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Cryptic lineage differentiation among Indo-Pacific bottlenose dolphins (*Tursiops aduncus*) in the northwest Indian Ocean

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ABSTRACT

Phylogeography can provide insight into the potential for speciation and identify geographic regions and evolutionary processes associated with species richness and evolutionary endemism. In the marine environment, highly mobile species sometimes show structured patterns of diversity, but the processes isolating populations and promoting differentiation are often unclear. The Delphinidae (oceanic dolphins) are a striking case in point and, in particular, bottlenose dolphins (*Tursiops* spp.). Understanding the radiation of species in this genus is likely to provide broader inference about the processes that determine patterns of biogeography and speciation, because both fine-scale structure over a range of kilometers and relative panmixia over an oceanic range are known for *Tursiops* populations. In our study, novel *Tursiops* spp. sequences from the northwest Indian Ocean (including mitogenomes and two nuDNA loci) are included in a worldwide *Tursiops* spp. phylogeographic analysis. We discover a new '*aduncus*' type lineage in the Arabian Sea (off India, Pakistan and Oman) that diverged from the Australasian lineage ~261 Ka. Effective management of coastal dolphins in the region will need to consider this new lineage as an evolutionarily significant unit. We propose that the establishment of this lineage could have been in response to climate change during the Pleistocene and show data supporting hypotheses for multiple divergence events, including vicariance across the Indo-Pacific barrier and in the northwest Indian Ocean. These data provide valuable transferable inference on the potential mechanisms for population and species differentiation across this geographic range.

1. Introduction

During the Pleistocene, rapid and dramatic climatic fluctuations generated extensive environmental change that would have influenced the temporal and spatial distribution of taxa over glacial cycles (Hofreiter and Stewart, 2009; Stewart et al., 2010). In the marine environment, fluctuations in sea level changed coastal topography and caused patterns of isolation between areas of available habitat (e.g. Gaither and Rocha, 2013). Oscillations in climate also affected oceanographic processes, such as the reduction and intensification of monsoon systems associated with upwelling (Wang et al., 1999a), which could have contributed to the spatio-genetic structure and taxonomic variation in marine species. In the coastal waters of the northwest Indian Ocean there is high productivity off the Arabian

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Peninsula (Singh et al., 2011; Banse and McClain, 1986; Bauer et al., 1991; Burkill, 1999; Kindle and Arnone, 2001) and freshwater influx from rivers (e.g. the Indus delta), carrying large amounts of organic material (Longhurst, 2006). This, unique, heterogeneous environment has the potential to promote habitat dependencies or resource specialisations (e.g. Hoelzel, 1998b).

In this study we focus on the radiation of diversity in the genus *Tursiops*, in the sub-family Delphininae. Species within this group radiated recently, making genetic resolution difficult due to incomplete lineage sorting (retention of ancestral polymorphisms) and other confounding factors (e.g. Amaral et al., 2012a). Species within this group have high dispersal ability yet often exhibit genetic structure over unexpectedly small spatial scales (e.g. Natoli et al., 2004; Natoli et al., 2008; Andrews et al., 2010). Various studies have shown that genetic sub-division within these delphinid species is often associated with environmental heterogeneity (e.g. Bilgmann et al., 2008; Natoli et al., 2005; Natoli et al., 2008; Andrews et al., 2010; Mendez et al., 2011) and/or historical climatic or geological events (e.g. Amaral et al., 2012b; Moura et al., 2013; Louis et al., 2014; Moura et al., 2014). As top predators, the pattern of genetic differentiation between populations of coastal delphinids may provide an insight into the broader ecological changes happening in the coastal waters of the Indian Ocean over time (see Fontaine et al., 2007). Evolutionary endemism of marine mammal species has been documented in the region previously (e.g. Jefferson and Van Waerebeek, 2002; Mendez et al., 2011; Minton et al., 2011; Amaral et al., 2012b; Mendez et al., 2013; Pomilla et al., 2014).

The taxonomy of bottlenose dolphins, *Tursiops* spp. has been the subject of much discussion (e.g. IWC, 2016). Although more work is needed (see Reeves et al., 2004), resolution is improving, with the genus receiving much taxonomic attention in recent decades (e.g. Mead and Potter, 1990; Ross and Cockcroft, 1990; Hoelzel et al., 1998; Wang et al., 1999b, Möller and Beheregaray, 2001; Kemper, 2004; Natoli et al., 2004, Charlton-Robb et al., 2011, 2015; Moura et al., 2013; IWC, 2016). The genus encompasses at least two species, the common bottlenose dolphin, *T. truncatus* and the Indo-Pacific bottlenose dolphin, *T. aduncus* (LeDuc et al., 1999; Wang et al., 1999b; 2000). There is recent support for a third species, the Burrunan dolphin, *T. australis*, from southern Australia (Charlton-Robb et al., 2011) and further division within the *T. aduncus* group to include distinct lineages off South Africa, Australasia (Natoli et al., 2004; Moura et al., 2013) and possibly Bangladesh (Amaral et al., 2017). Analysis of mtDNA from the *T. aduncus* holotype specimen (Red Sea) revealed it to be a match for the South African *T. aduncus* (Perrin et al., 2007). Within the *T. truncatus* lineage, further division into regional ecotypes occupying coastal or pelagic habitat is recognised (Mead and Potter, 1995, Hoelzel et al., 1998; Torres et al., 2003). Regional patterns suggest that offshore *T. truncatus* can provide a source for colonizing coastal habitats (Tezanos-Pinto et al., 2009, Richards et al., 2013), though the broader pattern suggests a relatively recent radiation of the offshore populations (see Moura et al. 2013).

