We note that in the Chinese region, both species were found to share similar levels of haplotype and nucleotide diversity. No significant deviation from mutation-drift equilibrium was detected (Table 4), except for a negative value of Fu's F_s in China/Taiwan ($P = 0.049$).

Overall, a highly significant level of population differentiation was detected among *T. truncatus* haplotype frequencies in the Pacific Ocean (global $F_{ST} = 0.106$, $P < 0.0001$; $\Phi_{ST} = 0.111$, $P < 0.0001$; exact test result, $P < 0.0001$). Pairwise comparisons using exact tests of sample differentiation indicated that all regions were significantly different from one another, including after sequential Bonferroni correction ($P < 0.05$). This pattern of regional differentiation was confirmed by F_{ST} and $\Phi_{ST,}$ although some pairwise comparisons were not significant with these measures (Table 5). We note in particular that no significant differentiation was detected between the *T. truncatus* from Solomon Islands and populations from China/Taiwan, New Caledonia, New Zealand, and Palmyra Atoll when using Φ_{ST}. Applying the sequential Bonferroni correction did not affect the results substantially, only confirming nonsignificant differentiation between the Solomon Islands *T. truncatus*-like form and other regions based on F_{ST}. This absence of differentiation could be due to the small sample size in the Solomon Islands ($n = 7$). Furthermore, no significant differentiation was found between Palmyra Atoll and Kiribati.

Regional Diversity and Differentiation of the "Pacific" T. aduncus

Contrary to *T. truncatus*, large differences in mtDNA diversity were found among the regional populations of the "Pacific" *T. aduncus* (Table 4). The highest haplotype and nucleotide diversities were found in China/Taiwan, followed by the Solomon Islands. However, while haplotype diversity was fairly similar between these two, nucleotide diversity was found to be substantially higher in China/Taiwan. The lowest haplotype diversity was found in New Caledonia, but haplotype diversity in east Australia was only slightly higher. On the other hand, the level of nucleotide diversity in New Caledonia was extremely low in comparison to the other regions, with only two haplotypes with one base pair difference among 70 individual samples. None of the Tajima's and Fu's tests showed significant deviation from neutral expectation.

