Genomic Divergence and the Evolution of Ecotypes in Bottlenose Dolphins (Genus *Tursiops*)

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Abstract

Climatic changes have caused major environmental restructuring throughout the world's oceans. Marine organisms have responded to novel conditions through various biological systems, including genomic adaptation. Growing accessibility of next-generation DNA sequencing methods to study nonmodel species has recently allowed genomic changes underlying environmental adaptations to be investigated. This study used double-digest restriction-site associated DNA (ddRAD) sequence data to investigate the genomic basis of ecotype formation across currently recognized species and subspecies of bottlenose dolphins (genus *Tursiops*) in the Southern Hemisphere. Subspecies-level genomic divergence was confirmed between the offshore common bottlenose dolphin (*T. truncatus truncatus*) and the inshore Lahille's bottlenose dolphin (*T. t. gephyreus*) from the southwestern Atlantic Ocean (SWAO). Similarly, subspecies-level divergence is suggested between inshore (eastern Australia) Indo-Pacific bottlenose dolphin (*T. aduncus*) and the proposed Burrunan dolphin (*T. australis*) from southern Australia. Inshore bottlenose dolphin lineages generally had lower genomic diversity than offshore lineages, a pattern particularly evident for *T. t. gephyreus*, which showed exceptionally low diversity. Genomic regions associated with cardiovascular, musculoskeletal, and energy production systems appear to have undergone repeated adaptive evolution in inshore lineages across the Southern Hemisphere. We hypothesize that comparable selective pressures in the inshore environment drove similar adaptive responses in each lineage, supporting parallel evolution of inshore bottlenose dolphins. With climate change altering marine ecosystems worldwide, it is crucial to gain an understanding of the adaptive capacity of local species and populations. Our study provides insights into key adaptive pathways that may be important for the long-term survival of cetaceans and other organisms in a changing marine environment.

Key words: species divergence, adaptive radiation, environmental adaptation, comparative genomics, parallel evolution, phylogenomics.

Significance

Bottlenose dolphins (genus *Tursiops*) have repeatedly evolved inshore and offshore ecotypes worldwide, contributing to a highly contentious taxonomy in the genus. Advances in genomic techniques allow revisiting phylogenetic relationships, exploring the genomic basis of ecotype formation and the role of adaptive divergence in bottlenose dolphin speciation. We found levels of genomic divergence among four Southern Hemisphere lineages that support the subspecies classification of inshore bottlenose dolphins from the southwestern Atlantic Ocean (SWAO) (*T. t. gephyreus*) within *T. truncatus*, and suggest a similar level of classification may be warranted within *T. aduncus* for inshore bottlenose dolphins from southern Australia. We discovered hundreds of genes likely involved in the adaptive divergence of inshore and offshore dolphins in response to different selective pressures between the two environments, providing insights into key adaptive pathways potentially important for adaptation of dolphins in changing environments.

Introduction

Climatic change and the opening of novel niche spaces have been important drivers in the evolution of species (Stroud and Losos 2016). This has often led to population divergence by creating physical barriers to gene flow and/or opposing selective pressures on populations in different habitats. In the marine environment, sympatric and parapatric evolution is not uncommon (e.g., Crow et al. 2010), and can be driven by local adaptation to different niches (i.e., incipient speciation). Where adaptive differentiation is paired with neutral processes, such as mutation and genetic drift, this can result in genomic divergence and speciation (Nosil and Feder 2012). Colonization of similar niche spaces with comparable selective pressures in different regions can, in some cases, result in parallel evolution (Stern 2013). This refers to the formation of similar traits in lineages derived from a recent common ancestor (Wood et al. 2005). The independent rise of similar traits can occur through identical, independent mutations in different populations or species, through selection on a polymorphic allele present in both populations or species from shared ancestral history, and/or through the introduction of an allele into a population *via* introgression (Stern 2013). Phenotypic parallelism does not necessarily stem from changes in the same genomic loci, and, therefore, it is important to investigate the genomic underpinnings of these adaptations to establish the extent and causes of parallel evolution. Parallel genetic evolution has been documented in several species of teleosts (e.g., Jones et al. 2012; Le Moan et al. 2016) but is difficult to study in species where mapping of quantitative trait loci is not available. Sampling of thousands of loci across the genome of nonmodel species, however, now enables tests for selection to establish some regions of the genome that are putatively under selection, or linked to targets of selection, within populations or species. Comparison can then be made across lineages to establish if parallel evolution has possibly occurred. This framework can be particularly useful in studying the radiation and adaptation of nonmodel species, including that of cetacean species.

Cetaceans provide an excellent opportunity to study evolutionary adaptations, particularly the role of environmental discontinuity and associated differences in selective pressures in the formation of new species and ecotypes. There is a vast diversity of cetacean families and species today, with further subdivision within many of these species because of adaptation and specialization to environmental niches. While adaptations associated with the initial aquatic transition of cetaceans have been well documented (see Thewissen et al. 2009), secondary, microevolutionary adaptations are only beginning to be investigated, especially at the genomic level. The most well-studied case is that of the killer whale (*Orcinus orca*), where distinctive differences among sympatric and allopatric ecotypes are evident (e.g., Foote et al. 2009; Moura et al. 2014). Ecotypes are defined here as populations within a species that have evolved heritable variation in physiology, morphology, behavior, and/or life history characteristics due to environmental differences (see Le Moan et al. 2016). Several cetacean species exhibit ecotypic differentiation, including bottlenose dolphins (genus *Tursiops*), which have repeatedly evolved into inshore and offshore ecotypes around the world (e.g., Hersh and Duffield 1990; Hale et al. 2000; Perrin et al. 2011), providing a unique opportunity to study parallel evolution in marine mammals.

Inshore (i.e., all nearshore, coastal, estuarine, and brackish environments) and offshore forms of bottlenose dolphins typically differ in several traits. This includes body size (Ross and Cockcroft 1990), skull and skeleton morphology (Costa et al. 2016), fin size and shape (Félix et al. 2018), diet (Wang et al. 2000), coloration (Diaz-Gamboa et al. 2018), parasite load (Walker 1981), level of population genetic diversity (Fruet et al. 2017), and social behaviors (Costa et al. 2015). The typical characteristics of these ecotypes, however, are not consistent on a worldwide scale (see Hale et al. 2000; Kemper 2004; Charlton-Robb et al. 2011; Costa et al. 2016; Wickert et al. 2016). Similarities in the features that characterize the "inshore environment" compared to the "offshore environment" create comparable selective pressures across inshore

habitats, resulting in cases of phenotypic parallelism in the inshore bottlenose dolphin ecotype. By investigating the underlying genomic basis of ecotype formation, it is possible to examine the extent to which phenotypic parallelism is underpinned by genotypic parallelism, potentially revealing additional adaptive differences. With several marine species, including teleosts and other marine mammals, exhibiting inshore and offshore ecotypes (e.g., Lowther and Goldsworthy 2011; Le Moan et al. 2016), this framework may be useful in deducing how the inshore environment has contributed to genomic divergence and adaptation across cetaceans and other marine vertebrate species.

While inshore-offshore bottlenose dolphin ecotypes have been recorded in all oceans, the extent of divergence likely differs depending on the age of the divergence. In Australian waters, the offshore ecotype has been recognized as the common bottlenose dolphin (*T. truncatus*) and the inshore ecotype as the Indo-Pacific bottlenose dolphin (*T. aduncus*; Hale et al. 2000; Möller and Beheregaray 2001). In southern Australia, inshore bottlenose dolphins have been recently described as the Burrunan dolphin (*T. australis*) (Charlton-Robb et al. 2011), based on morphological and genetic evidence (Charlton et al. 2006; Möller et al. 2008), although the species is not officially recognized by the Committee on Taxonomy of the Society for Marine Mammalogy (2022). In the SWAO the offshore ecotype has been recognized as *T. t. truncatus*, while the inshore ecotype has been classified as the Lahille's bottlenose dolphin, *T. t. gephyreus* (Costa et al. 2016; Committee on Taxonomy of the Society for Marine Mammalogy 2022). While *T. t. gephyreus* is currently recognized as a subspecies of *T. truncatus*, there is contention around whether species-level classification is warranted (Wickert et al. 2016; Hohl et al. 2020). In the Northern Hemisphere, both inshore and offshore ecotypes are currently classified as *T. t. truncatus*, despite being genetically, morphologically, and physiologically divergent (e.g., Mead and Potter 1995; Hoelzel et al. 1998; Lowther-Thieleking et al. 2015; Oudejans et al. 2015), but recent work suggests that the coastal ecotype of the northwestern Atlantic should be considered a separate species, *T. erebennus* (Costa et al. 2022). Incomplete lineage sorting, inconsistent patterns in morphology, and potential hybridization with other delphinid species have resulted in extensive confusion in the taxonomy of the Delphinidae family, of which *Tursiops* is a member (Amaral et al. 2012; Moura et al. 2013). As such, the phylogenetic relationships and taxonomy of the genus *Tursiops* are being revisited using genomic techniques. A recent comprehensive study of the phylogenomic relationships within this genus based on over 25,000 genetic markers proposed a subspecies level classification for the Burrunan dolphin under *T. aduncus* (Moura et al. 2020). Due to the ongoing controversy surrounding this taxon, the Burrunan dolphin will hereafter be referred to as the southern Australian bottlenose dolphin lineage, or simply SABD. A subspecies in the context of cetaceans is defined as "a population, or collection of populations, that appears to be a separately evolving lineage with discontinuities resulting from geography, ecological specialization, or other forces that restrict gene flow to the point that the population, or collection of populations, is diagnosably distinct" (Taylor et al. 2017, pp. 17). We use the term "ecotype" to differentiate between inshore and offshore bottlenose dolphin populations (regardless of species or subspecies classification), while "subspecies" refers to genomic divergence which suggests that the two populations are evolving separately, which may or may not have begun with an initial ecotypic differentiation. With the divergence of species and subspecies in this genus seemingly associated with adaptation to new habitats and niche spaces, an in-depth investigation of ecological features that may be driving adaptation and evolution in this genus is warranted.

