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Genetic Differentiation among Recently Diverged Delphinid Taxa Determined Using AFLP Markers

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Abstract

In the mid-1990s, a new common dolphin species (*Delphinus capensis*) was defined in the northeast Pacific using morphological characters and mitochondrial DNA (mtDNA) sequences. This species is sympatric with a second species, *Delphinus delphis*; morphological differences between the two are slight and it is clear they are closely related. Does the phenotypic distinction result from only a few important genes or from large differences between their nuclear genomes? We used amplified fragment length polymorphism (AFLP) markers to broadly survey the nuclear genomes of these two species to examine the levels of nuclear divergence and genetic diversity between them. Furthermore, to create an evolutionary context in which to compare the level of interspecific divergence found between the two *Delphinus* taxa, we also examined two distinct morphotypes of the bottlenose dolphin (*Tursiops truncatus*). A nonmetric multidimensional scaling analysis clearly differentiated both *Delphinus* species, indicating that significant nuclear genetic differentiation has arisen between the species despite their morphological similarity. However, the AFLP data indicated that the two *T. truncatus* morphotypes exhibit greater divergence than *D. capensis* and *D. delphis*, suggesting that they too should be considered different species.

Until the mid-1990s, only one species of common dolphin (*Delphinus delphis*) was recognized; the species was considered to have a worldwide distribution in temperate, subtropical, and tropical waters. However, geographic variants have been recorded since this species was described in 1758 (Evans 1994). In 1994, a new species, *Delphinus capensis*, was described from coastal waters of the northeast Pacific using both morphological (Heyning and Perrin 1994) and genetic data (Rosel et al. 1994). These two *Delphinus* species exist sympatrically in northeast Pacific coastal waters, although *D. delphis* is found farther offshore in pelagic waters as well (Heyning and Perrin 1994). Heyning and Perrin's (1994) morphological study indicated two distinct species based on color patterns, external morphology, and cranial characters. While characters related to rostral length are fixed between the two species, other morphological features exhibit only modal differences, although no mature intermediate specimens were found (Heyning and Perrin 1994). Rosel et al. (1994) confirmed genetic separation: the two species share no haplotypes in either mitochondrial DNA (mtDNA) control region or cytochrome *b* sequences. Control region sequence divergence between these species is relatively low, estimated at 1.11%. In comparison, control region sequence divergence between two other delphinine taxa, *Stenella frontalis* and *Stenella attenuata*, is 6.2% $\pm$ 1.6% (SE) (Bero D, unpublished data).

Similar to the *Delphinus* species, two sympatric *Tursiops truncatus* morphotypes in the western North Atlantic have

Table 1. Species, collection location, number of specimens (*N*), and collection date for individuals used in this study (excluding "unknown" blind-run samples)

Species	Location	*N*	Sampling dates
Delphinus delphis			
	Eastern Tropical Pacific	6	1978–1982
	Black Sea	8	n/a
	Western North Atlantic	8	1998–2000
	Northeast Pacific (California)	21	1988–1991
Delphinus capensis			
	Northeast Pacific (California)	17	1986–1991
Tursiops truncatus (coastal morphotype)			
	Western North Atlantic	8	1998–2000
	Gulf of Mexico	3	1999
T. truncatus (offshore morphotype)			
	Western North Atlantic	5	1998–1999

also been differentiated recently using mtDNA sequences and microsatellite loci (Hoezel et al. 1998; Rosel PE, unpublished data). These coastal and offshore groups exhibit morphological and ecological distinctions as well, although neither is the Indo-Pacific species *Tursiops aduncus* (Mead and Potter 1995). Whether other similarly differentiated morphotypes of *T. truncatus* in various regions of the world represent analogous taxa remains unresolved, so the two morphotypes are still pooled and designated as one species (Rice 1998).

Although both the *Delphinus* and *Tursiops* taxa can be distinguished from each other using the mtDNA control region, robust phylogenetic reconstructions of all members of their subfamily, Delphininae, are more difficult. The mtDNA control region is too variable within, and not divergent enough among, these species to aid in construction of well-supported phylogenies (Bero 2001; Dizon et al. 2000). Similarly mtDNA cytochrome *b* sequences have been unable to support robust phylogenies describing the evolutionary relationships among all the species in this subfamily (LeDuc et al. 1999). Our inability to construct reliable phylogenies for this subfamily has hampered investigations of the evolution of these species. In addition, conservation and management problems can arise from the lack of a reliable species identification method (Dizon et al. 2000). Since some species in the subfamily Delphininae cannot be distinguished at mtDNA loci with statistical support (Bero 2001; LeDuc et al. 1999), a reliable genetic marker for species identification among the delphinine species is needed.

The amplified fragment length polymorphism (AFLP) assay is a recently developed technique that can elucidate hundreds of genetic markers useful for species identification and phylogenetics (Buntjer et al. 2002; Vos et al. 1995). The method augments restriction fragment length polymorphism (RFLP) analysis with the amplification power of the polymerase chain reaction (PCR). The novel assay utilizes the known sequence of restriction enzyme cut sites; using these sites, the AFLP method allows for ligation of synthetic oligonucleotide adapters to restriction fragments of the genome. Using the AFLP primers complementary to the synthetic adapters, fragments from across the genome can be amplified with no prior knowledge of genomic sequence (Vos et al. 1995). Increased statistical power stemming from the great number of markers produced is one strength of the AFLP method. AFLP markers are highly reproducible (Ajmone-Marsan et al. 1997; Bagley et al. 2001) and comigration of nonhomologous markers is rare (Ajmone-Marsan et al. 2002; Buntjer et al. 2002). In addition, AFLP markers are derived mainly from the nuclear genome, offering both paternal and maternal genetic history. Buntjer et al. (2002) also suggest that AFLP markers have relatively slow coalescence due to the point mutations, insertions, and deletions from which their presence or absence is derived. Because of this evolutionary property, AFLP markers are appropriate for surveying nuclear genetic variation among species (Buntjer et al. 2002). Gatesy and O'Leary (2001) also suggest that the robustness of molecular phylogenies is augmented when multiple markers from across the genome are utilized.