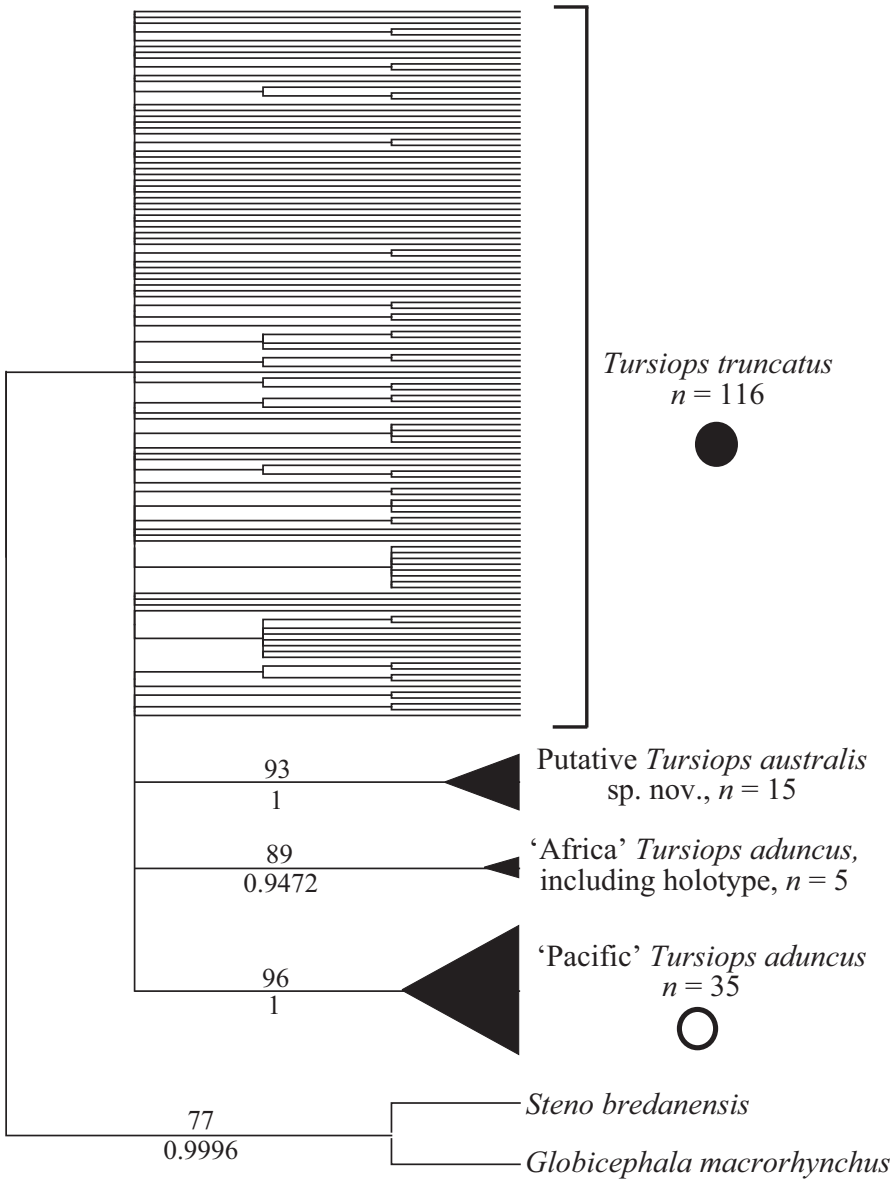

Figure 2. Maximum-likelihood phylogenetic reconstruction (70% bootstrap consensus tree) of mtDNA control region haplotypes of *Tursiops* sp. Numbers above branches indicate bootstrap values (of 1,000 simulations) obtained from maximum likelihood analyses. High posterior probability support values (>0.95) from Bayesian reconstruction are shown below branches. Placements of the Melanesian *T. aduncus*-like form and *T. truncatus*-like form haplotypes are indicated by a white or a black circle, respectively.

Table 3. Mean gross divergence (below diagonal), net divergence (above diagonal) and number of fixed nucleotide differences (showing in brackets below the diagonal) of mtDNA control region haplotypes (364 bp) between phylogenetic units of *Tursiops* sp. and the two *Tursiops* forms in Melanesia. Underlined values along the diagonal represent the within-group mean diversity. Values in italics indicate the lowest mean distances for Melanesian *T. aduncus*-like and *T. truncatus*-like among the four phylogenetic units of *Tursiops*.

	"Pacific" *T. aduncus*	"Africa" *T. aduncus*	*T. truncatus*	*T. australis* nov. sp.	Melanesian *T. aduncus*-like	Melanesian *T. truncatus*-like
"Pacific" *T. aduncus*	0.016	0.043	0.044	0.060	*0.002*	0.045
"African" *T. aduncus*	0.054 (5)	0.006	0.029	0.036	0.048	0.031
T. truncatus	0.067 (2)	0.046 (1)	0.028	0.031	0.048	*0.001*
putative *T. australis* nov. sp.	0.073 (10)	0.044 (9)	0.050 (3)	0.011	0.063	0.036
Melanesian *T. aduncus*-like	*0.015* (0)	0.055 (11)	0.067 (4)	0.073 (14)	0.009	0.049
Melanesian *T. truncatus*-like	0.065 (5)	0.046 (3)	*0.026* (0)	0.053 (5)	0.066 (6)	0.023

Table 4. Indices of mtDNA diversity and neutrality tests in *T. aduncus* and *T. truncatus* within different regions of the Pacific Ocean. h is the haplotype diversity and π is the nucleotide diversity.

