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Mitochondrial sequence divergence among Antarctic killer whale ecotypes is consistent with multiple species

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Recently, three visually distinct forms of killer whales (*Orcinus orca*) were described from Antarctic waters and designated as types A, B and C. Based on consistent differences in prey selection and habitat preferences, morphological divergence and apparent lack of interbreeding among these broadly sympatric forms, it was suggested that they may represent separate species. To evaluate this hypothesis, we compared complete sequences of the mitochondrial control region from 81 Antarctic killer whale samples, including 9 type A, 18 type B, 47 type C and 7 type-undetermined individuals. We found three fixed differences that separated type A from B and C, and a single fixed difference that separated type C from A and B. These results are consistent with reproductive isolation among the different forms, although caution is needed in drawing further conclusions. Despite dramatic differences in morphology and ecology, the relatively low levels of sequence divergence in Antarctic killer whales indicate that these evolutionary changes occurred relatively rapidly and recently.

Keywords: Antarctica; killer whale; *Orcinus orca*; phylogenetics

1. INTRODUCTION

As top marine predators, killer whales (*Orcinus orca*) are known to consume a wide range of food items, from small fishes to the largest whales (Ford 2002). Although generally considered to be monotypic, in some areas such as the northeast Pacific, up to three sympatric ecotypes of killer whale are recognized, which specialize on different prey types and show at least some degree of reproductive isolation (Hoelzel & Dover 1991; Barrett-Lennard 2002; Hoelzel *et al.* 2002, 2007). According to two recent papers (Pitman & Ensor 2003; Pitman *et al.* 2007), there are also three visually distinct forms of killer whales in Antarctic waters, which may be separate species; these were designated types A, B and C (figure 1). Type A has the typical black and white coloration; it inhabits ice-free waters and appears to prey mainly on cetaceans, particularly Antarctic minke whales

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(*Balaenoptera bonaerensis*). Type B is grey, black and white, with a larger eyepatch and a distinct dorsal cape; it forages in pack ice and feeds on pinnipeds. Type C is similar to type B in appearance but with a narrow, oblique eyepatch; it is most frequently encountered in dense pack ice where it specializes on fishes.

Owing to the lack of skeletal material, it is not clear how these ecotypes compare to previously described 'new' species of Antarctic killer whales (Mikhalev *et al.* 1981; Berzin & Vladimirov 1982), but the evidence for their distinctness now includes differences in colour patterning, body size, feeding habits and habitat (Pitman & Ensor 2003; Pitman *et al.* 2007). Understanding the degree and pattern of differentiation among these forms will be important not only for resolving their systematics and evolution, but also for identifying the roles that killer whales play in the Antarctic ecosystem.

As part of a preliminary study of genetic variation in Antarctic killer whales, we compared sequence variation in the mitochondrial control region using samples from all three forms. We selected this marker because it has been used globally and has no issues with respect to inter-laboratory comparisons. For speciation questions, we were looking for fixed or nearly fixed differences. We recognize that phylogenetic relationships among these ecotypes cannot be fully resolved without including nuclear markers and research is ongoing to identify appropriate markers. Although inconclusive, this paper provides additional evidence for multiple species of killer whales in Antarctica and offers some insight into killer whale systematics and evolution.

2. MATERIAL AND METHODS

Sequences were generated from 80 killer whale biopsy samples from Antarctic waters;¹ an additional sequence was included from Hoelzel *et al.* (2002) which we identified as from a type A from unpublished photographs. In total, 74 samples were assignable to ecotype based on at-sea identifications or photographs: these included 9 type A, 18 type B, 47 type C and 7 type-undetermined samples were from collectors unfamiliar with the different types. All type B samples were taken from the waters near the Antarctic Peninsula save for two from the Falkland Islands; all type C samples came from the Ross Sea and type A from various locations around Antarctica. The sequences were generated according to the methods described by Zerbini *et al.* (2006).