AFLP markers have been used on other vertebrate species to answer population-level and phylogenetic questions (Ajmone-Marsan et al. 1997, 2001, 2002; Albertson et al. 1999; Buntjer et al. 2002; Herbergs et al. 1999). Buntjer et al. (2002) found hundreds of polymorphic markers among nine bovine species, one-third of which were polymorphic within species. Phylogenetic trees of the Bovini tribe built from these AFLP markers yielded high bootstrap values and resolved topologies (Buntjer et al. 2002).

The two common dolphin species seem ideal for assessing the AFLP assay on a delphinine genome. Mitochondrial haplotype differences are fixed (Rosel et al. 1994), but it is unknown to what degree this differentiation is represented in the nuclear genome. Although the species are morphologically disparate, the phenotypic distinction may result from only a few important loci rather than large differences between the nuclear genomes. In this study, AFLP markers are used to determine the presence and extent of detectable nuclear differentiation between the two closely related *Delphinus* species. Since *Delphinus* samples included in the Heyning and Perrin (1994) morphological study and Rosel et al. (1994) mtDNA survey are used in the AFLP assay, direct comparison of the datasets is possible. In addition, we compared the differences found with AFLP markers between the two *T. truncatus* morphotypes to add scale to the evolutionary divergence of the *Delphinus* species.

Materials and Methods

Sampling

California *D. delphis* (21) and *D. capensis* (17) skin tissue samples were collected from individuals stranded south of Point Conception, California, or via skin dart biopsy (Table 1). Eastern tropical Pacific (ETP) *D. delphis* (6) skin tissue samples were obtained from animals caught incidentally in the yellow-fin tuna purse seine fishery. Black Sea *D. delphis* (8)

skin tissue samples were also from fisheries-related incidental catch. California and ETP sample tissues were stored at –20°C or –80°C from the time of collection. Black Sea tissue samples were stored in NaCl-saturated 20% dimethyl sulfoxide from the time of collection. The California samples are part of the specimen base used in the Heyning and Perrin (1994) morphological study and include the same individuals used in the Rosel et al. (1994) mtDNA analysis. All ETP and six Black Sea individuals were also included in the mtDNA analysis (Rosel et al. 1994). Western North Atlantic *D. delphis* skin samples were collected during National Marine Fisheries Service (NMFS) biopsy cruises (6) or from stranded animals (2) on the U.S. east coast. Tissue from the Atlantic animals was stored in NaCl-saturated 20% dimethyl sulfoxide from the time of collection.

Three *T. truncatus* skin tissue samples were collected from stranded animals on the U.S. Gulf of Mexico coast. The remaining *T. truncatus* samples (13) were collected via skin dart biopsy in the western North Atlantic. Tissue was stored in NaCl-saturated 20% dimethyl sulfoxide from the time of collection. Within the *T. truncatus* sample set, 5 of the 16 individuals have been identified as the distinct offshore morphotype and the remaining 11 identified as the coastal morphotype using mtDNA sequences (Rosel PE, unpublished data). These *T. truncatus* morphotypes are both distinct from *T. aduncus* found in Indo-Pacific waters (Curry 1997; LeDuc et al. 1999; Rosel PE, unpublished data).

Finally, the potential utility of AFLP markers in species identification was tested via a blind experiment. Three skin samples each of California *D. capensis* and *D. delphis* were provided by the NMFS Southwest Fisheries Science Center bearing only the numbers 01 to 06 for identification. All six samples were treated as "unknowns" and analyzed in the same fashion as all the other samples.

DNA Extraction and AFLP Markers

DNA from California, ETP, and Black Sea samples was extracted before the inception of this study (see Rosel et al. 1994). DNA from the remaining samples was extracted according to a standard proteinase K method, as described in Rosel and Block (1996), using half the volume (250 µl) of extraction buffer. DNA concentrations (ng/µl) were assessed on a Hoefer DyNA Quant 200 fluorometer.

The AFLP assay was run according to the protocol of Vos et al. (1995) and PE Applied Biosystems (1997) with a few exceptions. During the AFLP process, nuclear DNA was first digested with two restriction enzymes simultaneously, *Eco*RI as the rare cutter and *Taq*I as the frequent cutter. *Taq*I was used as the frequent cutter enzyme rather than *Mse*I; *Taq*I increases resolution because it creates fewer fragments in larger, guanine/cytosine-rich vertebrate genomes (Vos and Kuiper 1996). Double-stranded synthetic adapters (Table 2) were ligated to the "sticky ends" of the resultant fragments to serve as templates for PCR primers. Two rounds of PCR (preselective and selective) using primers complementary to the synthetic oligonucleotide adapter sequence amplified and labeled the fragments. The second, selective PCR incorporated primers with three selective bases that extended into the fragment beyond the adapter sequence; different combinations of these selective primers selected out different fragment sets, depending on which three selective bases were chosen for both forward and reverse primers (Herbergs et al. 1999; Vos et al. 1995). *Eco*RI-selective primers (Table 2) were fluorescently labeled for detection on an ABI 310 PRISM genetic analyzer.

