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GENETIC EVIDENCE ON THE DEMOGRAPHY OF SPECIATION IN ALLOPATRIC DOLPHIN SPECIES

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Abstract.—Under a neutral model, the stochastic lineage sorting that leads to gene monophyly proceeds slowly in large populations. Therefore, in many recent species with large population size, the genome will have mixed support for monophyly unless historical bottlenecks have accelerated coalescence. We use genealogical patterns in mitochondrial DNA and in introns of four nuclear loci to test for historical bottlenecks during the speciation and divergence of two temperate *Lagenorhynchus* dolphin species isolated by tropical Pacific waters (an antitropical distribution). Despite distinct morphologies, foraging behaviors, and mitochondrial DNAs, these dolphin species are polyphyletic at all four nuclear loci. The abundance of shared polymorphisms between these sister taxa is most consistent with the maintenance of large effective population sizes (5.09×10^4 to 10.9×10^4) during 0.74–1.05 million years of divergence. A variety of population size histories are possible, however. We used gene tree coalescent probabilities to explore the rejection region for historical bottlenecks of different intensity given best estimates of effective population size under a strict isolation model of divergence. In *L. obliquidens* the data are incompatible with a colonization propagule of an effective size of 10 or fewer individuals. Although the ability to reject less extreme historical bottlenecks will require data from additional loci, the intermixed genealogical patterns observed between these dolphin sister species are highly probable only under an extended history of large population size. If similar demographic histories are inferred for other marine antitropical taxa, a parsimonious model for the Pleistocene origin of these distributions would not involve rare breaches of a constant dispersal barrier by small colonization propagules. Instead, a history of large population size in *L. obliquidens* and *L. obscurus* contributes to growing biological and environmental evidence that the equatorial barrier became permeable during glacial/interglacial cycles, leading to vicariant isolation of antitropical populations.

Key words.—Antitropical, biogeography, *Lagenorhynchus*, lineage sorting, mitochondrial DNA, nuclear gene tree.

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Conceptual views on the genealogical distinctiveness of species have grown increasingly sophisticated (Baum and Shaw 1995; Maddison 1997; Palumbi et al. 2001), but our empirical knowledge of genealogical differentiation at the species level derives mostly from mitochondrial DNA (mtDNA) data (Avise 2000). In a recent review of mtDNA patterns reported for sexual vertebrates, more than half of the putative sister species had large genetic distances separating strongly supported species clades (Avise and Walker 1999). The mtDNA results provide comforting genealogical support to the classical view of species as qualitatively distinct taxa (Mayr 1957), as might be expected if reproductive isolation was an important bridge to both genealogical monophyly and speciation. However, the view provided by mtDNA is not representative of most of the genome and may bias perceptions of evolutionary diversification in two ways. First, as molecular measures of evolutionary distinctiveness are applied more widely in taxonomic and conservation studies, perceptions of genealogical differentiation acquire practical consequences (Palumbi and Cipriano 1998). For example, in conservation biology a working definition for evolutionary significant units (ESU) codifies the perceived importance of genealogical distinctiveness by making mtDNA monophyly a primary criterion of ESU recognition (Moritz 1994). In molecular systematics, the analysis of species relationships without sampling intraspecific variation is justified by the assumption, often untested, of gene monophyly within species. If the monophyly assumption is unfounded, species relationships may be incorrectly inferred. Second, mtDNA studies leave the demographic context of differentiation relatively unexplored because single loci offer low precision on estimates of historical population size (Hilton and Gaut 1998; Edwards and Beerli 2000; Wall 2000) and because the relatively shallow coalescent time for this molecule limits the temporal window for demographic inferences (Knowles et al. 1999).

Do polyphyletic gene lineages persist in species long enough after divergence for molecular analysis to commonly uncover them? This is a familiar question about the expected rate of lineage sorting leading to gene monophyly over time (Tajima 1983; Palumbi et al. 2001) and relates to expectations for gene tree–species tree agreement (Pamilo and Nei 1988; Takahata 1989; Wu 1991; Hudson 1992). However, few empirical studies have moved from a phylogenetic consideration of lineage sorting (Moore 1995; Ruvolo 1997) with a focus on gene tree topology to a sampling of individuals and loci suitable for analyzing the historical processes shaping patterns and rates of genetic differentiation between species (Wang et al. 1997; Nagl et al. 1998; Kliman et al. 2000).