We also compared these Antarctic samples with north Pacific killer whale sequences found in GenBank (accession numbers DQ399074–DQ399082 and DQ851147–DQ851148), as well as sequences in Hoelzel *et al.* (2002). These latter sequences that include the aforementioned Ross Sea sequence were reconstructed from the table of variable sites in Hoelzel *et al.* (2002).

3. RESULTS

The 81 Antarctic samples yielded 16 haplotypes. With the addition of the published sequences, the total dataset consisted of 35 unique haplotypes defined by 36 variable sites (table 1, electronic supplementary material). Although the dataset incorporates sequences from disparate parts of the world, the two most divergent sequences differed by only 1.2%, supporting the observations of Hoelzel *et al.* (2002) regarding the low levels of mitochondrial variation in killer whales. Figure 2 depicts phylogenetic relationships among these haplotypes. The two ice-inhabiting forms (B and C) are closely related and constitute a monophyletic clade. There were three

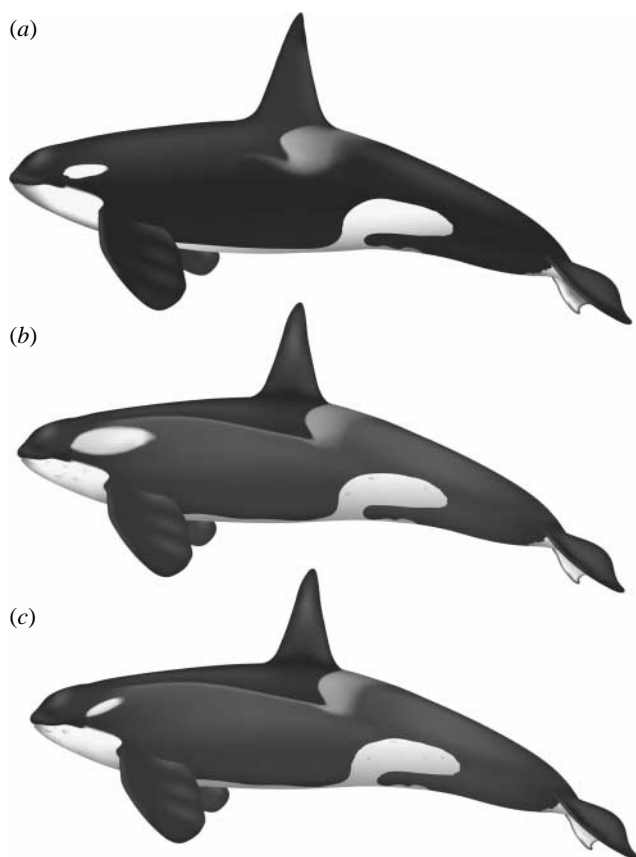


Figure 1. Three different ecotypes of Antarctic killer whales: (a) type A, (b) type B and (c) type C. Type designation from Pitman & Ensor (2003); not to scale. Illustration by U. Gorter.

fixed differences (table 1, electronic supplementary material; sites 479, 539 and 943) between types A and B/C. These included two base substitutions and a single one-base insertion/deletion. There was a single fixed-base difference (site 978) between type C and the other two types. Type A showed a pattern of CATT for sites 479, 539, 943 and 978, respectively, while type B showed TG-T and type C showed TG-C for the same sites. Among the seven samples of undetermined ecotypes, four showed type A sequence pattern and two showed type B pattern; the remaining sample (haplotype 7, closely related to the B/C clade) was unique and had TATT for the four sites.

4. DISCUSSION

Three fixed differences between type A and the two ice-inhabiting forms is congruent with previous suggestions of separate species status. The genetic pattern suggests that for females, at least, there is no gene flow between them. However, the genetic evidence for reproductive isolation between types B and C (one fixed difference) is notably weaker; the variation within ecotypes is equivalent to the differences between them. This single fixed difference is therefore also consistent with simple geographical variation within a single species, especially considering that types B and C were sampled at geographically disparate locations. Determining the relationship between types B and C, and indeed among all the

types, will require more extensive sampling, especially from areas where they co-occur (i.e. east Antarctica), as well as data from independent genetic markers.