Table 2. AFLP adapter and preselective primer oligonucleotide sequences

Primer	Sequence
*Eco*RI adapter	5′-CTCGTAGACTGCGTACC-3′
*Eco*RI adapter	5′-AATTGGTACGCAGTCTAC-3′
*Taq*I adapter	5′-GACGATGAGTCCTGAC-3′
*Taq*I adapter	5′-CGGTCAGGACTCAT-3′
*Eco*RI + 1 preselective	5′-GACTGCGTACCAATTCA-3′
*Taq*I + 1 preselective	5′-GATGAGTCCTGACCGAA-3′

A preliminary optimization study was performed to evaluate the efficiency of 25 selective primer combinations chosen at random from a possible 64 (Table 3). These primer combinations were tested on six *D. delphis*, two *Stenella frontalis*, and two *T. truncatus* samples, all from the western North Atlantic (data not shown). Twenty of the 25 selective primer combinations tested generated scorable polymorphic markers (Table 3). The remaining five selective primers, all labeled with HEX fluorescent dye, resulted in poor-quality electropherograms, which we did not attempt to optimize. These primers were excluded from the present study.

An initial 30 µl restriction enzyme reaction contained 200 ng of whole genomic DNA, 5 units of *Eco*RI (New England Biolabs, Beverly, MA), and 100 µg/ml bovine serum albumin (BSA) in 1× *Eco*RI buffer. After an hour incubation at 37°C, 15 units of *Taq*I (New England Biolabs, Beverly, MA) in 10 µl of *Eco*RI 1× buffer were added to the mixture, which was then incubated at 65°C for two more hours. After the digestion was complete, 5 pmoles of *Eco*RI adapters, 50 pmoles of *Taq*I adapters (Table 2), and 1 unit T4 DNA ligase (New England Biolabs, Beverly, MA) in 10 µl 5× (for a final concentration of 1× in 50 µl) T4 DNA ligase buffer with ATP were added. The ligation reaction was then incubated 3 h at 37°C.

The restriction digest and ligation was followed by two PCRs designed to amplify specific subsets of markers. Normally the restriction-ligation products are diluted prior to the preselective PCR round (Vos et al. 1995). However, since studies of cetacean genetics often incorporate skin tissue from stranded animals, we needed to address the potential effect of degraded DNA on the robustness of the AFLP method (approximately 11% of our samples had degraded DNA). Departing from the Vos et al. (1995) assay, we bypassed the dilution of the restriction-ligation products for all samples, which increased electropherogram quality. Furthermore, poor-quality DNA samples showed reduced amplification of larger fragments due to the degradation of the DNA. To stabilize amplification of larger fragments in

Table 3. Selective primer combinations chosen (marked with X) for use in the AFLP assay[a]

	*Taq*I							
*Eco*RI	AAC	AAG	ACA	ACT	AGA	AGT	ATC	ATG
AAC, 6-FAM	X	X			X	X	X	X
AAG, TET		X						
ACT, 6-FAM	X	X	X					
AGA, TET		X	X	X	X		X	
ATC, 6-FAM	X		X		X			
ATG, TET				X	X			

[a] Rows contain selective trinucleotide extensions attached to the 3′ end of the fluorescently labeled *Eco*RI primer—5′-GACTGCGTACCAATTC–NNN-3′. Columns contain selective trinucleotide extensions attached to the 3′ end of the *Taq*I primer—5′-GATGAGTCCTGACCGA–NNN-3′.

poor-quality samples, bovine serum albumin (BSA; final concentration 0.16 mg/ml) was added to both preselective and selective PCRs. Thus, for the preselective amplification, 5 μl of restriction-ligation product were added to a mixture containing 75 ng of each preselective primer containing an additional selective 3′ nucleotide (Table 2), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.16 mg/ml BSA, and 1 unit of *Taq* polymerase (Gibco/Invitrogen, Carlsbad, CA) in 1× PCR buffer in a total volume of 50 μl. The cycling profile consisted of 30 repetitions of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s, with a final 10 min hold at 72°C to ensure complete extension of larger fragments. These products were diluted 10-fold in $TE_{0.1}$ pH 8.0 and used as the template for the selective PCR. For the selective PCR, 5 μl of diluted preselective PCR products were added to 20 μl of 1× PCR buffer containing 5 ng of selective (three selective 3′ nucleotides), fluorescently-labeled *Eco*RI primer, 30 ng of selective (three selective 3′ nucleotides) *Taq*I primer (Table 3), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.16 mg/ml BSA, and 0.4 units of *Taq* polymerase (Gibco/Invitrogen, Carlsbad, CA). Touchdown PCR was used to ensure highly specific primer-template binding: 13 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 60 s, where the annealing temperature dropped 0.7°C with each repetition, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s, with a final 7 min hold at 72°C. One microliter of the final labeled product was then loaded with an internal size standard (GeneScan-500, Applied Biosystems, Foster City, CA) onto an ABI 310 PRISM genetic analyzer for fragment detection. Fragment size was determined from the standard using GeneScan 3.1 software (Applied Biosystems, Foster City, CA).