Lineage sorting is the process by which ancestral allelic lineages become fixed in two sister species, either by drift or selection, to produce a reciprocally monophyletic gene tree (Neigel and Avise 1986). Based on coalescent theory, polymorphisms that existed in an ancestor are expected to persist in daughter populations for about $4N$ generations after the onset of reproductive isolation, where N is the effective

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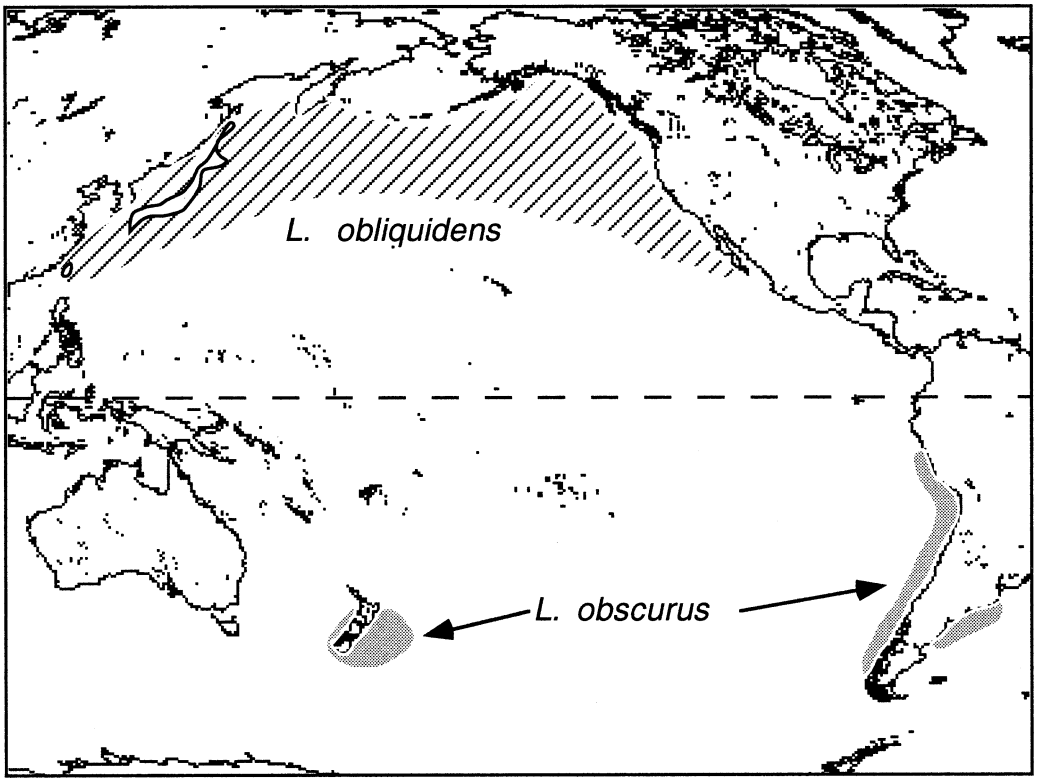

FIG. 1. Distribution map for *Lagenorhynchus obliquidens* and *L. obscurus* in the Pacific.

population size of the daughter populations (Nei 1987, p. 395). Thus, for a given divergence time, historical population size is the key factor determining whether a species is genetically unique at most loci and whether genes are expected to accurately trace the species phylogeny. Animal population sizes range over seven orders of magnitude or more (Soule 1976; Nevo et al. 1984). If the effective size/census size ratio averages 0.1 in plants and animals (Frankham 1995), then the approach to monophyly at nuclear loci may require as few as 400 generations or as many as 4×10^9 in populations of constant size. At the extreme, large populations undergoing rapid speciation, such as in some marine species (Palumbi 1994), could create intermingled genealogical tracings containing very little phylogenetic information among species. For example, nuclear DNA polymorphisms are shared among 12 species of Lake Victoria haplochromid cichlids that probably diverged after Lake Victoria formed 12,000 years ago (Nagl et al. 1998). The Lake Victoria cichlids also shared these polymorphisms across a 1.4 million year divergence with their riverine ancestors (Nagl et al. 1998). The absence of fixed differences at multiple loci is itself informative about population demography during cichlid diversification, indicating that the effective population sizes of cichlid species remained large ($\sim 10^5$) throughout the history of their adaptive radiation. There are many other examples of shared neutral polymorphisms documented between closely related species, but in only a few cases have genealogical patterns been inferred at multiple loci and used to infer historical demography (Takahata and Nei 1985; Hey and Kliman 1993; Takahata and Satta 1997; Wang et al. 1997; Hilton and Gaut 1998; Li et al. 1999; Kliman et al. 2000).