	No. samples	h	π (%)	Tajima's D P-value	Fu's F_s P-value
T. truncatus					
China/Taiwan	33	0.913 ± 0.034	1.663 ± 0.904	0.277	0.049
Hawaii	118	0.866 ± 0.016	1.978 ± 1.034	0.653	0.683
Kiribati	23	0.830 ± 0.048	1.284 ± 0.729	0.340	0.692
New Zealand	206	0.907 ± 0.007	2.334 ± 1.199	0.905	0.848
Palmyra Atoll	11	0.909 ± 0.066	2.059 ± 1.176	0.528	0.614
Solomon Islands	7	0.952 ± 0.095	2.157 ± 1.311	0.207	0.342
New Caledonia	18	0.908 ± 0.044	1.970 ± 1.084	0.792	0.491
"Pacific" *T. aduncus*					
China/Taiwan	31	0.920 ± 0.028	1.580 ± 0.865	0.921	0.085
East Australia	185	0.580 ± 0.030	0.512 ± 0.327	0.710	0.781
Solomon Islands	12	0.873 ± 0.089	0.973 ± 0.605	0.396	0.197
New Caledonia	70	0.503 ± 0.014	0.140 ± 0.133	0.975	0.808

The AMOVA shows highly significant global differentiation in mtDNA haplotype frequencies in the "Pacific" *T. aduncus* (global $F_{ST} = 0.270$, $P < 0.0001$; $\Phi_{ST} = 0.355$, $P < 0.0001$; exact test result, $P < 0.0001$). All pairwise comparisons under F_{ST}, Φ_{ST}, and exact test ($P < 0.01$) were found to be significant, even after applying sequential Bonferroni correction (Table 5).

Table 5. Genetic differentiation in *Tursiops truncatus* (a) and *Tursiops aduncus* (b) from different regions of the Pacific, based on mitochondrial DNA control region sequence data and pairwise *F*-statistics. F_{ST} with *P*-values are below the diagonal; Φ_{ST} with *P*-values are above the diagonal. Level of significance after sequential Bonferroni correction is shown as follows: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns: $P > 0.05$.

(a)

	China/Taiwan	Hawaii	Kiribati	New Caledonia	New Zealand	Palmyra Atoll	Solomon Islands
China/Taiwan	–	0.086***	0.352***	0.124**	0.101***	0.329**	0.037 ns
		$P = 0.0000$	*$P = 0.0000$*	$P = 0.0003$	$P = 0.0000$	*$P = 0.0000$*	$P = 0.1654$
Hawaii	0.103***	–	0.281***	0.115***	0.058***	0.236**	0.046*
	$P = 0.0000$		*$P = 0.0000$*	$P = 0.0004$	$P = 0.0000$	$P = 0.0000$	$P = 0.1344$
Kiribati	0.120***	0.142*	–	0.308***	0.182***	0.075 ns	0.306*
	$P = 0.0000$	$P = 0.0000$		$P = 0.0000$	$P = 0.0000$	*$P = 0.0540$*	$P = 0.0006$
New Caledonia	0.088**	0.110***	0.110**	–	0.086***	0.213*	0.062 ns
	$P = 0.0002$	$P = 0.0000$	$P = 0.0001$		$P = 0.0021$	*$P = 0.0005$*	$P = 0.1333$
New Zealand	0.091***	0.111***	0.123***	0.087***	–	0.140**	0.045 ns
	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$		$P = 0.0022$	$P = 0.1210$
Palmyra Atoll	0.081*	0.111*	0.050 ns	0.077*	0.092**	–	0.204 ns
	$P = 0.0022$	$P = 0.0017$	$P = 0.0770$	*$P = 0.0053$*	*$P = 0.0003$*		*$P = 0.0055$*
Solomon Islands	0.070 ns	0.100 ns	0.118 ns	0.065 ns	0.075 ns	0.071 ns	–
	$P = 0.0201$	$P = 0.0111$	$P = 0.0103$	*$P = 0.0385$*	$P = 0.0164$	$P = 0.0442$	

(b)