Scoring and Reproducibility

Resulting electropherograms were scored for polymorphic peaks using Genotyper 2.1 software (Applied Biosystems, Foster City, CA). Peaks were scored as dominant markers, present or absent (1 = present, 0 = absent). Bins 1 bp in size were created for each dominant marker category. We developed a conservative scoring protocol to protect against potential problems associated with uneven amplification among samples and poor amplification of larger fragments for degraded DNA samples. To filter out background, peaks less than 100 fluorescence units were never scored. Markers with evidence of "false-negative" peaks (small, unscorable peaks in a size bin where other samples had larger, scorable peaks) were discarded from all samples. This conservative screening prevented the potential introduction of artifacts into the data due to uneven amplification among samples. Markers used in the analysis were sized between 75 and 300 bp in order to ensure reliable size resolution from the standard curve. The samples with the poorest-quality DNA determined the final marker size range for each primer combination; if samples with poor-quality DNA exhibited weak or missing monomorphic peaks, polymorphic scoring was halted for all samples at the last detectable monomorphic marker, even if it was smaller than the 300 bp upper size limit. This technique prevented the scoring of peaks missing due to DNA degradation rather than genetic variation.

AFLP markers have been shown to be highly reproducible, even across laboratory settings and detection systems (Ajmone-Marsan et al. 1997). In order to verify that this is true for the delphinid genome as well, four individuals (two *Tursiops*, two *Delphinus*) were reprocessed (starting with the AFLP enzyme digestion) after the end of the study. The samples chosen ranged from intermediate to high molecular weight in DNA quality. Each individual was rerun at five selective primer combinations (randomly selected without replacement; i.e., all 20 primer pairs were used in total on the four individuals). The resulting polymorphic bands were then compared to those scored in the study using the same scoring methodology.

Data Analyses

Binary characters, representing the presence or absence of a polymorphic marker, were compiled for each individual and primer combination. This master data matrix was used as the basis for all analyses. In order to confirm that a sample of 60 *Delphinus* individuals could express the majority of potential polymorphic markers, a rarefaction-like analysis was performed. Individuals were added to the scoring process in random order (blind-run samples were excluded). The number of new polymorphic markers was plotted against the number of individuals scored and a logarithmic regression was fit to the scatter plot (JMP 3.2.6; Sall 1999).

A matrix of genetic Jaccard similarity values was created using NTSYSpc (Rohlf 2000), where $J_{xy} = (a)/(a + b + c)$ and where a is the number of polymorphic markers shared by individuals x and y, b is the number of markers present in x but absent in y, and c is the number of markers present in y but absent in x (Jaccard 1908). The Jaccard measure is robust because the calculation does not assume homology among absent bands; this is appropriately conservative considering absent markers may arise from different mutations (Ajmone-Marsan et al. 2002). An ordination technique, nonmetric multidimensional scaling (NMDS), was employed to create a three-dimensional representation of relationships among

individuals as indicated by the Jaccard similarity values. The NMDS analysis was executed using NTSYSpc (Rohlf 2000); three sets of principal coordinate analysis values were used as an initial configuration for better fit. Goodness-of-fit was measured with a stress value ranging from zero to one, where a value of zero indicates perfect fit and a value of one represents a meaningless relationship between the NMDS coordinates and the similarity matrix. The NMDS analysis was performed on the *Delphinus* species alone, the *T. truncatus* groups alone, and all species together.

In order to test whether the NMDS coordinates revealed significant differences between the nuclear genomes of *D. delphis* and *D. capensis*, the squared Euclidean distance between the centroids of the NMDS species clouds was calculated (NTSYSpc 2.11a; Rohlf 2000). This value was then compared to a null distribution of squared Euclidean distances created by 1000 random permutations of the binary data matrix. One-thousand randomized binary data matrices were created from the original data matrix using the permute command in the SEQBOOT module of PHYLIP (Felsenstein 1995). Jaccard similarity matrices were then calculated from these datasets using NTSYSpc. NMDS analysis was performed on the 1000 matrices of Jaccard values; principal coordinates analysis results were used for the initial matrix configurations (NTSYSpc; Rohlf 2000). The three-dimensional NMDS coordinates from each replicate were used to create the null distribution of squared Euclidean distances between centroids against which the value derived from the true dataset was tested (JMP 3.2.6; Sall 1999; method adapted from France 1993).

A neighbor-joining phylogram (Saitou and Nei 1987) for all *Delphinus* individuals rooted with *T. truncatus* was built from Jaccard distance values ($1 - J_{xy}$) using the NTSYSpc njoin module (Rohlf 2000) as well as using total character distance in PAUP* 4.0b10 (Swofford 2000). The total character distance tree was bootstrapped 1000 times using PAUP* 4.0b10 (Swofford 2000).

Finally, each of the six "unknown" samples was assigned a putative species identification based on the resulting NMDS plot and neighbor-joining tree. The AFLP-based species identifications were then compared to those made at the Southwest Fisheries Science Center using mtDNA sequences and skull morphology.

Results

The AFLP assay produced 208 polymorphic markers among 66 *Delphinus* individuals (10.40 $\pm$ 4.91 polymorphic markers per primer combination, mean $\pm$ SD). Among the *Delphinus* samples, each individual exhibited, on average, 30.39 $\pm$ 5.49 (mean $\pm$ SD) polymorphic markers when all 20 primer sets were surveyed. When the *Tursiops* samples were scored concurrently with the *Delphinus*, the assay produced 272 polymorphic markers among all 82 individuals (13.60 $\pm$ 5.24 polymorphic markers per primer combination, mean $\pm$ SD); each individual exhibited 56.05 $\pm$ 8.93 (mean $\pm$ SD) polymorphic markers over all primer sets. Eighteen AFLP markers demonstrated fixed differences between *T. truncatus* and the two *Delphinus* species. DNA quality affected the magnitude and quality of large-fragment (>200 bp) markers. Our conservative scoring practices, instituted as a result of the DNA quality discrepancy, reduced the number of total polymorphic markers available for this study, but increased accuracy. The reproducibility test demonstrated 97.79% accuracy among the reprocessed samples and their initial AFLP assays when scored in pairs only. AFLP fingerprints for each reassayed pair were 100% identical when scored in concert with all 82 original samples using the scoring practices described in the methods section.

