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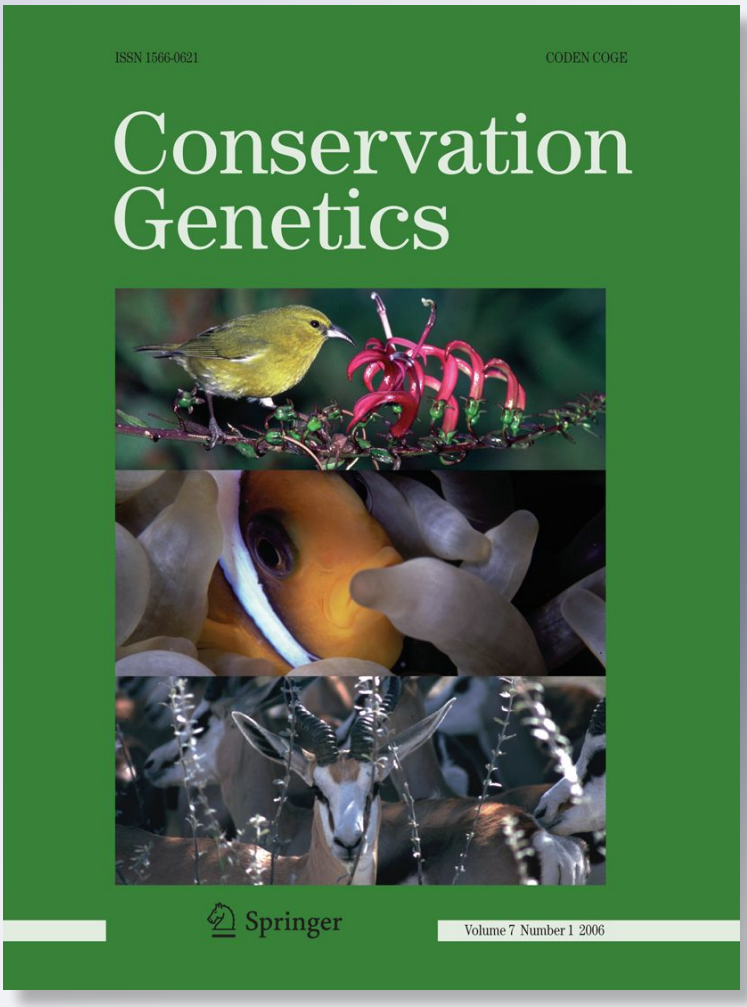

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SHORT COMMUNICATION

Multiple lines of evidence for an Australasian geographic boundary in the Indo-Pacific humpback dolphin (*Sousa chinensis*): population or species divergence?

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Abstract The taxonomic status of humpback dolphins (genus *Sousa*, sub-family Delphininae) is unresolved. While the classification of this genus ranges from a single to three nominal species, the International Union for Conservation of Nature and the International Whaling Commission only recognise a 'two-species' taxonomy (*S. teuszii* in west Africa, and *S. chinensis* in the Indo-Pacific). Under the IUCN (2008), *S. chinensis* is listed as 'near threatened', but is only considered as a 'migratory' species in Australia. Taxonomic resolution of the genus *Sousa* is needed to define particular conservation status and develop appropriate management actions. Using phylogenetic analyses of 1,082 bp of mitochondrial and 1,916 bp of nuclear DNA, we provide multiple lines of genetic evidence for the genetic distinction of *S. chinensis* in China and Indonesia from *S. chinensis* in Australia. The separation of Australian *Sousa* from *Sousa* of Southeast Asia requires a review of their current conservation status and respective management actions.

Keywords Speciation · Humpback dolphins · *Sousa* · ESU · Conservation · Phylogenetics

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Introduction

Phylogeographic and phylogenetic methodologies have long been used to address critical questions about geographic history, genetic lineages and biodiversity (Avise 2000; Bloomquist et al. 2010; Hickerson et al. 2010; Wolf et al. 2009). In the last two decades, the growing application of molecular techniques to phylogenetic reconstruction has lead to the review and identification of many cryptic taxa (e.g., dolphins *Orcaella heinsohni* and *Sotalia* sp.), Beasley et al. 2005, Caballero et al. 2007; lizards (*Bachia squamata* and *Bachia gymnophthalmidae*), Rodrigues et al. 2007; elephants (*Loxodonta africana* and *Loxodonta cyclotis*), Roca et al. 2001). Further, methodological advances in the field of phylogeography have contributed to a greater understanding of how demographic, environmental, and genetic factors interact to drive population genetic variation and structure (Hickerson et al. 2010; Knowles 2009). Despite such methodological advances, defining the relationship between population and species histories, as well as the conditions for and mechanisms of molecular speciation, remain a challenge (Wolf et al. 2009).