	China/Taiwan	New Caledonia	Solomon Islands	Australia
China/Taiwan	–	0.451***	0.220**	0.369***
		$P = 0.0000$	*$P = 0.0012$*	$P = 0.0000$
New Caledonia	0.317***	–	0.702***	0.267***
	$P = 0.0000$		*$P = 0.0000$*	$P = 0.0000$
Solomon Islands	0.101***	0.372***	–	0.579***
	$P = 0.0004$	$P = 0.0000$		$P = 0.0000$
Australia	0.288***	0.252***	0.325***	
	$P = 0.0000$	$P = 0.0000$	$P = 0.0000$	

Discussion

Taxonomic Status of Melanesian Tursiops

To clarify the taxonomic status of the two forms of bottlenose dolphins found in Melanesia, we reconstructed one of the most comprehensive data sets of mtDNA control region sequences available to date from the genus *Tursiops*. Phylogenetic reconstructions and measures of evolutionary distances clearly support the distinctiveness of "Pacific" *T. aduncus*, "African" *T. aduncus, T. truncatus*, and putative *T. australis* sp. nov. We note, however, the absence of support for a monophyletic clade of *T. truncatus*, which is most likely explained by a high level of mtDNA diversity in *T. truncatus* and low phylogenetic signal of the short consensus sequence of the mtDNA control region. Attempts to resolve the *T. truncatus* clade would benefit from sequences of the complete mitochondrial genome (*e.g.*, Morin *et al.* 2010) and inclusion of additional nuclear markers. Similarly, it would help resolve the evolutionary relationships within *Tursiops*, which, not surprisingly, we were not able to achieve with this analysis. However, as pointed out earlier, the purpose of our study was to provide clear evidences regarding the phylogenetic placement of the two forms of *Tursiops* found in Melanesia in comparison to the four groups represented in the reference data set.

The inclusion of our new *Tursiops* sequences from New Caledonia and the Solomon Islands confirms the assumptions based on appearance and habitat. The sequences from dolphins classified as *T. aduncus*-like fall under the same phylogenetic unit as the "Pacific" *T. aduncus* while the *T. truncatus*-like are confirmed to belong to the species *T. truncatus*. Based on available information, *T. truncatus* is found in sympatry or parapatry throughout the range of *T. aduncus* (Wang and Yang 2009, Wells and Scott 2009). Interestingly, coastal or inshore subtropical populations of *T. truncatus* are frequently observed outside the Indo-(west) Pacific range, in particular in the Atlantic, where there is no *T. aduncus* (*e.g.*, Parsons *et al.* 2006, Baird *et al.* 2009). On the other hand, wherever *T. aduncus* is distributed, there are no reports of coastal populations of *T. truncatus*. This pattern is confirmed in Melanesia and supports the hypothesis that the absence of *T. aduncus* could leave *T. truncatus* with a chance to fill an empty ecological niche in coastal environments (Tezanos-Pinto *et al.* 2009).

Preliminary Findings on the Population Structure and Genetic Diversity of T. truncatus *in Melanesia*

Here, we show that *T. truncatus* from New Caledonia have limited mtDNA gene flow with China/Taiwan to the north, New Zealand to the south, and east-Kiribati/Palmyra Atoll/Hawaii to the east. Limits to gene flow between these regions and the Solomon Islands were less obvious, but these results are probably due to a small sample size for the latter. Future studies might reveal levels of population structure similar to that seen for New Caledonia. Overall, this species is widely distributed across Oceania as well as in Australasia (Wells and Scott 2009) and many regions are not represented in this study. It is therefore not possible to identify the exact boundaries of the population to which the Solomon Islands and New Caledonia *T. truncatus* belong. They could be part of a large oceanic and nomadic population, restricted to Melanesia, or even to a smaller area, as suggested by exact tests of population differentiation between New Caledonia and the Solomon Islands. However, because of the small sample size available for *T. truncatus* in the Solomon Islands ($n = 7$), we are unable to draw conclusions on the level of connectivity within Melanesia.