Figure 1 illustrates the number of new polymorphic markers discovered as each new *Delphinus* individual was scored. The number of new polymorphic markers diminished with increasing numbers of individuals scored. This curve flattened out at 50 individuals, showing that most of the observed AFLP variation between *D. delphis* and *D. capensis* was represented by 50 animals. When analyzed separately, each species exhibited a similar pattern (data not shown).

NMDS analysis illustrated clear differentiation between the *D. delphis* and *D. capensis* nuclear genomes (Figure 2A). Two distinct species clouds can be seen in three dimensions. The ETP animals 1Dd01 and 1Dd02 were separated from other California and ETP *D. delphis* (squared Euclidean distance = 6.18) while remaining distant from the *D. capensis* species cloud as well (squared Euclidean distance = 6.12). The squared Euclidean distance of 1.41 between the species cloud centroids was significant when compared to a null distribution created by 1000 random iterations of the data matrix, allowing rejection of the null hypothesis of nuclear homogeneity ($P < .001$).

NMDS analysis also revealed that the *T. truncatus* morphotypes form two separate clusters (Figure 2B). Surprisingly the squared Euclidean distance between the *T. truncatus* morphotype cloud centroids was 2.86, twice the distance between the *Delphinus* species. Since the *T. truncatus* sample size is small and not over a wide geographic range, the significance of this distance was not tested statistically. Finally, when all individuals of both species were evaluated together, the distance between the sister *Delphinus* taxa and *T. truncatus* was much greater than the distance between either set of sister taxa (Figure 2C).

A neighbor-joining phylogram rooted with *T. truncatus* (Figure 3) did not reveal reciprocal monophyly between the *Delphinus* species, but relationships within the tree still hold meaning. The topology within *Delphinus* and the robustness of the genus clade itself is not affected by the *Tursiops* rooting; the topology within each genus is identical on an unrooted tree (data not shown). Although nested within the *D. delphis* samples, clade A contains all but one of the *D. capensis* individuals assayed. One *D. capensis* individual, 2Dc12, sits outside clade A. This animal was identified as *D. capensis* using morphology and mtDNA prior to this study. Sample 2Dd09, obtained by biopsy in the field, was originally identified at sea as *D. delphis*. Its placement within clade A (with *D. capensis*) cast suspicion on the initial species identification. We consequently sequenced the control

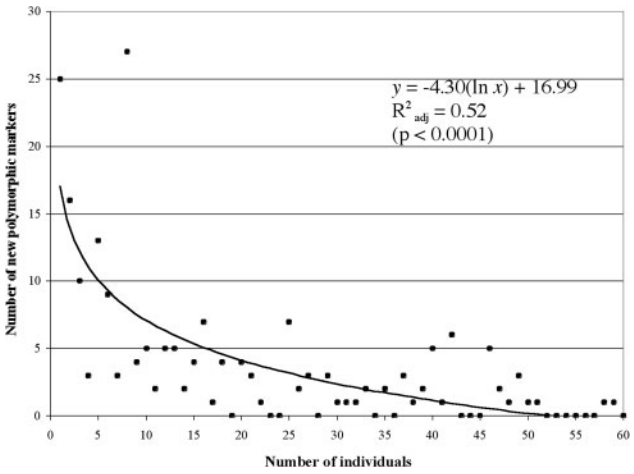

Figure 1. Number of new polymorphic markers plotted against the number of individuals scored within the genus *Delphinus*.

region of the mitochondrial genome for this individual to explore the issue. The mtDNA sequence confirmed 2Dd09's place in the *D. capensis* clade, and the sample has been subsequently reclassified as *D. capensis*.

Upon bootstrap resampling, the neighbor-joining tree lost nearly all node resolution within *Delphinus*, including the node leading to the *D. capensis* samples (Figure 3, node A). Six nodes maintained bootstrap values greater than 50%: node B joins six of the eight Atlantic *D. delphis* assayed, one of the only groupings with a relatively high bootstrap value. Node E groups three of eight Black Sea individuals. Of the remaining nodes with bootstrap values above 50%, node C clusters a suspected mother-calf pair from the Atlantic, while node D connects a pair of Black Sea animals. Only one pair of *D. capensis* clustered significantly, coupled by node F. Two ETP samples, 1Dd01 and 1Dd02, consistently clustered (node G) to the exclusion of all other samples. Within the *T. truncatus* clade, two separate groups were supported by bootstrap analysis. These clusters correspond to the coastal and offshore forms of the species.

The AFLP markers allowed us to correctly identify all of the blind-tested California *Delphinus* samples to species. Each sample was assigned a species designation according to its placement within a NMDS species cloud, since positions of unknown samples in the neighbor-joining tree were inconclusive (data not shown). The samples D02, D05, and D06 fell within the boundaries of the *D. capensis* species cloud on the three-dimensional NMDS plot, while D01, D03, and D04 fell within the boundaries of the *D. delphis* species cloud on the three-dimensional NMDS plot (Figure 2). These species identifications agreed with the identifications made using morphology and mtDNA.