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Humpback dolphins (genus *Sousa*) belong to the subfamily Delphininae (LeDuc et al. 1999) and has a widespread coastal distribution, which ranges from East Africa to Australia (Parra and Ross 2009). The taxonomy of this genus is not well established and current views range from recognition of only a single species, *S. chinensis*, to three nominal species: *S. chinensis* (Pacific Ocean), *S. plumbea* (Indian Ocean), and *S. teuszii* (Atlantic Ocean) (Parra and Ross 2009). Frère et al. (2008) showed that South African, Chinese and Australian populations of Indo-pacific humpback dolphins (*S. chinensis*) each formed a robust monophyletic clade. However, *S. chinensis* was paraphyletic, with *S. chinensis* from China and *S. plumbea* from South Africa more closely related to the Atlantic humpback dolphin *S. teuszii* than to Australian *S. chinensis*. Frère et al.'s (2008) study was only based on a small 383 bp fragment of mitochondrial DNA (mtDNA) and did not contain any samples between Hong Kong and northern Australia.

Presently, the International Union for Conservation of Nature (IUCN) and the International Whaling Commission (IWC) only recognise a 'two-species' taxonomy (*S. teuszii* in West Africa, and *S. chinensis* in the Indo-Pacific). The genus *Sousa* is listed in Appendix I of CITES as 'most endangered' and is listed under the IUCN (2008) as 'near threatened' for *S. chinensis* and as 'vulnerable' for *S. teuszii*. In contrast, the Australian Environment Protection and Biodiversity Conservation Act (1999) lists *S. chinensis* as a non-threatened 'migratory' species. The term 'non-threatened migratory species' is a default status given by the Australian Environment Protection and Biodiversity Conservation department to any cetacean species. This status remains unchanged until sufficient proofs have been provided to warrant a change of status classification.

Taxonomic resolution of the genus *Sousa* is needed to determine particular conservation status and develop appropriate management actions. In this study, we extended Frère et al.'s (2008) research by conducting a phylogenetics analysis of the genus *Sousa* across Australasia using a total of two mtDNA regions (1,081 bp) and three nuclear intron regions (1,916 bp). Moreover, we included a new *S. chinensis* sample from Indonesia, filling the gap between China and Australia. Here, we test the validity of recognising the Australian *Sousa* as an Evolutionarily Significant Unit (ESU; Moritz 1994), and as a distinct species, which would require a review of their current conservation status and management plan.

Materials and methods

Sampling

A total of 33 DNA samples were available for this study. Apart from three biopsy samples collected from free-ranging *Sousa* in Darwin Harbour (Northern Territory, Australia), the remaining 30 samples were obtained from dead stranded animals, or victims of shark nets in West Africa ($n = 1$, Mauretania), China ($n = 19$, Hong Kong), Indonesia ($n = 1$, Berau Archipelago), and Australia ($n = 10$, Moreton Bay (2), Great Sandy Straight (2), Townsville (2), Western Australia (2), and Darwin (1)). All samples except the five samples collected from Indonesia and Darwin were used in Frere et al.'s (2008) study. Tissue samples were preserved in either a salt saturated 20% DMSO solution or 95% ethanol. Genomic DNA was extracted using standard methods (Davis et al. 1986). Location and collection details for all samples used in this study are listed in online resource 1. Sample acquisition in Southeast Asia was severely hampered in this genus for several reasons. Firstly, apart from China and Australia, research groups found in Malaysia and Indonesia do not currently collect DNA material. Secondly, dart biopsying to collect tissue samples is prohibited in most Southeast Asian countries, meaning that any genetic studies conducted in these countries have to rely entirely on stranded samples. Thirdly, the evasive nature of Indo-Pacific dolphins makes them a very difficult species to sample at best of times.