How general is the pattern of slow lineage sorting when measured against the rate of speciation? One predictable exception may involve allopatric populations derived by founder speciation if colonization across a dispersal barrier generally causes a bottleneck in effective population size (Carson and Templeton 1984). Under these conditions lineage sorting may be rapid enough to cause gene monophyly at most loci prior to the next speciation event. Genetic estimates of historical population size provide a valuable test of the assumption that long-distance colonization involved few individuals (Vincek et al. 1997). In addition, recently diverged allopatric populations provide useful models for empirical studies of historical demography. Recent divergence assures that information has not been lost by a fixation of all ancestral polymorphisms at all loci, and allopatry minimizes the likelihood that recent gene flow affected genealogical patterns.

Here, we evaluate the pattern of genealogical differentiation in recently isolated marine taxa to understand the relationship between adaptive divergence, population size, and genetic polyphyly. The dolphin species we studied have a typical antitropical distribution, that is, they are isolated in temperate latitudes of the northern and southern oceans by warm equatorial waters (Fig. 1). In the Northern Hemisphere, Pacific white-sided dolphins (*Lagenorhynchus obliquidens*) inhabit temperate offshore waters across the Pacific Ocean (Brownell et al. 1999). In the central North Pacific *L. obliquidens* has a peak breeding period from March to May (Fer-

raro and Walker 1996). In the Southern Hemisphere, dusky dolphins (*L. obscurus*) live in disjunct coastal populations around New Zealand, South America, and South Africa (Brownell and Cipriano 1999). On the coast of Peru *L. obscurus* has a peak breeding period in September and October, offset by several months from the peak in the North Pacific *L. obliquidens* (Van Waerebeek and Read 1994). These species are sister taxa based on cranial (Miyazaki and Shikano 1997) and mtDNA characters (Cipriano 1997; LeDuc et al. 1999). Divergence to the level of species is indicated by differences in reproductive phenology as well as morphologies and behaviors related to feeding (Miyazaki and Shikano 1997; Brownell and Cipriano 1999; Brownell et al. 1999), traits assumed here to be adaptive.

An affinity for temperate waters by *L. obscurus* and *L. obliquidens* appears to be ancestral because all other members of the genus share this characteristic (Ridgeway and Harrison 1999). Given that these species are adapted to cold water, their recent divergence (Cipriano 1997; see below) suggests that colonization from one hemisphere to the other either involved a one-time dispersal event across the consistently warm tropics, perhaps entailing a population bottleneck, or resulted from extended dispersal by many individuals during oceanographic disruptions of equatorial currents in the Pleistocene, followed by vicariant isolation (Lindberg 1991).

We tested whether nuclear DNA sequences are polyphyletic, and if so, whether extreme reductions in historical population size accompanied divergence in mitochondrial, morphological, and behavioral characters by analyzing intron variation among multiple independent loci in the allopatric *Lagenorhynchus* dolphins. By employing a phylogenetic approach to identify ancestral allele clades, as well as analytical methods to compare patterns across loci (Wakeley and Hey 1997; Wang et al. 1997), the persistence of ancestral variation could be discerned and used to estimate population sizes before and after speciation.

Materials and Methods

Samples of *L. obscurus* were obtained from New Zealand and Peru, and *L. obliquidens* was sampled in the eastern North Pacific. The closest congeneric relative to this species pair is not fully resolved (Cipriano 1997; LeDuc et al. 1999), so the unambiguously allopatric North Atlantic white-sided dolphin (*L. acutus*) was used as an outgroup.