It seems paradoxical that the types B and C can appear very different in external morphology, with no recorded intermediates, and yet be nearly identical in their control region sequences, a gene noted for its high levels of variation and rapid evolution. One could argue that unless intermediate phenotypes are found, the most parsimonious explanation is that these nevertheless represent different species, arising from a very recent divergence. However, caution dictates that any conclusion about reproductive isolation also requires data from other genetic markers and morphology, and on seasonal movements and breeding patterns; these are not currently available.

Whether or not interbreeding among the different types occurs, the pattern of genetic variation may say something about the relative plasticity of non-molecular characters. The largest degree of sequence differentiation, seen between types A and B/C, coincides with the most obvious morphological differences (body size and coloration), as well as the differences in habitat preference (open water versus ice). The body size differences alone are substantial: type C reaches a maximum of 6 m while type A attains 9 m, 50% larger (Pitman *et al.* 2007). Among all three, the differences in prey preferences and morphology are all fairly dramatic regardless of the degree of genetic differentiation. There are some parallels between the Antarctic killer whales and the better studied north Pacific killer whales. In both, there are three known largely sympatric ecotypes (resident, transient and offshore in the north Pacific), and divergence in both communities seems to focus on specializations in habitat (inshore versus offshore) and prey (fishes versus mammals). However, morphological divergence among Antarctic forms is much more dramatic than among the north Pacific ecotypes. For both regions, osteological comparisons are needed, but efforts are hindered by a paucity of specimens.

Figure 2 also provides a context for examining this variation relative to samples from other geographical regions. Despite low levels of variation within the types, types B and C contribute to killer whale variation on a global scale as the sole representative of an entire clade. By contrast, type A killer whales from Antarctica are interspersed throughout the rest of the tree among those from other regions. In spite of the small sample size, type A killer whales thus show the highest level of sequence variation. This may be due to multiple populations being sampled. If type A killer whales represent seasonal visitors that follow migrating minke whales into Antarctic waters (Pitman & Ensor 2003), it is possible that this sample set represents multiple breeding populations from low latitudes of the different ocean basins.

How distinct then are Antarctic types B and C in a global context? If one were to categorize killer whales from outside Antarctica according to the coloration designations used herein, all the killer whales in figure 2 from outside Antarctica for which we have documentation would be considered as type A.