Here we aim to investigate the genomic basis of ecotype formation in bottlenose dolphins (genus *Tursiops*) in the Southern Hemisphere using a double-digest restriction site-associated DNA sequencing (ddRADseq) dataset of 18,060 filtered single nucleotide polymorphisms (SNPs). Our study includes several of the recognized and proposed lineages from the Southern Hemisphere, including for the first time comparisons of the inshore and offshore dolphins of southern and eastern Australia to those in the SWAO. We hypothesize that genomic differentiation will be high among the putative lineages and ecotypes, highlighting divergent evolutionary trajectories for inshore and offshore dolphins, and the two inshore Australian lineages. Adaptation to opposing environments is expected to be driving genomic differentiation between ecotypes, while responses to similar selective pressures in the inshore environments may be reflected in parallel evolution of their populations. A number of the sampled populations and lineages inhabit waters in close proximity to urbanized areas and are therefore, subject to human-related stressors, such as pollution, bycatch, overfishing, tourism, boat strikes, and habitat degradation (e.g., Charlton-Robb et al. 2015; Fruet et al. 2016a). Climate change is also posing a significant threat to dolphins, with oceans becoming warmer and more acidic, and climate extremes, such as marine heatwaves, increasing in frequency (Poloczanska et al. 2013). Rising sea surface temperature and salinity due to climate change have been identified as the most significant threats to marine mammals in southern Australian waters (Robbins et al. 2017). Well-informed management and conservation strategies are needed to ensure that these populations are not negatively affected by human activities to an irreversible extent. A crucial step is to clarify species and subspecies levels of genomic differentiation among regions, as well as to identify populations of high conservation

concern. Studying how these dolphins have evolved in response to different selective pressures allows a better understanding of how they may continue to diverge and adapt to present and projected climatic changes.

Results

A total of 375 biopsy samples collected at 30 locations in the Southern Hemisphere were ddRAD sequenced. The samples encompass three ocean basins and all currently recognized species and subspecies of bottlenose dolphins in the Southern Hemisphere, as well as SABD in inshore waters of southern Australia (supplementary table S1, Supplementary Material online). The *Tursiops* dataset comprised over 1.1 billion raw sequence reads. Individuals with <500,000 reads were removed, leaving an average of 3,274,483 reads per individual (±2,758,909). The raw *Tursiops* dataset consisted of 196,751 SNPs. After a series of rigorous filtering steps, 18,112 SNPs and 353 individuals were retained (supplementary table S2, Supplementary Material online). These loci were then mapped to the *T. aduncus* reference genome, with a 99.71% alignment rate. The final *Tursiops* dataset available for analysis thus consisted of 18,060 SNPs (supplementary table S2, Supplementary Material online), with an average of 6.6% missing data (± 5.6%) per individual, and average of 30.35× depth of coverage per locus per sample. The *Tursiops* + *Delphinus* dataset, which included the nine common dolphins used as the outgroup for phylogenomics, consisted of 386 individuals and 223,408 SNPs, with an average of 3,121,368 reads per individual (± 2,741,764) (supplementary table S2, Supplementary Material online). After filtering, 362 individuals were retained, with an average of 7.0% missing data (± 6.2%) per individual, and average of 29.74× depth of coverage per locus per sample. No common dolphins were removed during the filtering process. The 18,338 SNPs retained after filtering were then aligned to the reference genome at a rate of 99.69%, with 18,282 SNPs retained for phylogenomic analysis (supplementary table S2, Supplementary Material online).

Genomic Variation

Genomic diversity was estimated for each sampling site and then averaged across each of the four lineages to minimize the effect of small sample size in some localities and to better understand overall trends in diversity. *T. t. gephyreus* had substantially lower genomic diversity than the other taxa across all measures. Nonetheless, this lineage does not appear to have high levels of inbreeding (supplementary table S3, Supplementary Material online). Relatively high genomic diversity was estimated for the other inshore lineages, but offshore *T. t. truncatus* from across the Southern Hemisphere recorded slightly higher genomic diversity on average. The number of private alleles (PA) was also the lowest for *T. t. gephyreus*. *T. t. truncatus* on the other hand, had the highest number of PA, while *T. aduncus* had substantially more than SABD (supplementary table S3, Supplementary Material online).

Genomic Divergence

Phylogenomics

A clear initial split between *T. aduncus*/SABD and *T. t. truncatus*/*T. t. gephyreus* was evident and supported by bootstrap values of 100% in the phylogenomic tree generated in RAxML (fig. 1; see supplementary fig. S1, Supplementary Material online for full phylogeny including admixture individuals). This is consistent with the two currently recognized species in the Southern Hemisphere (Committee on Taxonomy of the Society for Marine Mammalogy 2022). There was subsequent strong genomic separation within each of these clades, with a similar level of divergence between SABD and *T. aduncus*, and *T. t. truncatus* and *T. t. gephyreus*, supported by bootstrap values of 99% or higher. Subpopulation divergence corresponding to geographical regions was also evident within each lineage. Branch lengths were considerably shorter within the *T. t. gephyreus* lineage than for the other three lineages, suggestive of more recent evolution (fig. 1).

Population Genomic Structure

Several different methods were used to assess genomic divergence among and within the four lineages (*T. t. truncatus, T. t. gephyreus, T. aduncus*, and SABD). Substantial differentiation among taxa was revealed by principal component analysis (PCA), with PC1 (22.76% of variance) splitting SABD/*T. aduncus* from *T. t. truncatus*/*T. t. gephyreus* (fig. 2). PC2 showed the division between the two inshore Australian bottlenose dolphin lineages, SABD and *T. aduncus*, and more subtle divergence between *T. t. truncatus* and *T. t. gephyreus* (9.86% of variance). When the PCA was run again with only *T. t. truncatus* and *T. t. gephyreus* individuals to investigate this differentiation further, there was a clear separation of the two taxa (supplementary fig. S2, Supplementary Material online). Fourteen individuals from ten locations showed admixed membership or full assignment to a taxon inconsistent with the sampling location and/or observed morphology (see *Admixture* (supplementary fig. S3*A–J*, Supplementary Material online) and PCA results (fig. 2); supplementary table S4 and supplementary fig. S1, Supplementary Material online). These individuals represent only 3.6% of the total dataset of 386 dolphins. Their presence is probably due to migration, recent admixture, or shared ancestral polymorphism (Moura et al. 2020). The divergence between the four lineages was further supported by analysis of molecular variance (ANOVA) with