DNA markers

We amplified a total of 1,082 bp of maternally inherited mitochondrial DNA, comprised of a 494 bp fragment of the control region using the primers dlp1.5 (Baker et al. 1993) and dlp5 (Krützen et al. 2004) and a 585 bp fragment of the mitochondrial DNA cytochrome b gene region using primers L14724 (Amaral et al. 2007) and Reverse Cytochrome B2 (5′-GGAATAGCAGGTGAACGGC-3). A partial intron from each of the three nuclear genes were amplified: buglycan (BNG, 686 bp) and cytotoxic T-lymphocyte associated serine esterase 3 (CTLA, 305 bp) from (Lyons et al. 1997); and muscle actin (ACT, 925 bp) from (Palumbi and Baker 1994). Each of the three nuclear genes are located on a different chromosome in humans (Lyons et al. 1997) and are unlinked in cetaceans (Dalebout et al. 2008). Unfortunately, we were unable to successfully amplify the Mauretanian sample for any the nuclear markers, likely due to sample degradation. PCR products were purified using ExoSAP-IT (Affymetrix), sequenced with the BigDyeTM Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and separated on an ABI 3730 DNA Sequencer (Applied Biosystems). The sequences were visualised and edited using Geneious Pro version 5.0.

Genetic data analysis

Phylogenetic relationships were reconstructed using Bayesian and maximum likelihood methods. Appropriate

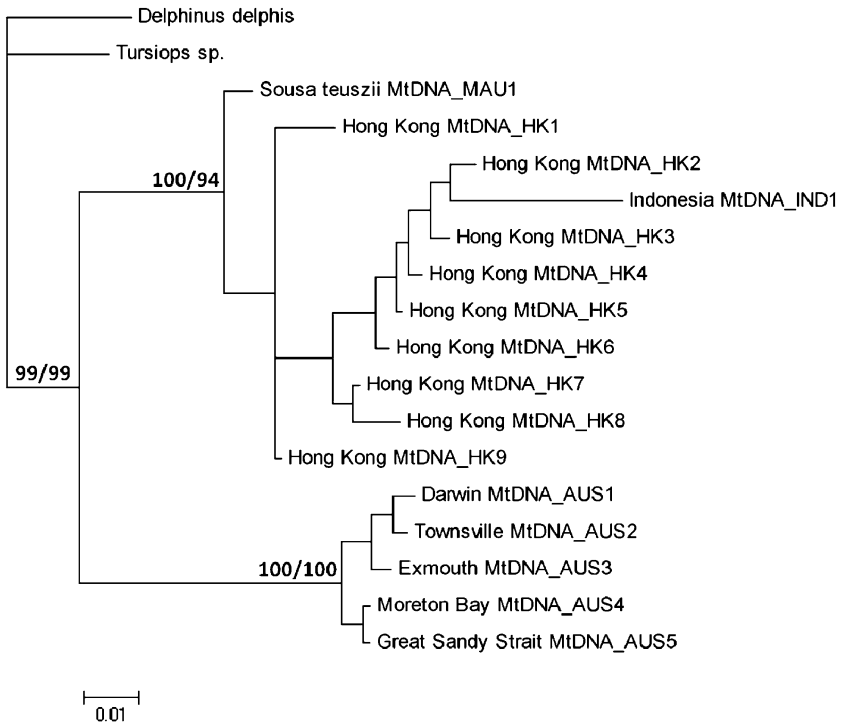

Fig. 1 Bayesian reconstruction of relationships among *Sousa* spp. based on the combined (1,081 bp) mtDNA control (495 bp-evolutionary model HKY + G) and cytochrome b region (586 bp-evolutionary model TrN + G) from four regions: Australia (Queensland, Northern Territory, and Western Australia), China (Hong Kong), Indonesia (Berau Archipelago), and West Africa (Mauretania). Haplotypes without a species designation are putative *S. chinensis*. Maximum likelihood and Bayesian methods converged on the same tree. Bayesian posterior probability scores are shown first followed by ML bootstrap scores. Terminal nodes are labelled with haplotype codes as in online resource 1 and 2. Sequences from *Delphinus delphis* (HQ699820–HQ699837) and *Tursiops* sp. (EU557092.1) were used as outgroups

models for patterns of DNA substitution for each gene regions were selected using jModelTest 1.1 (Posada 2008) using the Akaike Information Criterion corrected for small sample size (AICc) as recommended in Posada and Buckley (Posada and Buckley 2004). Two combined datasets were produced by concatenating data for the mitochondrial or nuclear sequences. The appropriate evolutionary model was used for each gene region in analyses detailed in Figure legends 1 and 2.

