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Analysis of mitochondrial DNA clarifies the taxonomy and distribution of the Australian snubfin dolphin (*Orcaella heinsohni*) in northern Australian waters

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Abstract. Conservation management relies on being able to identify and describe species. Recent morphological and molecular analyses of the dolphin genus *Orcaella* show a species-level disjunction between eastern Australia and South-east Asia. However, because of restricted sampling, the taxonomic affinities of the geographically intermediate populations in the Northern Territory and Western Australia remained uncertain. We sequenced 403 base pairs of the mitochondrial control region from five free-ranging *Orcaella* individuals sampled from north-western Western Australia and the Northern Territory. Low net nucleotide divergence (0.11–0.67%) among the Australian *Orcaella* populations show that populations occurring in the Northern Territory and Western Australia belong to the Australian snubfin (*O. heinsohni*) rather than the Asian Irrawaddy dolphin (*O. brevirostris*). Clarifying the distribution of *Orcaella* is an important first step in the conservation and management for both species; however, an understanding of the metapopulation structure and patterns of dispersal among populations is now needed.

Additional keywords: distribution, mitochondrial DNA, northern Australia.

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Introduction

Accurate taxonomic identification of organisms is essential to making informed decisions about conservation and implementation of protective legislation (Mace 2004) and for undertaking on-ground management. Recently, the Australian snubfin dolphin (*Orcaella heinsohni*) was described as a new species and is currently the only cetacean known to be endemic to Australian waters, although the species may also occur in Papua New Guinean waters (Beasley *et al.* 2002, 2005). The species was split from the Asian Irrawaddy dolphin (*O. brevirostris*) on morphological characteristics and 17 fixed nucleotide differences in a 403 base-pair (bp) segment of the mitochondrial control region (mtDNA; Beasley *et al.* 2005). The genetic component of the study by Beasley *et al.* (2005) was based on 24 samples from South-east Asia and four samples from Australia (three from north-eastern Queensland and one from the Northern Territory). Since the publication of the study of Beasley *et al.* (2005), uncertainties have arisen about the collection location of the sample from the Northern Territory (i.e. Darwin). The Darwin sample was received by South-west Fisheries Science Centre, USA, by a secondary party and the origin of the sample has not been confirmed through museum

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records (K. M. Robertson, pers. comm., Protected Resources Division, South-west Fisheries Science Centre, National Marine Fisheries Services, National Oceanic and Atmospheric Administration).

Orcaella populations in the Northern Territory and Western Australian waters are approximately midway between the known distributions of *O. brevirostris* and *O. heinsohni*. Additionally, with changing sea levels for ~90% of the past 250 000 years, and the formation of the land-bridge between Australia and New Guinea during the Pleistocene, *Orcaella* populations on the eastern coast of Queensland would have been isolated at varying times from populations in the Northern Territory and Western Australia (Voris 2000). Given the doubt surrounding the 'Darwin' sample in the study of Beasley *et al.* (2005), and the historical separation of eastern coast Queensland from northern Australian waters, it remains unclear whether these northern Australian populations are composed of *O. brevirostris*, *O. heinsohni* or a mixture of both species.

In the present study, as part of an ongoing multi-species coastal dolphin program, we analysed mtDNA sequence data from *Orcaella* individuals from the Northern Territory and north-western Western Australia. Our results corroborate the findings of Beasley *et al.* (2005) and confirm the presence of the Australian snubfin dolphin across northern Australia.

Materials and methods

Tissue sampling

During 2008, the following two areas were targeted for collection of biopsy samples from free-ranging *Orcaella*: Cobourg Peninsula (Northern Territory) and Roebuck Bay (Western Australia) (Fig. 1, Table 1). Skin samples were collected using a biopsy system specially designed for small cetaceans (PAXARMS, Krützen *et al.* 2002).

Sampling was attempted only during calm sea conditions (Beaufort <2), with no swell. Biopsy sampling of *Orcaella* presents some challenges, in comparison with sampling other coastal dolphins. The species is wary of vessels (Dhandapani 1992; Parra and Corkeron 2001; Kreb 2004) and typically their surfacing patterns are unpredictable. Individuals tend to maintain a low profile on surfacing and inhabit turbid in-shore waters (Parra and Corkeron 2001; Parra *et al.* 2002); thus, sampling opportunities are limited. We were able to approach *Orcaella* schools to within sampling distance (2–12 m) more successfully