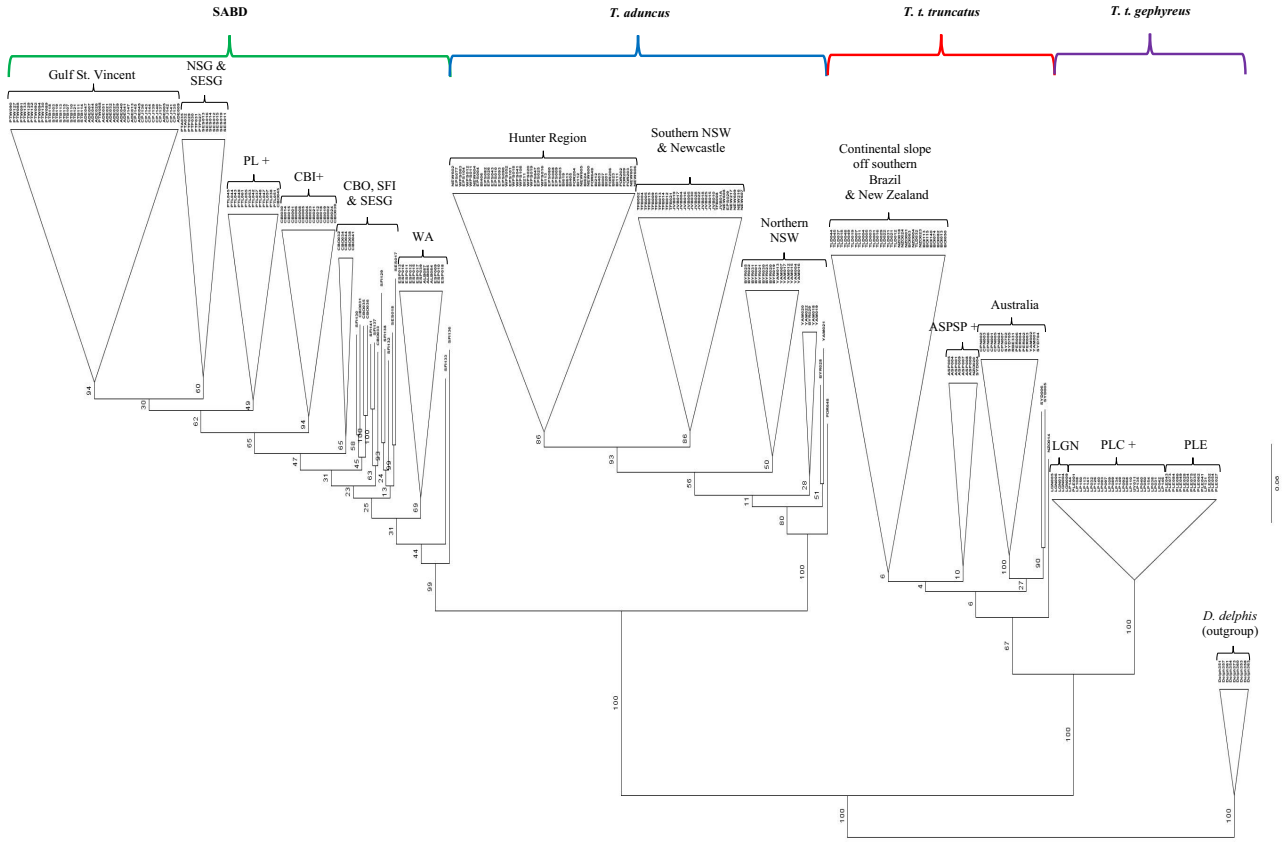

Fig. 1.—*RAxML* maximum-likelihood tree with 1,000 RELL bootstraps based on 18,282 SNPs, displaying phylogenomic relationships among *Tursiops* species across the Southern Hemisphere and including nine (Australian) common dolphins (*Delphinus delphis*) as outgroup. Population-level divergences are shown. Abbreviations are explained in supplementary table S1, Supplementary Material online. A plus sign (+) represents that the clade does not solely consist of the majority location specified.

42.81% of variance ($P < 0.001$) explained by among lineage divergence, compared to just 4.20% ($P < 0.001$) of variance explained among populations within the four putative taxa (supplementary table S5, Supplementary Material online). Fine-scale subpopulation division within each of the lineages was detected by *Admixture* analysis (supplementary fig. S3*A–J*, Supplementary Material online), in a pattern consistent with the results of the phylogenomic analysis. Estimates of F_{ST} were, in general, moderate to high among sampling localities, with an average of 0.3604 (fig. 3). This particularly highlighted the divergence of *T. t. gephyreus* from all other taxa. When averaged among lineages, the mean of estimates between *T. t. gephyreus* and *T. t. truncatus* was substantially higher than those between *T. aduncus* and SABD (fig. 3).

Genomic Basis of Ecotype Formation

Candidate Loci Detection

To identify signature of selection between ecotypes, we ran two outlier detection methods, which identified a total of 325 outliers as candidates for selection between *T. t. truncatus* and *T. t. gephyreus*, 1,126 outliers between *T. t. truncatus* and *T. aduncus*, and 842 outliers between *T. t. truncatus* and SABD. The lists of candidate loci were then compared to identify SNPs that were present in all three, being potentially implicated in parallel genomic evolution of the inshore ecotype across the Southern Hemisphere. This resulted in a total of 142 candidates for parallel evolution. Fourteen annotated candidate genes were highlighted as having an F_{ST} value in the top 10%. Genotype frequencies for these candidates revealed stark differences between the inshore and offshore lineages. Across the three inshore lineages homozygosity of the top candidate loci was markedly more common than in the offshore animals (fig. 4). For 11 of the 14 top candidates, this reflected near-fixation of the major allele in each of the inshore putative taxa. In the offshore dolphins, on the other hand, heterozygosity and the representation of the minor allele were much higher (fig. 4).

Arlequin and RandomForest identified 12 early stage evolution candidate loci. Genotype frequencies were

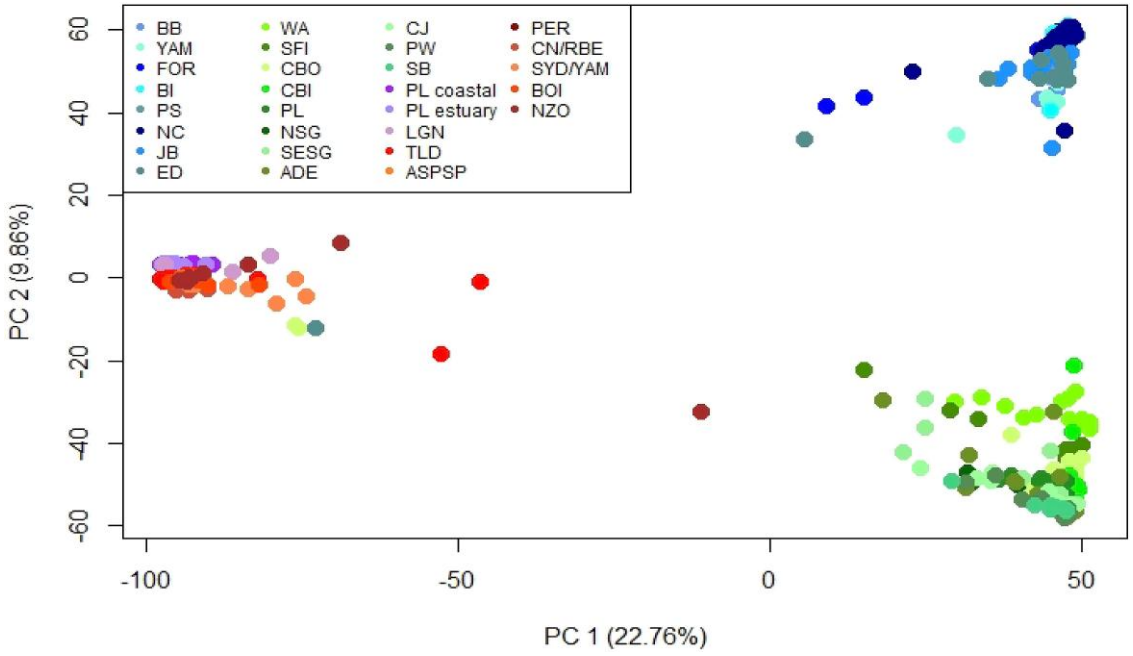

Fig. 2.—Differentiation among bottlenose dolphins (*Tursiops* spp.) from across the Southern Hemisphere based on 18,060 SNPs as estimated by PCA. Sampling locations are colored as per putative lineage: *T. aduncus* (blue shades), SABD (green shades), *T. t. gephyreus* (purple shades), *T. t. truncatus* (red shades). Sampling location abbreviations are explained in supplementary table S1, Supplementary Material online.

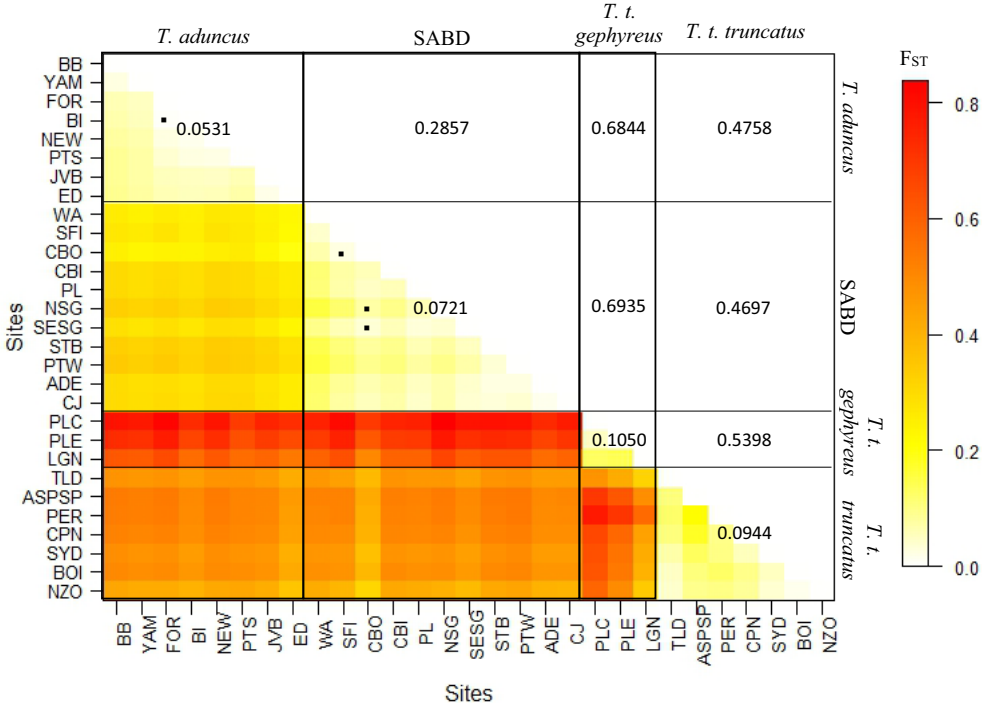

Fig. 3.—Heat map of pairwise genomic differentiation (F_{ST}) between sampling sites of bottlenose dolphins (*Tursiops* spp.) across the Southern Hemisphere based on 18,060 SNPs. Values on the diagonal represent the average F_{ST} value for comparisons within each putative lineage, while those in the top half of the matrix represent the average value of pairwise comparisons between each lineage. Nonsignificant F_{ST} values at the B-Y corrected alpha value of 0.0076 are marked with a black square (■). Transitions between putative lineages are marked by black lines. The global F_{ST} was 0.3604. Sampling location abbreviations are explained in supplementary table S1, Supplementary Material online.