Patterns of divergence within bottlenose dolphins, and reconstructions of ancestral biogeography, suggest a coastal and Australasian origin for the *Tursiops* genus (Moura et al., 2013). The South African *T. aduncus* (hereafter referred to as the holotype lineage) and the Australasian lineage diverged during the Pleistocene ~327 Ka (Moura et al., 2013). To date, few phylogenetic studies have incorporated genetic data from bottlenose dolphins in the northwest Indian Ocean. A study by Särnblad et al., (2011) showed that coastal bottlenose dolphins off Oman ($n = 4$) grouped with the holotype lineage of *T. aduncus*. Sightings data from the broader region suggest the presence of both coastal and pelagic *Tursiops* species; the latter recognized as *T. truncatus* based on morphology (Ponnampalam, 2009; Minton et al., 2010) and mtDNA markers ($n = 13$) (Curry, 1997; Ballance and Pitman, 1998). As fisheries related mortalities (IWC, 1999; Collins et al., 2002; Anderson, 2014), pollution (Preen, 1991; IWC, 1999; Freije, 2015) and habitat fragmentation (IWC, 1999; Baldwin et al., 2004) continue to threaten regional populations; clarification of the taxonomic status of *Tursiops* sp. in this region has become a conservation concern.

In the present study we combine new *T. aduncus* mitogenomic sequences from the northwest Indian Ocean with the mitogenome dataset generated by Moura et al., (2013). In addition, a dataset consisting of *T. aduncus* and *T. truncatus* samples from the northwest Indian Ocean and sequences from five mtDNA loci and two nuDNA loci were analysed to improve representation from the region and include bi-parentally inherited markers. We investigate whether ancestral distributions and divergence times at key phylogenetic nodes, particularly within the *T. aduncus* lineage, coincide with historic climatic events throughout the Pleistocene. In particular, we test the hypothesis that historical climate transitions during the Pleistocene are consistent with the timing and pattern of differentiation. Understanding this will provide important insight into the processes underlying the evolution of diversity in mobile marine taxa.

2. Material and methods

2.1. Sample acquisition and DNA extraction

Among the 98 samples included in phylogenetic reconstructions, representing various regional populations and putative species, new regions were represented by Oman, collected from strandings ($n = 1$) or free-ranging ($n = 7$) individuals and from strandings in Pakistan ($n = 2$; see Table S1). Samples from India ($n = 11$) were provided by the Environmental Specimen Bank (es-BANK) of Ehime University, Japan. All mitogenome sequences generated by Moura et al., (2013) and two generated by Xiong et al., (2009) were incorporated into the study (see Table S1 for locations and Table S2 for Accession Numbers). Fig. 1(a) and (b) shows the geographic locations of samples. DNA extraction was carried out on all tissue samples using phenol-chloroform DNA extraction protocols, as adapted from Hoelzel (1998a).

2.2. Mitogenome sequencing and assembly

Mitogenome sequences were generated from one Oman and two Pakistan samples following the protocols in Moura et al., (2013). DNA extractions were quantified using a Qubit Fluorometer (Life Technologies Inc.). Aliquots were made to a concentration of 10 ng/μl and randomly sheared to a range of 100–600 base pairs (bp) using a sonicator (Diagenode Biopruptor Pico). Fragment size distributions were checked on a Bioanalyzer (Agilent Technologies) and samples were concentrated to 20 μl using a centrifugal evaporator. Dual indexed sequencing libraries were then prepared following protocols adapted from Meyer and Kircher (2010). Capture-enrichment of mitogenomic DNA was then performed on the libraries (500 ng) using a target-enrichment kit (MYbaits, MYcroarray Inc.). Bait probes were synthesised (20,000 probes, 100 bp each, 2× coverage) with bait design based on an alignment of killer whale, *Orcinus orca,* mitogenomes (Accession Numbers GU187171, GU187200, GU187194, GU187181, GU187209). Captured libraries were quantified using qPCR and pooled in equimolar concentrations. The final sample pool was quantified using the KAPA Universal qPCR quantification kit (KAPA Biosystems), validated on a TapeStation 2200 (Agilent Technologies) and then sequenced on the Illumina HiSeq 2500 in rapid run mode using 150 bp paired-end reads.

After sequencing, adapters were trimmed using the Reaper tool in Kraken v. 13-274 (Davis et al., 2013) and de-multiplexing was carried out using the *process_radtags* program in Stacks v. 1.44 (Catchen et al., 2013). Reads for each individual were then transferred to Geneious v. 7.1.2 (http://www.geneious.com, Kearse et al., 2012) for quality trimming and assembly. Reads were mapped to a *T. aduncus* mitogenome reference sequence (GenBank Accession Number EU557092) using the algorithm available in Geneious. The Geneious map reader algorithm is a multi-step procedure which processes reads one at a time to match short sequences of 10–15 bp, 'words', to a reference sequence.