Bayesian analyses were performed using the program, MrBayes Vers. 3.1.2 (Huelsenbeck and Ronquist 2001) allowing for variable rates across gene regions. The Markov chain Monte Carlo search was run with four chains for 1,000,000 generations, with trees being sampled every 100 generations. The first 2,500 trees were discarded as burnin. Bayesian runs were replicated to ensure convergence of results. Clades with posterior probabilities of >0.95 were considered robust (Rannala and Yang 1996). Two indels were coded as binary characters for inclusion in Bayesian phylogenetic analyses. Maximum likelihood analyses were generated using the software GARLI (Version 0.96r587, 2009) and, for each combined dataset, ten independent searches were conducted using the recommended default options for all settings (Zwickl 2006). The ten tree topologies were compared by examining log likelihood values across searches and by computing distances between trees using the symmetric difference metric (Penny and Hendy 1985) implemented in PAUP 4.0b5 (Swofford 2003). Support for tree nodes was assessed using 100 bootstrap replicates.

Results and discussion

A total of 15 mtDNA haplotypes were identified among the 33 '*chinensis*' *s*amples (nine from Hong Kong, one from Indonesia and five from Australia, online resource 2). Each region showed a single nuclear allele; for the ACT and CTL gene regions, dolphins from Hong Kong and Indonesia shared an allele that differed from the Australian allele at one (ACT) or two (CTL) nucleotides (online resource 3). The BNG gene region showed unique alleles in each location; two SNPs (single nucleotide polymorphism) were shared by the Australian and Hong Kong alleles and a further two SNPs and a 4 bp deletion were found only in the Australian allele. Overall, we found that, at the nuclear

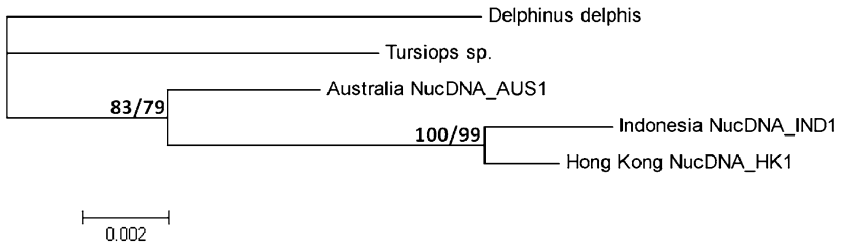

Fig. 2 Bayesian reconstruction of relationships among *Sousa* spp. based on the combined (1,916 bp) nucleotide sequences of three nuclear introns [BNG (686 bp-evolutionary model TPM2uf), CTLA (305 bp-evolutionary model HKY) and ACT (925 bp-evolutionary model TPM3uf)] from three regions: Australia (Queensland, Northern Territories, and Western Australia), China (Hong Kong), and Indonesia (Berau Archipelago). Alleles without a species designation are putative *S. chinensis*. Maximum likelihood and bayesian methods converged on the same tree. Bayesian posterior probability scores are shown first followed by ML bootstrap scores. Terminal nodes are labelled with allele codes as in online resource 1 and 3. Sequences from *Delphinus delphis* (HQ699806, HQ699811, and HQ699816) and *Tursiops* sp. HQ699805, HQ699819, and HQ699815) were used as outgroups

introns sequenced in this study, Australia shared no alleles with either China or Indonesia, whereas China and Indonesia shared the ACT and CTL alleles and showed the least variation at the BNG locus. Sequences representing all haplotypes and alleles have been deposited in GenBank (Accession numbers HQ699805–HQ699853).

The mitochondrial (1,082 bp) and nuclear (1,916 bp) DNA phylogenies inferred from Bayesian analyses are presented with posterior probabilities as well as maximum likelihood bootstrap scores (Figs. 1, 2). The two multigene phylogenetic analyses confirmed the genetic distinctiveness of Australian humpback dolphins. Both mitochondrial and nuclear DNA regions indicated that *Sousa* from Indonesia was more closely related to those from China than to *Sousa* from Australia (Bayesian posterior probability (BPP) 100% and maximum likelihood bootstrap scores (BS) 99%, Fig. 2). Further, in the mitochondrial phylogeny, the *S. chinensis* from China and Indonesia formed a robust clade with *Sousa teuszzi* (BPP 100% and BS 94%, Fig. 1) to the exclusion of *S. chinensis* from Australia. These results provide unequivocal evidence that *S. chinensis* from Southeast Asia are more closely related to *S. teuszzi* than they are to *S. chinensis* from Australia. These data clearly indicate that gene flow is strongly limited between Southeast Asia and Australia and that *Sousa* from these regions represent independent evolutionary units.