Discussion

This study is the first to apply the AFLP method to questions of cetacean evolutionary biology. The AFLP assay provides a large number of characters randomly amplified from the nuclear genome (Vos and Kuiper 1996). Compared to other organisms surveyed using the AFLP method, the delphinids investigated exhibit lower levels of polymorphism. Ajmone-Marsan et al. (1997) reported a mean of 15.5 polymorphic markers per primer combination within a breed of cattle, compared to the 10.40 $\pm$ 4.91 found in these two dolphin species. Herbergs et al. (1999) identified 8.5 polymorphic markers per primer combination within full-sibling families of the domestic chicken. Thus the level of polymorphism observed between the two *Delphinus* species using AFLP markers is comparable to levels found within species of other taxa. The rarefaction-like analysis (Figure 1) explores our power to detect most or all of the "alleles" at these 20 primer combinations. The apparent saturation in the curve indicates that a sample size of 50 individuals adequately represents the majority of the markers present in the primer pairs we surveyed.

The reduced genetic variation among cetacean species is not unique to this study. Nei's genetic distances calculated among toothed whale species from allozyme data are often as low as values that represent only population-level differentiation within other groups of animals (Shimura and Numachi 1987; Wada 1988). Similarly Schlötterer et al. (1991) found reduced variation at four microsatellite loci among five cetacean species when compared to other vertebrate species. Although the AFLP loci are anonymous, it is likely that many of these markers represent noncoding regions of the nuclear genome. The AFLP results, in concert with the Schlötterer et al. (1991) microsatellite data, suggest that the low genetic variability among cetacean species previously documented in the nuclear genome through allozyme studies is not limited to coding regions, but is, instead, a characteristic of the genome as a whole. Thus strong selective pressure on coding regions as a result of the high ecological specialization of cetaceans (Shimura and Numachi 1987) does not seem to be a plausible explanation for the low genetic differentiation seen among cetacean species. This lack of genetic diversity across cetacean species can be explained by a reduced mutation rate and/or recent rapid radiation of species; however, which of these alternatives (or combinations) is more plausible has yet to be determined (Schlötterer et al. 1991).

Similar to typical delphinine intraspecific mtDNA control region variation (Bero 2001), *D. delphis* seems to exhibit high nuclear intraspecific variation. Two ETP animals, 1Dd01 and 1Dd02, stand out from every other *D. delphis* in all AFLP analyses. Neither individual's mitochondrial control region sequence is abnormally distant from those of other California and ETP animals (Rosel et al. 1994). When 1Dd01 and 1Dd02 are removed from the NMDS plot, the squared Euclidean distance between the *Delphinus* species cloud centroids is even greater (1.45); the position of these unique individuals does not artificially inflate the distance between the species clouds used to reject our null hypothesis. These two samples may simply represent abundant intraspecific worldwide nuclear genetic variation within *D. delphis*.