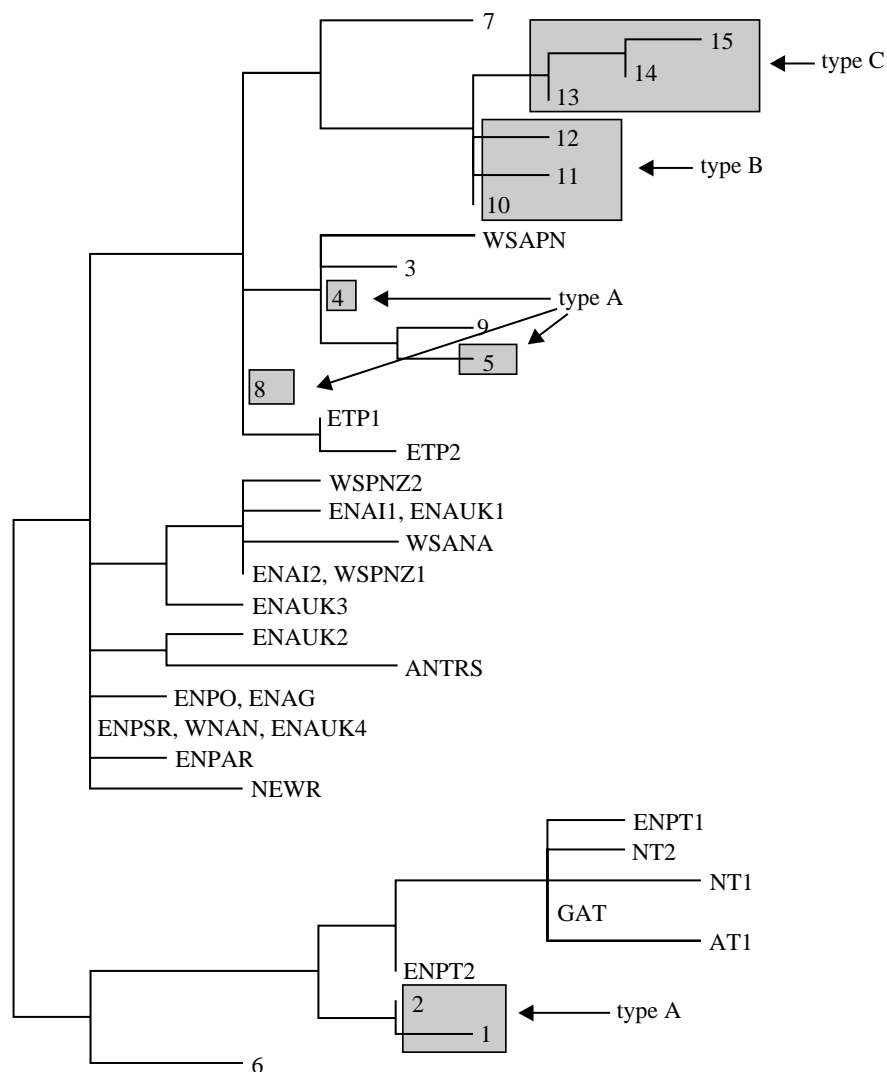


Figure 2. Global phylogenetic relationships of killer whale haplotypes (majority-rule consensus of the 1530 most parsimonious trees); haplotype designations are indicated in the electronic supplementary material. Shaded boxes indicate those haplotypes that were found in Antarctic whales of known ecotype.

Furthermore, all these haplotypes from other areas have CATT at the four diagnostic sites discussed above; the same pattern as in type A killer whales from Antarctica. In this sense, the diagnostic sites for types B and C are not area specific; the appearance and the genetic pattern are congruent on a global scale. In other words, the mutations that resulted in the diagnostic differences for types B and C appear not to have occurred in killer whales anywhere else. The congruence of these lines of evidence suggests that the divergence between these types represents a species boundary.

Although the existence of sympatric killer whale ecotypes has been recorded in different regions, on a global scale there does not appear to be congruence between ecotype and genotype (Hoelzel *et al.* 2002); specific prey specializations apparently had multiple independent origins. Whether or not any of these scenarios represent speciation events (either complete or incipient) remains an open question; some specialization may merely reflect behavioural preferences of particular family groups, and therefore perhaps reversible. Although data are lacking from many killer

whale populations around the world, ecological specialization and divergence does seem to be more prevalent at high latitudes, which may reflect higher densities of schooling fishes and mammals. The divergence from the more widespread type A to types B and C ecotypes seems to have occurred only once, as evidenced by the monophyly of the B/C clade, and seems to have taken place in Antarctica. Some uncertainty about the biogeography of this clade will persist until more is known about the distribution and seasonal movements of the two types.

Killer whales are arguably one of the best-studied and most eminently recognizable large animals on Earth, making the discovery of one or perhaps two new species a bit surprising. Recently, however, a number of studies have detected the presence of other previously unrecognized cetacean species based on morphological and genetic data (e.g. Dalebout *et al.* 2002; Wada *et al.* 2003; Beasley *et al.* 2005). Taken together, these findings indicate that cetacean diversity in the world's oceans may be substantially underestimated, and that there is still much to learn about even the largest of mammals.

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ENDNOTE

¹Samples collected by SWFSC were under MMPA permit no. 774-1437-05.

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