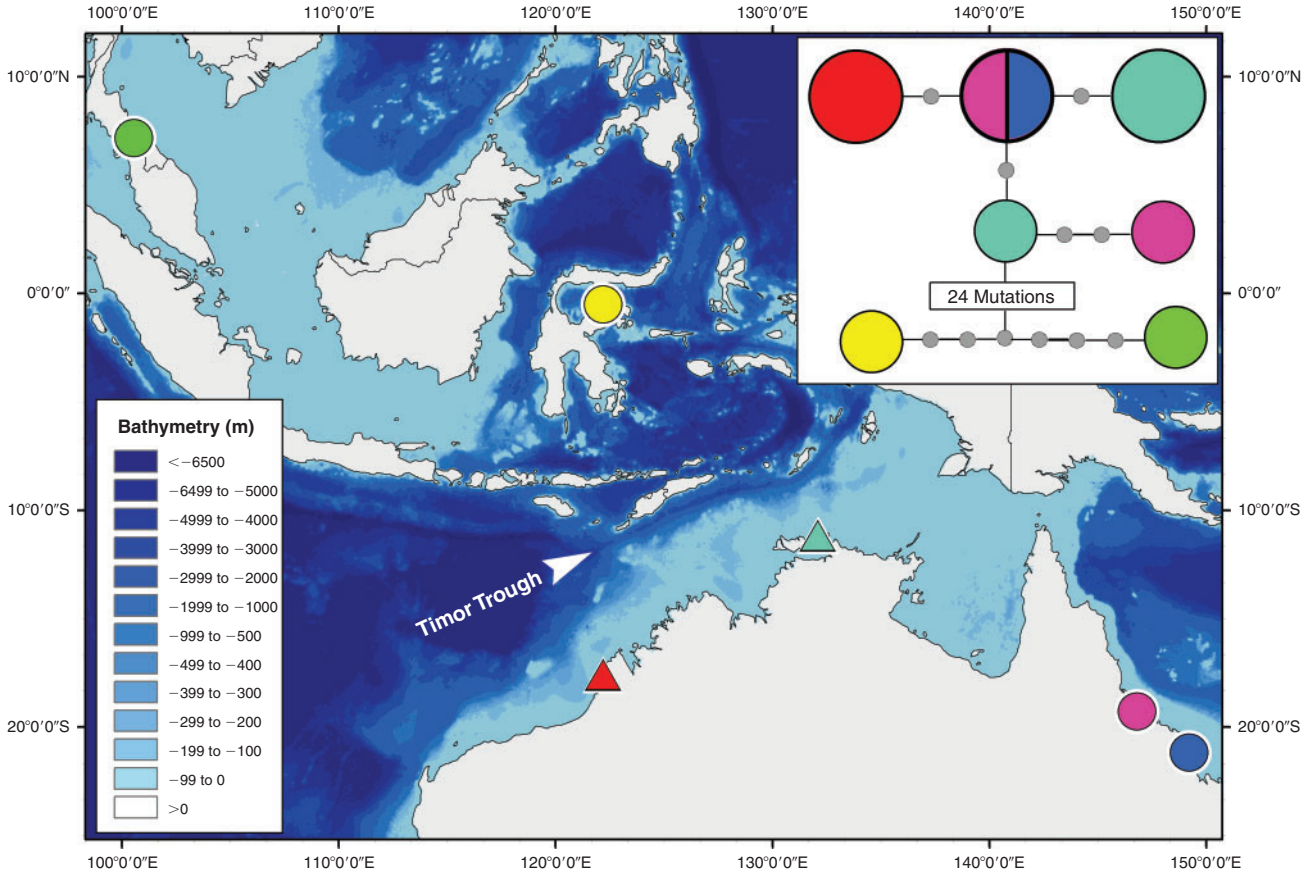

Fig. 1. *Orcaella* sample locations (coloured symbols on map) used to investigate taxonomy and distribution. Background map shows elevation, including bathymetry (darker shading = lower elevation). The white arrow indicates the position of the Timor Trough. The inset shows the haplotype network, colour-coded to match sample locations. Circle sizes in the network are proportional to the number of individuals with that haplotype. Grey circles represent single-step mutations.

when animals were socialising (i.e. dolphins in close proximity to one another with localised movements; high level of interactions – touching each other and rubbing bodies; fins and flukes breaking the water often; modified from Parra *et al.* 2006*a*). Dolphin schools are defined by relatively close cohesion; each dolphin within 100 m of each other and undertaking similar behavioural activities (Parra *et al.* 2006*a*).

It was during these socialising bouts that *Orcaella* schools maintained close cohesiveness (individuals usually <5 m apart) and allowed close approaches by our research vessel. Typically, the vessel was either stationary or moving very slowly (<2 km h^{-1}) when biopsy sampling was attempted. Sample location was recorded using a global positioning system (GPS) (±5 m). Tissue samples were stored in 20% dimethyl sulfoxide (DMSO) saturated with sodium chloride (Amos and Hoelzel 1991).