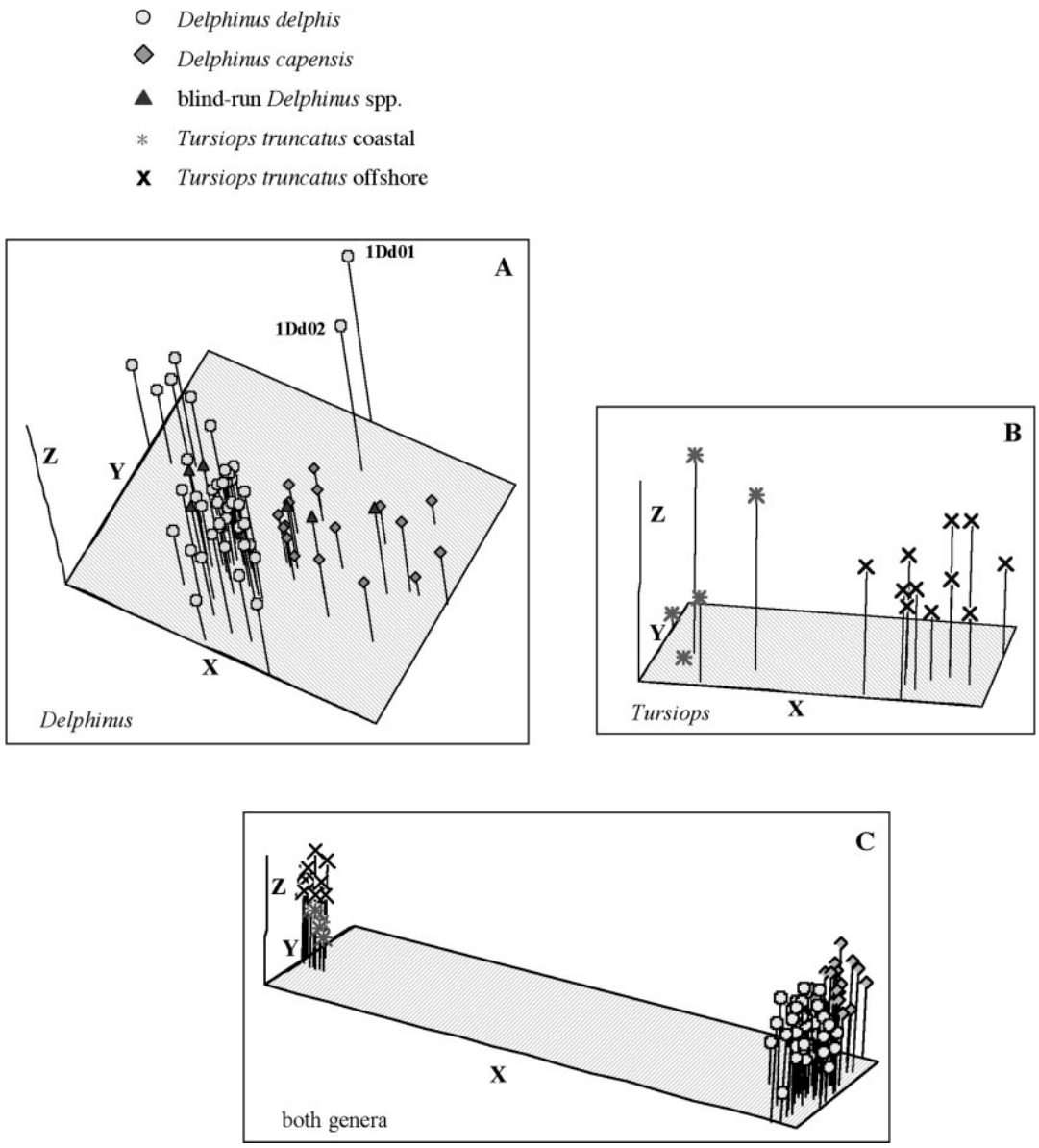

Figure 2. **(A)** Nonmetric multidimensional scaling analysis plot based on 208 polymorphic AFLP markers representing relationships among only *Delphinus* individuals in three dimensions. Stress value = 0.4. Unknown samples D01–D06 were identified based on placement within species clouds. **(B)** NMDS analysis plot of *T. truncatus* samples based on 272 AFLP markers. Two nonoverlapping clusters represent the coastal and offshore types. Stress = 0.13. **(C)** NMDS analysis plot depicting relationships among all individuals and taxa in three-dimensional space based on 272 polymorphic AFLP markers. Stress = 0.05.

Despite the chronic problem of high intraspecific and low interspecific levels of variation typical in delphinids, the AFLP technique is still powerful enough to resolve both sets of closely related *Delphinus* and *Tursiops* taxa. Application of NMDS analysis to the AFLP data reveals significant nuclear genetic separation between *D. delphis* and *D. capensis*. Since few morphological characters are fixed between the taxa and many other characters exhibit modal differences, morphology alone could not confirm the ubiquitous nature of the nuclear differentiation (Heyning and Perrin 1994). This NMDS analysis of AFLP markers demonstrates detectable, abundant nuclear differentiation between the sister species. However, *D. delphis* and *D. capensis* appear to be less separated than the *T. truncatus* morphotypes. The squared Euclidean distance between the *T. truncatus* morphotypes' NMDS analysis centroids (2.86) is more than twice the distance between the *Delphinus* species (1.41). The AFLP data suggest that the *Tursiops* morphotypes may be more genetically divergent than the *Delphinus* species. It should be noted, however, that the smaller sample size for the

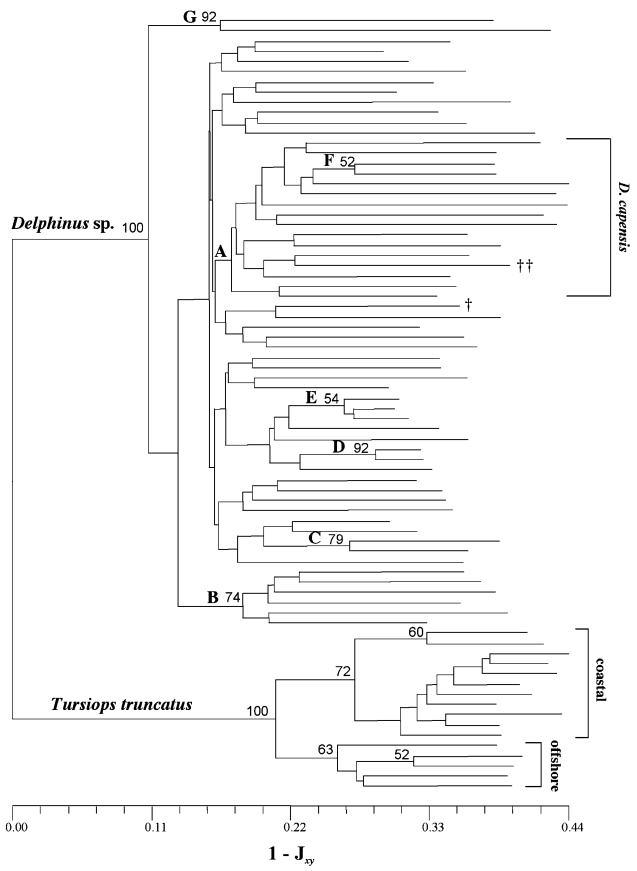

Figure 3. Neighbor-joining phylogram derived from the Jaccard distance matrix displaying relationships between *D. delphis*, *D. capensis*, *T. truncatus* coastal, and *T. truncatus* offshore. Bootstrap values ≥50 from 1000 iterations using total character distance are displayed above the nodes (topology of the neighbor-joining tree derived from total character distance was similar to that derived from the Jaccard distance). Clade A encompasses all *D. capensis* assayed except one (2Dc12, denoted with †). Sample denoted with †† (2Dd09) was identified as *D. delphis* in the field and reassigned a *D. capensis* species designation based on mtDNA control region haplotype and AFLP markers.

Tursiops morphotypes may not encompass all the genetic variation present within the species, potentially inflating the difference between the two on the NMDS plot.