The level of mtDNA diversity observed at both study areas is relatively high, as previously found in many populations of *T. truncatus* elsewhere (Natoli *et al.* 2004, Tezanos-Pinto *et al.* 2009). However, high mtDNA diversity does not necessarily imply that Melanesian populations form large, nomadic populations. Indeed, several relatively small and isolated populations have been described in both New Zealand and Hawaii, and all of them show a relatively high level of mtDNA diversity (Tezanos-Pinto *et al.* 2009, Martien *et al.* 2011). Although this pattern of diversity seems incompatible with the demography and isolation of such populations, it can still be explained by low levels of gene flow with a large oceanic population or with an extended network of small resident populations interconnected within a metapopulation framework (*e.g.*, Oremus *et al.* 2007).

Population Structure and Evolutionary History of T. aduncus *in Melanesia*

Similarly to *T. truncatus*, we found significant difference at the mtDNA level among "Pacific" *T. aduncus* in New Caledonia and in the Solomon Islands, as well as with the other surrounding regions represented in the data set, *i.e.*, east Australia and China/Taiwan. These findings should be confirmed by analyses of nuclear markers, but results from previous *T. truncatus* and *T. aduncus* studies suggest that this mtDNA pattern is likely to be concordant with reproductive isolation between these regions (Natoli *et al.* 2005, Sellas *et al.* 2005, Rosel *et al.* 2009, Wiszniewski *et al.* 2010, Ansmann *et al.* 2012). Finding evidence of population structure at a regional level in "Pacific" *T. aduncus* was not unexpected. Indeed, this species appears to be strictly coastal throughout its range and seems to form small resident populations that live in complex social systems, tending to favor strong philopatry (Wang and Yang 2009). These ecological factors are limiting the level of gene flow and can result in fine-scale population structure, as observed along the coast of east Australia (Wiszniewski *et al.* 2010).

In comparison to *T. truncatus*, the levels of mtDNA diversity were generally low in the "Pacific" *T. aduncus* populations, in particular at the nucleotide level. This is consistent with the demographic characteristics described from populations of this species, *i.e.*, low abundance and migration rate (Wang and Yang 2009). We note that despite a general pattern of lower nucleotide diversity than in *T. truncatus*, some substantial differences are still observed among regions in "Pacific" *T. aduncus*, with levels of diversity gradually decreasing from China/Taiwan, to the Solomon Islands, to east Australia and, finally, to New Caledonia (Table 4). This pattern is somewhat unexpected. Direct comparisons between these regions are complicated because of our general lack of knowledge on the demographic status. However, it is worthwhile to note a couple of interesting characteristics. First, when restricting the China/Taiwan data set to samples from Taiwan only, we found that the level of mtDNA diversity around this island remains high ($n = 14$, $h = 0.890 \pm 0.060$, $\pi = 1.574 \pm 0.901$). Interestingly, Taiwan is about the same size as the "Grande Terre" in New Caledonia and therefore, one could assume that the two regions shelter "Pacific" *T. aduncus* populations with fairly similar demographic characteristics. Under this assumption, it could have been expected that both regions held similar levels of diversity but instead, we found that mtDNA diversity in the latter is much lower (Table 4). The fact that the east Australia data set held lower mtDNA diversity than the sample from the Solomon Islands is also surprising (Table 4). Indeed, this data set encompass samples from nine distinct communities spread out over 1,060 km of Australian coastline (Wiszniewski *et al.* 2010), while all but two of the samples from

the Solomon Islands were collected from a small population northwest of Guadalcanal Island (Oremus *et al.* 2013*b*). Assuming that a larger overall population is represented in the east Australia data set than in the Solomon Islands, it would have been expected to find higher mtDNA diversity in the former if populations have been stable over a long period of time. It appears, therefore, that the current pattern of mtDNA diversity within *T. aduncus* populations of the west Pacific is substantially influenced by the recent evolutionary history of this taxonomic unit. Different colonization times and different demographic trajectories could explain the contrasting pattern of genetic diversity observed among these regions.