Laboratory procedures

DNA was extracted using Dneasy® Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. DNA extractions were quantified using electrophoresis. The same ~400-bp fragment of the control region used by Beasley *et al.* (2005) and Caballero *et al.* (2008) was amplified using primers H16498 (5′-CCTGAAGTAAGAACCAGATG-3′; Rosel *et al.* 1994) and L15812 (5′-CCTCCCTAAGACTCAAGG-3′).

Each amplification reaction contained 10–20 ng of DNA template, 0.125 mM dNTPs, 0.6 μM of each primer, 1.5 mM $MgCl_2$, 0.5 U Red Hot® DNA polymerase and 1 × Reaction Buffer IV (Applied Biosystems, Carlsbad, CA, USA), and sterile water to 25 μL. We used the following thermal profile for amplification: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 30 s. We also added a single 5-min 72°C extension step. Amplification products were purified using a Wizard® Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), and quantified by electrophoresis. Dye-terminating sequencing was performed on purified template using Big Dye Terminator v3.1 chemistry and protocols (Applied Biosystems). Sequencing products were analysed on an ABI 3130xl Genetic Analyzer (Applied Biosystems). Chromatograms were checked and edited using Sequence Scanner v1.0 (Applied Biosystems), and aligned and analysed using MEGA 4 (Tamura *et al.* 2007).

Analyses

We incorporated five previously published *O. heinsohni* and *O. brevirostris* sequences obtained from dolphins sampled from Queensland, Indonesia and Thailand into our dataset (Fig. 1, Table 1). We grouped samples into the following *a priori* populations for further analysis: Queensland, Northern Territory, Western Australia and South-east Asia (for the Indonesian and Thailand samples). To describe the magnitude of genetic divergence among these populations, we calculated net nucleotide divergence, Da, which is the nucleotide divergence between populations that accounts for within-population polymorphism (Nei and Miller 1990). We also calculated pairwise genetic distances among individual haplotypes. For both measures of genetic distance, we used Kimura's (1980) two-parameter model of DNA evolution that allows for variation in substitution rates with gaps (insertion–deletions) being ignored. Finally, we represented the relationship among haplotypes for all samples by constructing a haplotype network using the computer program TCS (Clement *et al.* 2000).

Results and discussion

Biopsy sampling

We obtained five tissue samples via biopsy sampling from three dolphins in Port Essington, Northern Territory, and two dolphins from Roebuck Bay, Western Australia (Fig. 1). The present study is the first published account of biopsy sampling from free-ranging Australian *Orcaella.*The small sample size reflects the difficulty in obtaining tissue samples from this shy and generally elusive dolphin. Unlike bow-riding dolphins, opportunities to approach *Orcaella* to within darting range are very limited. There were no other known tissue samples available from stranded or by-caught animals from this region.

Table 1. Details of the samples used in the study

Sample	Lat/Long	Population for analysis	GenBank #	Sequence (Variable sites with position number) 00000111111112222222233333333333 11348000233353677899901444455667 8 01367012014586536346797023719157 6
2905Qld[A]	−19.27, 146.80	Queensland	GQ922343.1	GAGGTTGAATGTAATAGTCAGTCGGTCAGCGA
2906Qld[A]	−19.27, 146.80	Queensland	GQ922344.1	.........C.....G..............
2907Qld[A]	−21.17, 149.20	Queensland	Not lodged[D]	.....
Co6COB[B]	−11.15, 132.07	Northern Territory	JN016533	......A......G..............
Co7COB[B]	−11.13, 132.11	Northern Territory	JN016534	......A......G..............
Co8COB[B]	−11.18, 132.08	Northern Territory	JN016530	.G.....
Ro1ROE[B]	−17.59, 122.17	Western Australia	JN016531	A..A.....
Ro4ROE[B]	−17.59, 122.20	Western Australia	JN016532	A..A.....
14360INDO[A]	−0.5, 117.0	SE Asia	GQ922337.1	AGA.CC.G..AGG.A.ACTGAATAACT.ATAG
EU121128.1[C]	'Thailand'	SE Asia	EU121128.1	..AA.C.GG.AGG.A.ACTGAATAACTGAT.G

[A]Beasley *et al.* (2005).
[B]The present study.
[C]Caballero *et al.* (2008).
[D]Sequence obtained from Beasley *et al.* (2005).