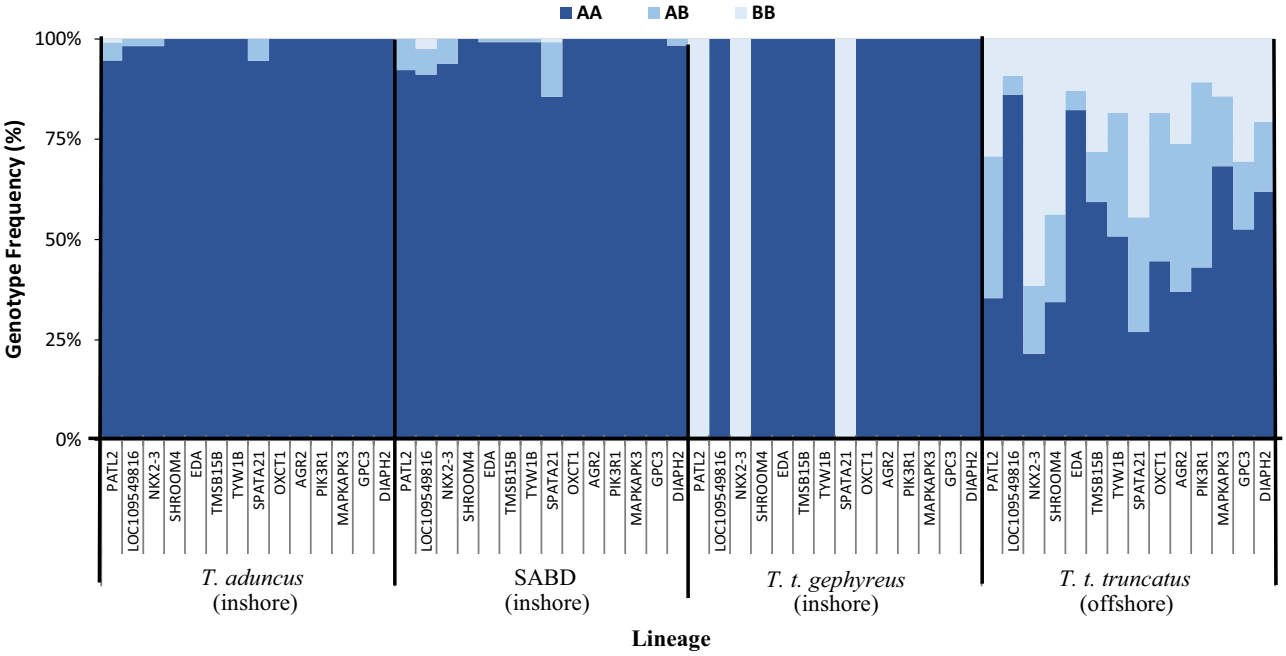

Fig. 4.—Genotype distribution for the parallel evolution candidate genes with F_{ST} values in the top 10% when comparing each inshore bottlenose dolphin lineage (*Tursiops* spp.) to offshore *T. t. truncatus* ($n = 14$).

plotted for each of the six annotated candidate genes, revealing that almost all sampled *T. t. gephyreus* individuals were major-allele homozygotes, with almost complete absence of the minor allele (fig. 5). Heterozygosity of candidates was substantially higher in the offshore SWAO dolphins on the other hand, primarily representing the frequency of the minor allele (fig. 5).

Functional Enrichment Analysis and Annotation

To better understand the potential functions of the candidate genes, a functional enrichment analysis, and gene annotation was carried out. Of the 18,060 loci, a total of 3,792 (20.99%) scored basic local alignment search tool (BLAST) hits and were mapped and annotated, 27 of which were candidates for parallel evolution. A functional enrichment analysis found 90 categories significantly over-enriched in the parallel evolution candidate set (supplementary table S6, Supplementary Material online). This included glycosaminoglycan metabolic process (GO:0030203), mesonephric duct morphogenesis (GO:0072180), carbohydrate transport (GO:0008643), and photoreceptor activity (GO:0009881), among many others (significance values provided in supplementary table S6, Supplementary Material online). Parallel evolution candidate loci were individually annotated, revealing 97 associated candidate genes (supplementary table S7, Supplementary Material online). Six candidate genes were identified from the early stage evolution candidate loci as above (supplementary table S8, Supplementary Material online).

Discussion

Large-scale environmental and oceanographic restructuring in the world's oceans since the Eocene has influenced the rapid diversification of cetaceans (Steeman et al. 2009). With climate change presently altering marine habitats worldwide, and to protect vulnerable populations and species, it is imperative to understand the principal drivers of genomic divergence and adaptation in marine organisms. The inshore-offshore pairs of bottlenose dolphin ecotypes in the Southern Hemisphere provide an excellent system to investigate genomic adaptation and diversification in delphinids. A genomic dataset was utilized to investigate some of the controversial phylogenomic relationships and genomic divergence within the genus *Tursiops*, as well as to explore potential environmental adaptation in these lineages. We found strong genomic differentiation between each putative lineage, suggesting that ecotypic differentiation can lead to incipient speciation. The signal of selection found in genes associated with modification to major bodily systems is indicative of the adaptation of inshore bottlenose dolphins to their respective habitats, which may also be affected by future environmental changes. The results highlight potentially critical adaptive pathways for cetaceans and possibly other marine vertebrates to successfully colonize new

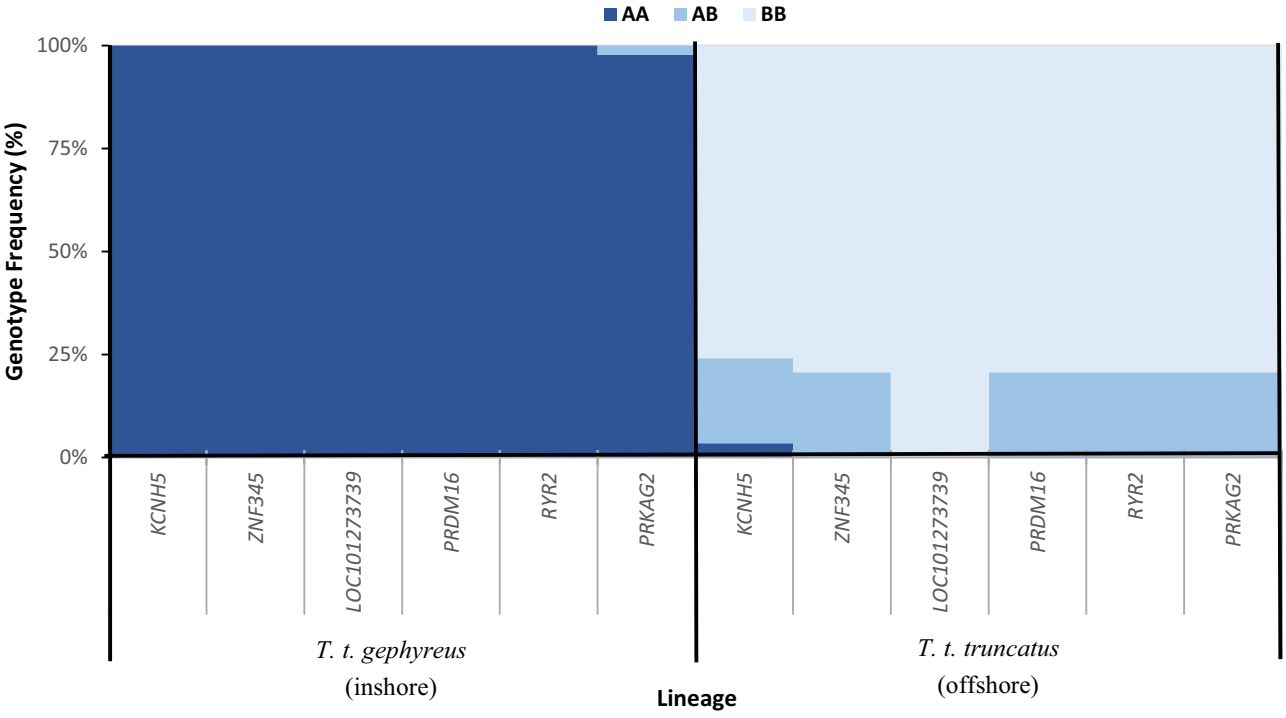

Fig. 5.—Genotype distribution for the six candidate genes implicated in early stage evolution between offshore *T. t. truncatus* and inshore *T. t. gephyreus* in the SWAO.

niche spaces, a process that is likely to become increasingly important with ongoing climate change.