In contrast to the strong patterns of genetic divergence found between animals from Southeast Asia and Australia, Jefferson and Van Waerebeek (2004) found little cranial osteological variation between animals from South east Asia, China, and Australia. Animals from Australia, however, showed on average longer skulls and fewer upper teeth than those in Southeast Asia (Jefferson and Van Waerebeek 2004). Differences between animals from China and Australia are also supported by other morphological differences such as skin coloration and dorsal hump (Parra and Ross 2009). For instance, the Australian humpback dolphins are pale grey with white spots around the ventral surface while the Chinese humpback dolphins are pink with darker grey spots and patches and gradually lighten with age to an almost pure white (Parra and Ross 2009). The small variation in cranial morphometric traits found between animals from Southeast Asia and Australia may reflect their evolution in similar environments. It is also possible that the time since isolation has not been sufficient to generate cranial morphological differences. As samples become available, future morphological studies should focus on measuring ecologically important traits that are likely to evolve more rapidly than slower evolving cranial osteological traits (Thompson 2009).

This study provided multiple lines of evidence that *S. chinensis* in China and South East Asia are genetically divergent to *S. chinensis* in Australia. Indeed, the phylogenetic species concept, in which species are usually recognised when lineages form robust monophyletic clades to exclusion of lineages representing other species (e.g., Milinkovitch et al. 2002) and when gene flow has stopped (Davis and Nixon 1992), suggests these may be recognised as different species. Although defining population versus species divergence may be seen as trivial, since intraspecific evolutionary change will more often than not gradually lead to speciation (Schluter 2009), incorrect classifications could lead to erroneous inferences with respect to evolutionary history and result in mismanagement (Moritz 2002).

The proper taxonomic identification of organisms is essential towards making informed decisions about conservation and implementation of protective legislation (Mace 2004). The recognition of the Australian *Sousa* as an Evolutionarily Significant Unit (ESU; Moritz 1994), and potentially as a distinct species in its own right, has important implications for their conservation and management. As demonstrated by our analysis, the genetic distinctiveness of *Sousa* from Southeast Asia and Australia indicates that, in order to conserve the genetic diversity of the genus *Sousa*, both national and international

conservation efforts are particularly significant and urgent. The broad-scale populations of *S. chinensis* in China and South East Asia and *S. chinensis* in Australia need to be managed separately and reassessments of their respective conservation status are required. Furthermore, an integrated approach to the conservation of *Sousa* in Australia and South East Asia will require the development of specific wildlife management plans tailored to the conservation issues and priorities of these regions.

Currently, Australia is recognised as a stronghold for humpback dolphins due to its sparsely developed northern coastline, in contrast to many other nations in the Indo-Pacific (Perrin et al. 2005). However, estimates of population size in local areas along the Queensland coast indicate that populations of this species are notably small making them particularly vulnerable to human-induced disturbances on coastal ecosystems (Cagnazzi et al. 2009; Parra et al. 2006a; Parra et al. 2004). Our initial genetic investigations coupled with ecological studies indicate that the conservation of Australian *Sousa* will depend on several key steps. First, the maintenance of high-quality habitat in areas that are already under some protection (e.g. Parra et al. 2006b). Second, there needs to be increased research efforts put towards the identification of critical habitats through population monitoring of unsurveyed areas. Third, the development of species distribution models at both a state and national scale. Fourth, the designation of protected areas supported by underlying population genetic structure studies, and last the inclusion of these habitats in the rezoning initiatives of protected areas throughout their range.

The difficulties of sampling these endangered animals and limited sampling effort in some regions means it is a challenge to determine the exact geographic location of the break between the Australian and the Asian clades of the Indo-Pacific humpback dolphin. Genetic divergence across the Wallace line, a biogeographic transitional zone between Asia and Australia, has been extensively documented in many taxa (e.g., fishes (*Scomberomorus commerson*), Sulaiman and Ovenden 2010; and dolphins (*Orcaella* sp.), Beasley et al. 2005). Beasley et al. (2005) argued that the Wallace line was implicated in the isolation and subsequent speciation of *Orcaella heinsohni* (snubfin dolphin) and *brevirostris* (Irrawady dolphin). However, the distribution of *S. chinensis* to the north of Australia is poorly understood. There are records of Indo-Pacific humpback dolphins in Papua New Guinea (Dawbin 1972), but there is an apparent gap in their distribution in the deep waters of the Indonesian archipelago (Jefferson and Van Waerebeek 2004). Future research efforts should resolve this lack of knowledge of the Indo-Pacific dolphin's distribution and its relationship to the genetic divide to assist conservation and improved management of Indo-Pacific humpback dolphins.

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