Table 2. Percentage pairwise nucleotide divergence for each *Orcaella* haplotype (the present study and Beasley *et al.* 2005)

Sample	Population		[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]
2905Qld	Queensland	[1]									
2906Qld	Queensland	[2]	0.8								
2907Qld	Queensland	[3]	0.0	0.8							
Co6COB	Northern Territory	[4]	0.5	1.3	0.5						
Co7COB	Northern Territory	[5]	0.5	1.3	0.5	0.0					
Co8COB	Northern Territory	[6]	0.3	0.5	0.3	0.8	0.8				
Ro1ROE	Western Australia	[7]	0.5	1.3	0.5	1.0	1.0	0.8			
Ro4ROE	Western Australia	[8]	0.5	1.3	0.5	1.0	1.0	0.8	0.0		
14360INDO	SE Asia	[9]	6.6	6.9	6.6	7.2	7.2	6.3	6.6	6.6	
EU121128.1	SE Asia	[10]	6.3	7.2	6.3	6.9	6.9	6.6	6.3	6.3	1.8

Table 3. Percentage net nucleotide divergence (Da) among *a priori* populations in the present study

Population	Queensland	Northern Territory	Western Australia
Northern Territory	0.11		
Western Australia	0.50	0.67	
South-east Asia	5.52	5.71	5.58

Haplotypes

A 403-bp section of the control region from our five samples revealed five variable sites, defining three haplotypes (Table 1). In the full analysis involving all sequences from 10 individuals, there were 32 variable sites (excluding gaps at two positions), which defined seven haplotypes.

Our study reports three novel haplotypes defined by only one or two point mutations (Table 1). Pairwise genetic distances among haplotypes are given in Table 2. Da values between South-east Asian and all Australian individuals from sampled areas were high, ranging from 5.5 to 5.7%. In contrast, Da among the Australian individuals from sampled areas was very low, ranging from 0.1% to 0.7%. Our sequences were clearly closely allied to *O. heinsohni* from Queensland. Table 3 shows Da values for our *a priori* groupings. These patterns are reflected in the haplotype network, which shows a relatively small number of mutational steps separating Australian haplotypes, compared with the differences between the Australian and South-east Asian haplotypes consisting of 24 and 25 nucleotide differences (Fig. 1).

Phylogeography

During the last 1.7 million years of the Pleistocene, it is thought that up to 28 stadial and interstadial cycles occurred, during which time the configuration of land and sea would have changed repeatedly (Shackleton and Opdyke 1973; Stringer and Gamble 1993). For northern Australia, these cycles caused repeated isolation of Queensland in-shore waters from those in the Northern Territory and Western Australia. In total, this configuration accounts for ~90% of the past 250 000 years (Voris 2000). It is therefore surprising that we observed low genetic differentiation between Queensland and Northern Territory/Western Australia (Da values from 0.11% to 0.67%) because this evidence suggests that there has been effective dispersal between these areas during periods of connectivity. In contrast, there has been continuous oceanic connectivity between Australia and South-east Asia throughout the Pleistocene. However, the narrow Timor Trough, which reaches depths of 1500–3200 m (Fig. 1) is likely to have provided a long-term and stable barrier to dispersal and gene flow for a near-shore shallow-water specialist such as *Orcaella* (Voris 2000; Parra 2006).

Conservation management

Conservation decision-making is inextricably linked to taxonomy (Mace 2004) and our results have helped resolve uncertainty about the taxonomy and distribution of *O. heinsohni* in Australian waters. *O. heinsohni* is listed as *Near Threatened* at an international level (Reeves *et al.* 2008); however, population trends at a national scale are unknown (Parra *et al.* 2006*a*; Reeves *et al.* 2008). Estimates for local areas in Queensland suggest that populations of *O. heinsohni* appear to be small and localised (Parra *et al.* 2006*a*). The low reproductive rate (i.e. maximum recorded age of 28 years: Marsh *et al.* 1989; estimated age at reproduction of 9 years and one calf every 2–3 years: Taylor *et al.* 2007), combined with the low numbers and continued threats (i.e. accidental catch in shark nets set for bather protection: Paterson 1990; Gribble *et al.* 1998) are some of the key reasons why the species is susceptible to local extinction. The Northern Territory and north-western Western Australia's maritime estate is situated in a region of high marine biodiversity and low human impact (Halpern *et al.* 2008). Despite encompassing some of Australia's remote and sparsely populated coastlines, the Northern Territory and north-western Western Australia face rapid development in some areas. As is common for remote areas, there is little baseline information with which to support marine planning and develop conservation plans in relation to coastal dolphins. Future studies on metapopulation structure and gene flow among different populations across their range, and taxonomic identification of potential populations (i.e. Papua New Guinea) are needed to establish conservation status and management actions at national and regional scales.

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