Genomic Variation

Genomic diversity in species can be affected by demographic history, including founder events (Ellegren and Galtier 2016). Inshore bottlenose dolphin populations have been repeatedly reported to have substantially lower genetic diversity than their offshore counterparts, which is thought to be a result of such founder events (e.g., Hoelzel et al. 1998). This was particularly apparent for *T. t. gephyreus*, consistent with previous low estimates of genetic diversity based on mitochondrial DNA (mtDNA) and microsatellite markers (Fruet et al. 2017; Costa et al. 2021). The genomic data supports the hypothesis of a strong founder event, likely after the Last Glacial Maximum (Fruet et al. 2017), which would also account for the strong genetic differentiation observed between this lineage and the adjacent *T. t. truncatus*.

The current hypothesis for *Tursiops* diversification and radiation includes a coastal Australasian origin for the genus, with subsequent colonization of the pelagic realm and then repeated movement back into inshore habitats as the genus spread throughout the world's oceans (Moura et al. 2013). We found that *T. t. gephyreus* had substantially lower genomic diversity, less private alleles and a phylogeny with shorter branch lengths compared to the other lineages, suggesting a more recent divergence from *T. t. truncatus* than for the Australian inshore lineages. The small population size (Fruet et al. 2016c), restricted geographical range (Costa et al. 2016; Wickert et al. 2016) and low genomic diversity makes the *T. t. gephyreus* lineage particularly susceptible to anthropogenic disturbances. The International Union for the Conservation of Nature (IUCN) has recently classified this subspecies as Vulnerable, citing a low number of mature individuals, high anthropogenic impacts, and declining habitat quality (Vermeulen et al. 2019). Ongoing habitat degradation and other human impacts, such as bycatch, are likely to have major negative consequences for these dolphins (Daura-Jorge and Simões-Lopes 2011; Fruet et al. 2012). Small cetaceans are mesopredators and, therefore, disturbances to their populations will undoubtedly have flow on effects to food webs of the ecosystems they inhabit.

Genomic Divergence

The taxonomy of cetaceans has long been a controversial topic. This is particularly true for the classification of *Tursiops* species and their close relatives within the subfamily Delphininae. Relatively recent species radiations have created discordance between mtDNA and nuclear DNA markers, fueling much of the debate (e.g., LeDuc et al. 1999; Möller et al. 2008; Moura et al. 2013). Clear genomic

divergence was evident among the four sampled bottlenose dolphin lineages. The differentiation between SABD and *T. aduncus*, which are both currently recognized as *T. aduncus*, was broadly similar to the level between *T. t. truncatus* and *T. t. gephyreus.* Along with previous findings of genetic differentiation and morphological and osteological dissimilarities between SABD and *T. aduncus* (Charlton et al. 2006; Möller et al. 2008), our findings suggest that these taxa are on separate evolutionary trajectories. There is however, ongoing confusion around the SABD's placement in the *Tursiops* genus (e.g., Jedensjö et al. 2020; Moura et al. 2020). Based on the definitions given for cetacean species, subspecies, and evolutionarily significant units (Taylor et al. 2017), a conservative subspecies classification is deemed most appropriate for SABD within *T. aduncus*. This refers to nearshore SABD from South Australia to southwestern WA, with future studies required to confirm the classification of nearshore bottlenose dolphins from Victoria and Tasmania, which were not included here. With many widely distributed, highly mobile cetacean species exhibiting strong population genetic structure at odds with their dispersal potential (see Hoelzel 2009), it is important to revisit the species classifications using genomic sequencing based on a comprehensive sample of localities and oceanic regions.

Conflicting evidence exists in the details surrounding the history of *Tursiops* divergence, with SABD initially suggested to be the ancestral lineage (Moura et al. 2013; Gray et al. 2018), but more recently found to be a sister group to *T. aduncus* (Moura et al. 2020). Support is provided here for the latter. The pattern of inshore-offshore-inshore colonization suggested by Moura et al. (2013) is also supported by the strong genomic divergence between *T. t. truncatus* and the Australian inshore lineages and longer branch lengths within each of these lineages than in *T. t. gephyreus*. The coastal Indo-Pacific form, *T. aduncus*, is divided into several genomic stocks (e.g., Amaral et al. 2017; Gray et al. 2018), and regional populations (e.g., Bilgmann et al. 2007b; Möller et al. 2007; Pratt et al. 2018). The offshore form (*T. t. truncatus*) on the other hand, appears to maintain relatively high gene flow throughout the Southern Hemisphere. Offshore bottlenose dolphins from across three ocean basins were found to be more genomically similar to each other than to their adjacent inshore populations (albeit one shared haplotype between *T. t. gephyreus* and offshore Atlantic animals was recently discovered in another study; Costa et al. 2022). This is despite sightings of mixed groups with inshore dolphins in some regions (e.g., Fruet et al. 2017). Pelagic connectivity seems to also extend between the two hemispheres, with recent genetic evidence of shared mtDNA haplotypes between animals of the North Atlantic Ocean and those from the St. Peter and St. Paul Archipelago (Oliveira et al. 2019), and those further south in the Brazilian coast (Costa et al. 2021). Here, we provide evidence for limited reproductive exchange between inshore and offshore bottlenose dolphins in SWAO (but see Oliveira et al. 2019; Costa et al. 2022), a conclusion reinforced by major morphological and osteological differences between them (e.g., Costa et al. 2016; Wickert et al. 2016; Fruet et al. 2017). With repeated inshore colonizations in the genus *Tursiops*, currently at varying stages of divergence, this system provides a unique opportunity to investigate adaptations of delphinids to the inshore environment.

Genomic Basis of Ecotype Formation

Large-scale environmental changes have driven several species radiations in the marine ecosystem over evolutionary history (Condamine et al. 2013), particularly as coastal habitats worldwide were released after the Last Glacial Maximum (e.g., Portnoy et al. 2014; Silva et al. 2014). This is thought to be the case for bottlenose dolphins, with inshore recolonization by the pelagic population likely occurring at that time (Moura et al. 2013). We identified potential adaptations involved in successful colonization of the inshore environment, which may be contributing to ecotypic divergence over time. We also found evidence for parallel evolution among inshore lineages in the Southern Hemisphere, as has been recently disclosed for those in the Northern Hemisphere (Louis et al. 2021). Similar selective pressures in inshore habitats appear to be driving strong directional selection in these lineages. In the inshore bottlenose dolphin ecotype in SWAO, this is observed by almost complete fixation of the major allele in all six early stage evolution candidate genes. In the offshore SWAO ecotype, however, major allele homozygotes were almost completely absent, and heterozygosity was substantially higher. This likely reflects divergent selective pressures between the two habitats and may indicate that balancing selection has a stronger role in the adaptation of the offshore than the inshore dolphins. This is potentially driven by the wide range of habitat types and environmental conditions experienced over the large home ranges of offshore dolphins (see Möller 2012). A very similar pattern was also detected for the top 10% of parallel evolution candidates. For almost all top candidates there was near fixation of the same allele for each inshore lineage across three ocean basins. The replication of the same pattern in all three sampled inshore bottlenose dolphin lineages is unprecedented in marine mammals and indicates that similar selective pressures across the inshore habitats may be creating parallelism in the adaptive responses of these dolphins. Recent findings of parallel adaptation of coastal lineages in the Northern Hemisphere suggest that this was facilitated by repeated selection on standing genetic variation in the pelagic population (Louis et al. 2021). Further investigation is warranted to determine if a similar mechanism is at play in the Southern Hemisphere.

Cardiovascular and Circulatory Systems

Several candidate genes were found to be associated with adaptation of the dolphins' cardiovascular and circulatory systems to inshore environments. This includes the early stage evolution candidate genes, *PRKAG2* and *RYR2*, and the parallel evolution candidates, *CACNA1B, JDP2, MYH11, NMRAL1, PDE1C, PDE9A*, *PLAT, PRKG1, RBM20, SEMA3E,* and *TBX1*. Briefly, these genes are involved in heart and blood vessel development and healthy functioning, heart muscle contraction, hemoglobin concentration, and in blood clotting (for specific gene functions and relevant literature, see supplementary table S9, Supplementary Material online). The *PRK* gene family appears to be particularly important, found twice here and previously implicated in the macroevolution of marine mammals to an aquatic lifestyle (Foote et al. 2015; Zhou et al. 2015). A change in diving behavior and associated physiology between inshore and offshore dolphins is among the potential causes for adaptation of the cetacean cardiovascular and circulatory systems. Although the diving behavior of inshore and offshore bottlenose dolphins has not been extensively documented in the Southern Hemisphere, offshore *T. t. truncatus* in the northwestern Atlantic Ocean have been found to dive to depths greater than 450 month (Klatsky et al. 2007). In the SWAO, they have been documented to belong to a higher trophic position than the epipelagic predator, the Atlantic spotted dolphin (*Stenella frontalis*), potentially indicating a high plasticity for predating upon deep-water prey to minimize niche overlap (Troina et al. 2021). It is, therefore, hypothesized that throughout their range the offshore ecotype dives to much greater depths than their inshore counterparts. Many deep-diving species and populations have been shown to have significantly higher blood volume, and hemoglobin and myoglobin concentration than their terrestrial and shallow-diving counterparts, including bottlenose ecotypes dolphins in the Pacific and Atlantic Oceans (Hersh and Duffield 1990; Kooyman and Ponganis 1998). Extended deep dives put significant stress on the body and often result in hypoxic conditions, which can lead to DNA damage (see Tian et al. 2016). Accordingly, we found three parallel evolution candidate genes that are involved in DNA damage response—*DYRK1A, UBE2E2,* and *USP10* (supplementary table S9, Supplementary Material online). At the macroevolutionary scale, deep-diving adaptations of cetaceans have been linked to positive selection of genes associated with cardiovascular system formation and regulation (McGowen et al. 2012; Nery et al. 2013; Foote et al. 2015), hypoxia tolerance (Tian et al. 2016), DNA repair and damage response (Zhou et al. 2013) and oxygen storage (McGowen et al. 2014). Adaptations of the cardiovascular system in relation to the evolution of hypoxia tolerance, and specifically the candidate gene *NMRAL1*, have also been found in high-altitude human populations (Simonson et al. 2015). Adaptation of the cardiovascular and circulatory systems, among many others, may, therefore, be crucial to the colonization of the inshore habitat by bottlenose dolphins and for dealing with changes to hypoxia-inducing behaviors in general.