In that regard, perhaps the most striking result of our study is the extremely low level of mtDNA diversity in New Caledonia. A sampling bias is unlikely since a large number of samples were available from that region and they were collected from many different social groups ($n = 42$ groups) around the country (Fig. 1). Furthermore, a few replicate samples initially present among the "Pacific" *T. aduncus* from New Caledonia were removed from the analysis on the basis of microsatellite loci genotyping and photo-identification data (results not shown). Therefore, we hypothesize that this low level of mtDNA diversity is the result of (1) a small population size and a complete absence of maternal gene flow from neighboring populations due to the relative isolation of New Caledonia and/or (2) a recent history of population bottleneck. The two scenarios are not exclusive but the occurrence of a bottleneck seems especially likely given the extreme reduction of mtDNA diversity. The presence of only two haplotypes separated by a single base pair suggests that a single female (and no more than a few at most) could be the ancestor of all "Pacific" *T. aduncus* in New Caledonia. These ancestors could either be the survivors of a recent population crash or the founding members of a recent colonization event. "Pacific" *T. aduncus* in New Caledonia are currently separated from neighbor populations by large areas of open water. The distance is larger between New Caledonia and east Australia (about 1,200 km to the east) than between New Caledonia and the Solomon Islands (about 900 km to the north) but multiple banks and shallow plateaus emerging throughout the Coral Sea might have provided opportunities for dispersal following a stepping-stone model. Supporting a scenario of colonization *via* the Coral Sea, we found that one of the two haplotypes in New Caledonia is shared with the most common haplotype among "Pacific" *T. aduncus* on the east coast of Australia, while there were no matching haplotypes between New Caledonia and the Solomon Islands. This result suggests that New Caledonia is or was connected to east Australia rather than the Solomon Islands despite the greater geographic distance of the former. We note that the presence of *T. aduncus* was recently described in the Chesterfield Archipelago, between Australia and New Caledonia (Oremus and Garrigue 2014).

Conservation implications for "Pacific" T. aduncus

The presence of "Pacific" *T. aduncus* in New Caledonia represents the most easterly known population for this species. It is still unclear whether "Pacific" *T. aduncus* occurs further east in Vanuatu, but cetacean systematic surveys conducted in this region failed to detect the presence of the species despite substantial effort at sea (over 135 h on effort primarily in coastal waters; MO, CG, personal observations). We note that with similar effort in the Solomon Islands or New Caledonia, several groups of *T. aduncus* would have very likely been encountered. New Caledonia might thus represent the eastern limit of distribution range for "Pacific"

T. aduncus, and as such, be considered as a peripheral population. Similarly to a trend observed in many peripheral populations across species (Eckert *et al.* 2008), "Pacific" *T. aduncus* in New Caledonia shows a lower level of genetic diversity (at least at the mtDNA level) than other, less isolated populations. Because of these characteristics, peripheral populations are thought to have a more precarious conservation status with an increased vulnerability to stochastic processes and a reduced resilience (*e.g.*, Andersen *et al.* 2011). The status of "Pacific" *T. aduncus* populations in New Caledonia should, therefore, be further investigated and monitored to prevent risks of extinction through efficient conservation efforts. We note also that study of the dynamics of peripheral populations can help to elucidate the process of range expansion (Hardie and Hutchings 2010), a field that remains largely unexplored in cetaceans.

"Pacific" *T. aduncus* in the Solomon Islands faces a different problem (Oremus *et al.* 2013*b*). The recent live-capture of a substantial number of individuals around the islands of Guadalcanal and Malaita has been of particular concern (Reeves and Brownell 2009). Our results show that "Pacific" *T. aduncus* in the Solomon Islands exhibit a fair amount of mtDNA diversity in comparison to surrounding regions. However, such indices do not provide sufficient information on the contemporary status of the population. For example, higher diversity in the Solomon Islands than in New Caledonia could be the result of the populations' recent evolutionary history more than a reflection of their current demography. In addition, significant local population structure indicates that the connectivity with surrounding regions is limited and thus, that the Solomon Islands "Pacific" *T. aduncus* are isolated at a regional scale. As such, repopulation of the areas impacted by the live capture is unlikely, with implications for the resilience of "Pacific" *T. aduncus* populations in the Solomon Islands.

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