The phylogenetic analysis does not yield monophyly of the two *Delphinus* species, although it does support monophyly of the two *Tursiops* morphotypes. Of interest is that LeDuc et al. (1999) also did not find reciprocal monophyly between the two *Delphinus* species in their phylogenetic analysis using mtDNA cytochrome *b* sequences. The similar pattern found with these two marker systems suggests the *Delphinus* species may be very recently diverged. Since the *Tursiops* morphotypes show greater differentiation in both phylogenetic and NMDS analyses than the two *Delphinus* species, these two morphotypes may represent different *Tursiops* species; in addition, mtDNA sequence analyses indicate that neither of these morphotypes is *T. aduncus* (Rosel PE, unpublished data). A thorough revision of *T. truncatus* will require an increased sample size from a wider geographic range, as well as an investigation of the relationship of these two *T. truncatus* morphotypes to the species *T. aduncus*.

The lack of reciprocal monophyly in the phylogenetic analysis of the AFLP markers contrasts with mtDNA control region data for the *Delphinus* species, but is not surprising: because of the effects of genetic drift, the development of monophyly can be four times slower at nuclear loci than mitochondrial genes (Birky et al. 1989). The nuclear monophyly of a species can be predicted using coalescence ratios; on average, nuclear loci are monophyletic when the mtDNA branch length leading to a species from the most recent common ancestor is three times greater than the average intraspecific mtDNA sequence diversity (Palumbi et al. 2001). *D. capensis* and *D. delphis* share no mtDNA control region haplotypes and are therefore reciprocally monophyletic at this locus (Rosel et al. 1994). Based on mtDNA control region data from Rosel et al. (1994), the coalescence ratios for *D. delphis* and *D. capensis* are estimated as 0.33 and 0.50, respectively. Similarly coalescence ratios based on mtDNA control region sequences for the *T. truncatus* taxa are estimated as 0.26 (offshore) and 0.51 (coastal) (Rosel PE, unpublished data). These low ratios suggest that a very small proportion (<1.0%) of nuclear loci should exhibit monophyly (Palumbi et al. 2001). In a strict sense, only fixed differences between two species reveal reciprocal monophyly. Since no AFLP loci show fixed differences between *D. capensis* and *D. delphis*, the actual nuclear data are consistent with low coalescence ratios. Even though the two *T. truncatus* forms are reciprocally monophyletic on the nuclear neighbor-joining tree, only two AFLP markers are fixed between the morphotypes. These fixed markers represent less than 1.0% of all the polymorphic sites surveyed; again, the low coalescence ratios predict a small proportion of nuclear loci should exhibit coalescence. The fact that the AFLP method detected this small percentage of loci for the *T. truncatus* taxa demonstrates the power of the assay. The results also suggest that targeting single nuclear genes for phylogenetic reconstruction would most likely reveal nonmonophyletic lineages in these species, as was found for *Lagenorhynchus* species (Hare et al. 2002). By virtue of the sheer number of loci, AFLP markers may be a better choice for building phylogenies using nuclear markers for the closely related taxa within the family Delphinidae.

The power exhibited by the AFLP assay stems from the large number of genomic markers generated; even in characteristically less divergent cetacean species, this method can be used to effectively differentiate closely related taxa. This differentiating potential of the AFLP assay suggests that AFLP markers may be able to provide an independent, robust estimation of the phylogenetic relationships among members of the subfamily (Delphininae) containing the *Delphinus* and *Tursiops* taxa. Phylogenetic analysis of the Delphininae has been hindered by a lack of markers with

such power; despite analyses using mtDNA markers, many of the phylogenetic relationships among the delphinine species remain unresolved (Bero 2001; LeDuc et al. 1999).

Together, the nuclear AFLP markers, mtDNA sequence data, and morphological characters illustrate differing aspects of the evolution of the *Delphinus* species. The mtDNA and morphological characters exhibit fixed differences because of a greater mutation rate and effect of genetic drift on mtDNA and likely selective pressure on morphological characters. The entire nuclear genome, represented by AFLP markers, lags behind, revealing smaller-scale, although significant differentiation. It was the fixed morphological and mtDNA differences between *D. delphis* and *D. capensis* that initiated the reclassification of the species (Heyning and Perrin 1994; Rosel et al. 1994). The results derived from AFLP markers add detail and scale to the picture. This method allows us to discriminate between the genomes of the two species, even though they exhibit no fixed nuclear differences. Since the *T. truncatus* morphotypes exhibit greater differentiation than the sister *Delphinus* species, the sympatric common dolphins may be the least divergent of the delphinine taxa. The resolution and quantification of these frequency-based differences between *D. delphis* and *D. capensis* offers us a baseline reference for nuclear differentiation between two recently diverged delphinine species.

Finally, the AFLP method may provide a new tool to aid in cetacean conservation and management. As demonstrated by the blind-run *Delphinus* samples and reclassified *D. capensis* individual (2Dd09), the suite of analysis techniques allows for identification of unknown samples. This ability is desirable for degraded strandings (although scoring of large fragments may be a problem), species difficult to differentiate in the field, exploration of hybridization, and forensic investigation. Even well-defined species within the subfamily Delphininae can be difficult to differentiate using molecular markers like mtDNA (Bero 2001; Dizon et al. 2000; LeDuc et al. 1999). Since AFLP markers allow us to distinguish between the young, closely related species *D. delphis* and *D. capensis*, the markers will most likely be useful for identifying unknowns to species and further resolving evolutionary relationships among other delphinids. AFLP markers proved to be a useful and appropriate tool for assessing genetic diversity among sister taxa; this molecular tool has application potential in other groups of closely related taxa to augment conservation and management.

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