Adipogenesis and Energy Production

Fat reserves are critical to the survival of animals, through their roles in thermoregulation (Speakman 2018), buoyancy (Hagen et al. 2000), metabolism, and energy production (Choe et al. 2016). Genome-level adaptations of this system can be dictated by long-term changes in temperature and diet. In cetaceans, the transition from a terrestrial to a fully aquatic lifestyle was coupled with major dietary changes and alteration of thermogenic requirements. Accordingly, several studies have found positively selected genes related to fat storage, lipid transport, metabolism, and fatty acid synthesis and transport in cetaceans (McGowen et al. 2012; Nery et al. 2013; Sun et al. 2013; Derous et al. 2019). Wang et al. (2015) documented positive selection in a member of the *PDE* gene family in cetaceans, associated with adipose tissue development. Two other members of this family were found here to be potentially involved in the parallel evolution of the inshore ecotype, including *PDE1C*, which has an important role in energy production (Han et al. 1999). Several other genes—*AGL, GPC3, JDP2, LOC101322629* (*COX8A*-like *Tursiops* gene), *NPC1*, *NSDHL, OXCT1,* and *RORA*—were discovered to be implicated in the parallel evolution of energy production pathways, as well as in adipogenesis, fat storage and several associated processes (supplementary table S9, Supplementary Material online). In addition, significantly over-enriched GO terms in the candidate gene dataset included glycosaminoglycan and aminoglycan metabolic, catabolic, and biosynthetic processes, carbohydrate transport and insulin binding, among others. *PRDM16*, which has a key role in deposition of brown adipose tissue (Seale et al. 2007, 2008) was also identified as an early stage evolution candidate and could be crucial to the long-term adaptation of organisms to cold temperatures (Cannon and Nedergaard 2004; Li et al. 2014). Adipogenesis, and lipid and glucose metabolism pathways were also shown to be under differential selection between killer whale (*Orcinus orca*) ecotypes found in differing climates and feeding on distinct diets (Foote et al. 2016), as well as between polar bears (*Ursus maritimus*) and brown bears (*U. arctos*; Liu et al. 2014). Temperature profiles can potentially differ between inshore and offshore habitats, for example, in the SWAO, inshore dolphins experience higher year-round temperature variability, and lower temperatures in the winter, than those in the offshore zone.

Coupled with discrepancy in diet and total body size between the two ecotypes (e.g., Charlton-Robb et al. 2011; Gibbs et al. 2011; Costa et al. 2016), this may create opposing thermogenic and energy production requirements for the dolphins. Genes associated with these processes may, therefore, become increasingly important for the survival of species with ongoing ocean warming under anthropogenic climate change. Fat is also an important part of the sensory system for odontocete cetaceans, with specialized fat stores involved in echolocation (Gabler et al. 2018). It is, therefore, possible that modification to adipogenic pathways could also be associated with changes in echolocation between bottlenose dolphin ecotypes.

Musculoskeletal System

Adaptations of the musculoskeletal system were of crucial importance to the colonization of the aquatic system by marine mammals (Zhou et al. 2018). Fittingly, several genes associated with muscle and bone development, particularly of the skull, were found to be candidates for parallel evolution. This included *GPC3, GTF2IRD1*, *MBNL3, PIK3R1,* and *SCUBE2* (supplementary table S9, Supplementary Material online). Skeletal studies of bottlenose dolphins have revealed that the inshore ecotype typically has fewer vertebrae than offshore dolphins (Hale et al. 2000; Kemper 2004; Wickert et al. 2016). *T. aduncus* and SABD have also been found to have shorter/smaller skulls than the offshore *T. truncatus* (Hale et al. 2000; Charlton-Robb et al. 2011), while the opposite is true in SWAO, with *T. t. gephyreus* having a longer/larger skull than offshore *T. truncatus* (Costa et al. 2016). These differences are thought to be associated with opposing requirements for maneuverability and the manipulation of prey (Hersh and Duffield 1990; Perrin et al. 2011), but many of the skeletal modifications reported have unknown adaptive functions. Differences in bone density have also been found among cetacean species, likely as an adaptation to diving depth and the associated buoyancy requirements (see Foote et al. 2015). Subsequently, Zhou et al. (2018) discovered several positively selected genes related to bone density in the common ancestor of cetaceans, identifying *PIK3R1* and another member of the *PIK* gene family (*PIK3CB)* to be highly correlated with different measures of bone compactness. While bone density changes have not been documented at the ecotype level previously, the repeated selection of *PIK3R1* across inshore lineages found here suggests this potential difference between ecotypes.

Brain Development and Nervous System

Delphinids have the largest relative cerebellum and overall brain size within the cetacean lineage, also being approximately ten times larger than terrestrial artiodactyls of similar body size (Ridgway et al. 2016; Ridgway et al. 2018). A larger brain requires a greater proportion of energy to be directed to the central nervous system and brain (Isler and Van Schaik 2006). Genes associated with brain and neural development and functioning, as well as lipid transport and metabolism, have been found to be positively selected in the evolution of *T. truncatus* (McGowen et al. 2012). We discovered several genes with functions related to the brain and nervous system possibly implicated in the evolution of the inshore ecotype. Specifically, *KCNH5* and *ZNF345* were identified as early stage evolution candidates, while *APH1B, CACNA1B, DYRK1A, EVL, NSG1*, *MSI2, NKX2-2, NRXN3, PARD3, PDE9A, PLXNA2, RORA,* and *SHROOM4* were parallel evolution candidates (supplementary table S9, Supplementary Material online). *SHROOM4* and several *KCN, ZNF,* and *CACN* genes have previously been documented to be involved in the evolution of marine mammals to the aquatic environment, particularly in regard to adaptation of the central nervous system (e.g., McGowen et al. 2012; Foote et al. 2015; Zhou et al. 2015, 2018). As a larger brain size requires more energy, *RORA* and several aforementioned candidate genes involved in energy production may be important in this adaptation. In birds, a larger brain has been reported to be important in colonizing new habitats by enabling enhanced innovation and adaptability (Sol et al. 2005). As inshore habitats typically show increased complexity and stark differences to the offshore realm, a large brain may be an important adaptation of these dolphins in ensuring successful colonization of the new niche space. Furthermore, inshore bottlenose dolphins are known to have more complex behavioral and social systems than seen in the offshore ecotype (Möller 2012), even exhibiting population-specific prey handling techniques and tool use (e.g., Krützen et al. 2005). These behaviors have all been previously implicated in the evolution of large brain size in mammals and birds (Marino 2005), and may, therefore, be playing an important role in driving adaptation of the nervous system and brain in inshore bottlenose dolphins. With the adaptation of this central bodily system implicated in the evolution of birds and both terrestrial and marine mammals, it is likely that this is a crucial step in the successful colonization of new habitats.

Conservation Implications

The Delphininae subfamily has perhaps the most complicated phylogeny in the cetacean lineage. The genus *Tursiops* particularly, has a very controversial taxonomic history, with up to 20 species previously described but only two formally recognized species currently (Hershkovitz 1966; Committee on Taxonomy of the Society for Marine Mammalogy 2022). We found genomic divergence within these lineages supporting previous findings of negligible reproductive exchange between SABD and Indo-Pacific bottlenose dolphins (*T. aduncus*) in Australian waters

(Möller et al. 2008; Charlton-Robb et al. 2011), and potentially between inshore (*T. t. gephyreus*) and offshore (*T. t. truncatus*) dolphins in SWAO (Fruet et al. 2017). Furthermore, the genomic divergence between SABD and *T. aduncus* was on a relatively similar level to that found between the proposed subspecies, *T. t. truncatus* and *T. t. gephyreus* (see Costa et al. 2016). It is therefore, proposed that a subspecies-level classification for SABD within *T. aduncus* is appropriate, as recently suggested by Moura et al. (2020). Inshore bottlenose dolphins typically reside in small, largely philopatric populations close to areas of high human disturbance (e.g., Daura-Jorge and Simões-Lopes 2011). It is particularly important to define these taxonomic relationships to ensure that management strategies are well-informed about their vulnerability and adaptive capacity. In the event of major population declines, knowledge of species ranges and their ability to replenish endangered populations or species is especially crucial. This is particularly exemplified by our finding of extremely low genomic diversity in the potentially reproductively isolated inshore SWAO dolphins, suggesting that this lineage is especially vulnerable to population declines. With ongoing environmental changes and increased human pressures throughout the world's oceans, especially in coastal waters, it is important to understand how marine organisms may respond and how this could shape patterns of speciation. Our results suggest that bottlenose dolphins have a vast capacity for adapting to changing selective pressures, but this is likely over an evolutionary scale of many thousands of years. Anthropogenically accelerated climate change may, therefore, pose a significant challenge to the adaptive capacity of these dolphins and other long-lived marine vertebrates. The findings presented here are an important step in understanding the vast scope of potential adaptive responses by marine organisms.

Future Directions

We present the first evidence of the possible parallel evolution of genes associated with several major physiological systems in inshore bottlenose dolphins of the Southern Hemisphere. Despite relatively high power to detect signatures of selection (Manel et al. 2016), the reduced-representation nature of ddRADseq yields low genomic coverage and may be biased toward hard sweeps, missing numerous loci involved in adaptation, particularly for species with short linkage disequilibrium (see Davey et al. 2011; Lowry et al. 2017). As a result, several important genes involved in the adaptive divergence of bottlenose dolphin inshore and offshore ecotypes are likely to have been missed here. The use of whole-genome sequencing in future studies would allow a more comprehensive overview of ecotype formation in bottlenose dolphins, and ecotypic differences in candidate genes associated with various bodily systems.

Although not discussed in detail, further research into ecotypic differences in genes also found here and associated with the gastrointestinal, sensory, osmoregulatory, immune, and reproductive systems is warranted. Future research should also investigate the history of ecotype divergence in the Southern Hemisphere using coalescent-based demographic reconstructions (see Hein et al. 2004) and apply genotype-environment association analyses at intraspecific level (Grummer et al. 2019; Barceló et al. 2022; Pratt et al. 2022) to test for competing hypotheses, such as allele surfing and drift associated to founder effects, that may mimic genomic signals of selection (Hoban et al. 2016). To further compliment this, studies should endeavor to include representatives of the South African inshore bottlenose dolphins (*T. aduncus*), and inshore and offshore populations from across the Northern Hemisphere (*T. truncatus,* including *T. t. ponticus*, and *T. aduncus*) to clarify subspecies level classifications. Enhanced collaboration between scientists across these study regions would allow the *Tursiops* phylogeny and patterns, as well as the underlying causes of genomic divergence, to be elucidated more completely.

Material and Methods

Sample Collection

Skin and blubber biopsy samples from free-ranging bottlenose dolphins (*Tursiops* spp.) were collected from 29 locations across three ocean basins in the Southern Hemisphere between 1998 and 2016 (fig. 6; supplementary table S1, Supplementary Material online), of which 375 samples were selected for use in this study based on sample quality, quantity, and representativeness. We used a hand-held biopsy pole (Bilgmann et al. 2007a), a remote biopsy gun system (Krützen et al. 2002), or a remote biopsy crossbow (Fruet et al. 2016b). Resampling of individuals was minimized by visually checking for biopsy wound marks on the animal's body and through identification of recognizable dorsal fin characteristics. No samples were obtained from dependent calves. Biopsy samples were preserved in either 90% ethanol or a salt-saturated solution of 20% dimethyl sulphoxide (DMSO) and stored at −20 °C or −80 °C upon return to the laboratory. Samples from Patos Lagoon, SWAO, were divided into two communities based on social and genomic structure between dolphins that show high residency in estuarine waters (Patos Lagoon's estuarine community) and those that strictly reside in coastal waters and do not enter the estuary (Patos Lagoon's coastal community) (Genoves et al. 2020, see Fruet et al. 2017).

Genomic Laboratory Methods

DNA Extraction

DNA was extracted from biopsy samples using a salting-out protocol (Sunnucks and Hales 1996) with modifications.

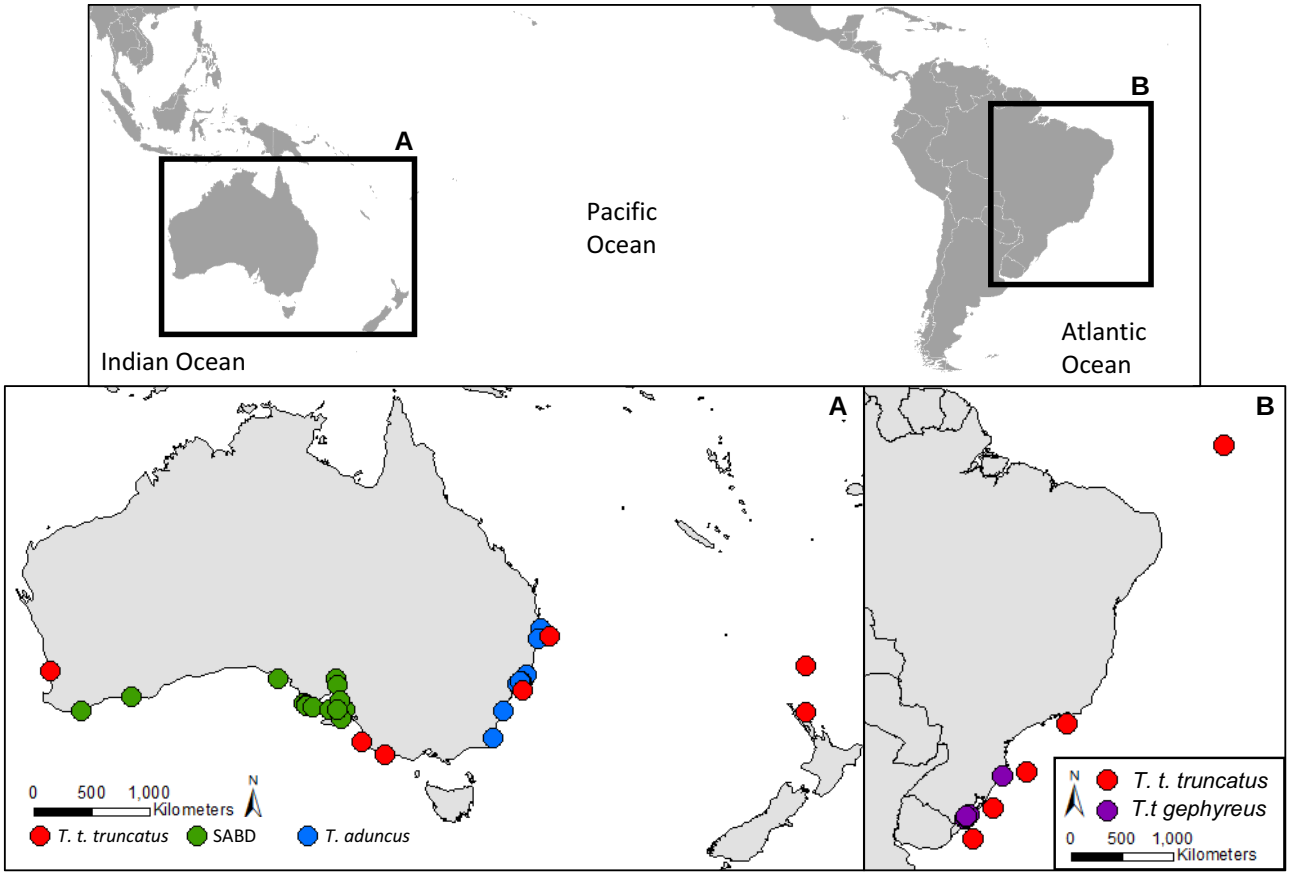

Fig. 6.—Sampling locations of *Tursiops* spp. across three ocean basins in the Southern Hemisphere.

DNA integrity was assessed by gel electrophoresis and purity was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Microsatellite data were used to confirm that no duplicate samples were used, and to remove closely related animals by selecting only one sample from any pair that had a relatedness estimate of ≥0.5 (i.e., theoretical value for first-order relatives). This was calculated in *GenAlEx* (Peakall and Smouse 2006, 2012) using the Queller and Goodnight's (1989) estimator. Microsatellite datasets were already available for many of the sampled locations (Wiszniewski et al. 2010; Fruet et al. 2014; Fruet et al. 2017; Pratt et al. 2018), including from the St. Peter and St. Paul Archipelago samples, Brazil (Fruet et al., unpublished data). Samples from Robe and Cape Nelson in southern Australia did not have an existing microsatellite dataset and therefore, seven loci (Tur80, Tur87, Tur105, TurE12, Tur142, Tur91, and Tur141) were amplified using the polymerase chain reaction (PCR) and genotyped as per conditions specified in Pratt et al. (2018).

ddRAD Library Preparation

Libraries were prepared in house (Molecular Ecology Lab at Flinders University (MELFU)) following a ddRADseq protocol modified from Peterson et al. (2012), as per Brauer et al. (2016). Four of the final multiplexed libraries consisted of 48 individually barcoded samples, while the other five libraries consisted of 96 samples. All libraries were sequenced at the South Australian Health and Medical Research Institute (SAHMRI) on an Illumina HiSeq2000 platform as single-end, 100 base pair (bp) reads. Details about library preparation are provided in Supplementary Material online (supplementary methods S1, Supplementary Material online).

Bioinformatics

The *dDocent* v.2.2.19 (Puritz et al. 2014) pipeline was used to demultiplex and process the raw data files, as per Brauer et al. (2016). *VCFtools* was used to filter the resulting variant call file (VCF) using custom BASH scripts for the filtering steps outlined in supplementary table S2, Supplementary Material online (modified from Brauer et al. 2016). Retained loci were then mapped against the *T. aduncus* genome, downloaded from the National Center for Biotechnology Information (NCBI) (GCA_003227395.1 ASM322739v1). Only loci that aligned to the genome were retained for analysis to exclude potential exogenous

sequences. This process was then repeated after the demultiplexing stage with the inclusion of nine common dolphins (*Delphinus delphis*), to be used as outgroup for phylogenomic analyses. Common dolphin sequences were available from Barceló et al. (2021) and were selected based on the quality of the data available, whilst ensuring that no first-order relatives were included. Details about bioinformatics are provided in Supplementary Material online (supplementary methods S2, Supplementary Material online).

Genomic Variation

Molecular diversity indices for dolphins at each sampling location, including the percentage of polymorphic loci (%PL) and expected and observed heterozygosity (H_E and H_O, respectively) were calculated at the locus level in *Arlequin* v. 3.5.2.2 (Excoffier and Lischer 2010). Wright's inbreeding coefficients (F_{IS}) for each sampling location were calculated as (H_E - H_O)/H_E (Wright 1922). The R package *PopGenKit* (Paquette 2011) and function *popgen* was used to determine the number of PA in each putative lineage (R version 3.6.1).

Genomic Divergence

Phylogenomics

A phylogenomic tree was generated in *RAxML* v.1.5 (Stamatakis et al. 2005) to investigate phylogenetic relationships within the genus *Tursiops*. This was run with nine Australian common dolphins (*D. delphis*) as outgroups, selected based on recent evidence of monophyly of the genus *Tursiops* (Moura et al. 2020). Fourteen individuals showed moderate to high (>20%) admixed membership to more than one lineage (see *Admixture* results; supplementary fig. S2*A–J*, Supplementary Material online). The presence of these individuals, which were found in samples from all lineages and across ten different locations, is probably due to migration, recent admixture, or shared ancestral polymorphism. These samples were subsequently removed from the phylogenomic analysis presented in the main text but results from the full dataset can be found in Supplementary Material online. *RAxML* was run using the GTRGAMMA model of evolution and 1,000 resampling estimated log-likelihood (RELL) bootstraps. The output was visualized in *FigTree* v.1.4.3 (Rambaut 2014), rooted with the outgroup.

Population Genomic Structure

Genomic divergence was assessed among and within the four lineages (*T. t. truncatus, T. t. gephyreus, T. aduncus*, and SABD). We use the term "population" when referring to a putative lineage. *Arlequin* was used to estimate pairwise genomic differentiation (F_{ST}) and corresponding significance levels among sampling locations based on 10,000 permutations. To account for multiple testing, significance levels were corrected using Benjamini and Yekutieli's (2001) method (B-Y correction) (see Narum 2006). This resulted in an alpha (α) level of 0.0076. F_{ST} values among and within the four putative lineages were averaged across sites. To establish the most statistically supported number of populations in the dataset, the model-based maximum-likelihood method in *Admixture* v.3.5.2.2 (Alexander et al. 2009) was run testing for population values from one to 25 (based on the number of sampling localities and putative populations). The lowest cross validation error value was used to determine the most likely number of populations (*K*) present in the dataset. We also used the nonmodel PCA via the *adegenet* R package (Jombart 2008; Jombart et al. 2010; Jombart and Ahmed 2011; Francois et al. 2015). Due to close association of *T. t. truncatus* and *T. t. gephyreus* individuals in the PCA, this analysis was rerun with just individuals from these two taxa to further investigate the subspecies level division proposed between these lineages. *Arlequin* was then used to carry out an ANOVA, testing the level of genomic variance explained by lineage division compared to sampling location.

Genomic Basis of Ecotype Formation

Candidate Loci Detection

Two outlier loci detection methods were used to investigate the genomic basis of ecotype formation in bottlenose dolphins. RandomForest was implemented in R using the *rfPermute* and *randomForest* packages (v.4.6-14) (Breiman 2001). The *na.roughfix* function was used to impute missing data before beginning the analysis. RandomForest was run with 125,000 trees and default settings for the proximity and importance parameters. The number of randomly chosen SNPs tested for each split of the tree (mtry) was set to the value that minimized the out-of-bag error rate and computational time (as suggested by Brieuc et al. 2018). The permutation method was used to calculate significance values for each SNP to statistically assess the likelihood of that SNP being a candidate for selection (see Brieuc et al. 2018). Candidate loci were selected by plotting importance value distributions and selecting those SNPs above the upper elbow of the distribution curve as candidates (e.g., Batley et al. 2019). The second method was the coalescent-based FDIST (Beaumont and Nichols 1996) run in *Arlequin* under the hierarchical island model with 100,000 simulations and 100 demes. The number of groups was set to the number of sampling locations, plus one. Using the *p.adjust* function in the R package *plyr* (Wickham 2011), *P*-values were false discovery rate (FDR) corrected to avoid biases due to multiple testing (Whittemore 2007). Loci with a FDR <10% were classified

as candidates for being under selection. Both methods were first run on pairwise comparisons of offshore *T. t. truncatus* with each of the three inshore lineages (*T. t. gephyreus*, *T. aduncus* and SABD). Outlier loci identified by both methods were combined into a single list for each pairwise comparison. Loci identified as outliers in all the three lists were selected for further analysis. We consider these loci as being putatively under selection in each instance of inshore ecotype evolution in the Southern Hemisphere and therefore, potentially implicated in parallel genomic evolution of the inshore bottlenose dolphin ecotype. They will hereafter be referred to as the "parallel evolution candidates". The two methods were then run to separately compare SWAO *T. t. truncatus* with *T. t. gephyreus*, as this is likely the most recent ecotypic divergence in the study region based on the short branch lengths in the phylogenomic results below and previous findings (Moura et al. 2013; Oliveira et al. 2019). Candidate loci identified between SWAO *T. t. truncatus* and *T. t. gephyreus* will therefore, potentially reveal adaptations key to the early stages of colonization of the inshore environment. These candidates will hereafter be referred to as the "early stage evolution candidates". Genotype frequencies were then calculated and plotted for all early stage evolution candidates and for parallel evolution candidates with an F_{ST} value in the top 10%. It is important to note that even after this hierarchical discarding approach was applied, it is possible that some of the loci identified may still represent false positives.

Functional Enrichment Analysis and Annotation

Flanking sequences for each SNP (300 bp either side) were extracted from the *T. aduncus* genome to carry out a functional enrichment analysis. A BLAST was performed using blastn (Altschul et al. 1990; Sayers et al. 2019) from the nucleotide database available through NCBI on the 601 bp sequences of all 18,060 loci, using an expectation (e) value of 1E-6. All "blasted" loci were then mapped and annotated in Blast2GO with an e-value of 1E-3 (Conesa et al. 2005). A functional enrichment analysis using a Fisher's exact test to look for over- or under-representation of gene otology (GO) annotation terms in the parallel evolution candidates was then conducted in Blast2GO using an alpha value of 0.05. This could not be repeated for the early stage evolution candidates due to a low number of loci. To further investigate the putative functions of the candidate loci and their associated genes, locus sequences were run in the NCBI web BLAST search against the *T. truncatus* genome assembly (NIST Tur_tru v1 Reference Annotation Release 101) (Altschul et al. 1990; Sayers et al. 2019). A threshold of an e-value of 1E-3 and an identity of >90% were used to select the most reliable candidates. Candidate genes were identified within 20 kilobases (KB) of the query sequence (as previously used for SABD; Batley et al. 2019). Putative gene functions were then investigated in UniProtKB using the Swiss-Prot database (Boutet et al. 2007; UniProt Consortium 2019).

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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Permits and Ethics Approvals

Southern/Western Australia

Biopsy samples were collected with Ministerial Exemption from Primary Industries Resources South Australia (PIRSA), exemptions #9902404, #9902648, #9902714, and #9902601, with permits #K25761-6, #E25889, and #E26171 from the Department of Environment, Water and Natural Resources (DEWNR), South Australia, #SF008961 from the Department of Environment and Conservation, Western Australia and #2008-0001 from the Department of Environment, Water, Heritage and the Arts (for sampling in Commonwealth waters). Animal ethics approvals were acquired from the Flinders University Animal Welfare Committee, projects #E310, #E375, and #E326.

Eastern Australia

Biopsy samples were obtained under licenses from the Department of Environment and Climate Change (license

Number: S10763) and Marine Parks Authority (Permit Number: PSGLMP 2008 / 003) and under approval by the Macquarie University Animal Ethics Committee (AEC Reference Number: 2007 / 013) as per Wiszniewski et al. (2010, 2012).

New Zealand

Samples were collected under Massey University, NZ permits and imported into Australia under Australian Quarantine and Inspection Service permit 0001172530 and the relevant CITES Appendix II permit (NZ013 to AU089) in March 2017.

Southwestern Atlantic Ocean

Samples were collected under regional permits (Brazil: SISBIO 16586-2issued to ER Secchi, SISBIO 24407-2 issued to PF Fruet) and transferred to Australia under CITES permits 11BR007432/DF and 2011-AU-647980.

Data Availability

Data are available in a repository and can be accessed via a DOI link. The data underlying this article are available in figshare, at DOI: 10.6084/m9.figshare.23354078.

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