Mitochondrial DNA and Nuclear Intron Data Collection

MtDNA cytochrome *b* (*cyt b*) sequences from a previous analysis (Cipriano 1997) were combined with additional sequences, all 496 bp long, to total 14 individuals from three *Lagenorhynchus* species. Previously developed primers were used to amplify most of an intron in actin (ACT; primers ACT-1 and ACT-7, Palumbi and Baker 1994), in Ca^{+}calmodulin-dependent kinase (*CAMK*, Lyons et al. 1997) and in lysosomal beta-hexosaminidase (*HEXB*, Lyons et al. 1997). *ACT* primers target the first intron in cytoskeletal actin, a member of a small gene family. *CAMK* primers amplify a complete intron from a tissue-restricted and multifunctional protein kinase that is single-copy in both human and mouse (Bland et al. 1994). *HEXB* primers correspond to sequences in exons 6 and 7 of the human single-copy locus (Proia 1988).

Butyrophilin (*BTM*) primers were designed in exon 3 (5′-GTCTCTGATGATGGGGAGTA) and exon 4 (5′-ATCTCT CCACTCTCTTGMAC) of a cow/human/mouse alignment. In the cow, *BTM* is a single-copy gene coding for a mammary-specific milk fat globule membrane glycoprotein (Davey et al. 1997).

For polymerase chain reaction (PCR) each 25-μl reaction used 0.6 units of Takara EX polymerase (Intergen, Norcross, GA) under recommended conditions. Annealing temperature was 55°C for actin and 57–60°C for the other three loci. All amplifications were in rapid-ramping machines (Perkin Elmer 9600, Wellesley, MA; or MJ Research PTC-100, Waltham, MA) and began with the addition of an enzyme mixture during a 1-min hot start at 80°C; denaturing for 2 min at 95°C; then 35 cycles of 94°C for 30 sec, annealing for 30 sec, and extension at 72°C for 2.0–2.5 min; followed by 5 min at 72°C. Amplification products were purified and directly cycle sequenced (FS dye-terminator and 377 sequencer; Applied Biosystems, Foster City, CA). At each locus, sequencing primers were designed at 350-bp intervals in each orientation.

Data Analyses

For nuclear loci in each individual, all double chromatogram peaks and disagreements between DNA strands were evaluated as potential heterozygous sites (Hare and Palumbi 1999). The single consensus diploid sequence for each individual was then added to a population alignment. Any polymorphic position in the population alignment was double-checked in all individuals by reinspecting the chromatograms, and confirmed with restriction assays on reamplified DNA when possible (Hare and Palumbi 1999). To obtain nuclear haplotype data for phylogenetic analysis, the cis/trans orientation of multiple heterozygous sites was determined in sequences from two loci (*BTM* and *CAMK*) by resequencing the original diploid template with allele-specific primers (Hare and Palumbi 1999).

Maximum-likelihood phylogeny reconstruction of mtDNA used two phocoenid outgroups (Rosel et al. 1995), empirical estimates of base frequencies, and the observed *Lagenorhynchus* transition/transversion ratio of 7.8 (fastDNAml 1.0.6, Olsen et al. 1994). Parsimony and neighbor-joining trees were inferred using PAUP* 4.0b4a (Swofford 1998). DnaSP 3.5 (Rozas and Rozas 1999) was used to calculate nucleotide diversity (Nei 1987, p. 256), Jukes-Cantor corrected sequence divergence (Jukes and Cantor 1969), and Tajima's *D* statistic (Tajima 1989). We simultaneously estimated the population mutation parameter θ ($= 4Nu$, where N is effective population size and u is the neutral mutation rate per site per generation) for two sister species and their common ancestor along with T, their divergence time in terms of $2N$ generations, under a strict isolation model using the method of Wang et al. (1997; ''WWH method'' hereafter). The WWH method accounts for the four-fold difference in effective population size between mtDNA and autosomal nuclear loci. All four nuclear loci were assumed to be autosomal.

Relative rates of nucleotide substitution were tested for each locus using RRTree version 1.1 (Robinson et al. 1998).

TABLE 1. Summary statistics for mitochondrial DNA and nuclear intron sequences in three dolphin species, *Lagenorhynchus acutus* (LAC), *L. obliquidens* (LOB), and *L. obscurus* (LOS). Locus abbreviations are given in the Materials and Methods. Ts/Tv, number of transitions divided by number of transversions for all sequences; λ, substitution rate per site per MY; *n*, number of alleles sampled; *k*, number of different alleles observed; *S*, number of variable nucleotide sites; π, average pairwise uncorrected sequence difference ± standard deviation; Δ*K*, substitution rate difference between LOS and LOB lineages, with negative values indicating a higher rate in LOS. None of the relative rate differences or Tajima's *D*-values are significantly different from zero ($P > 0.05$).

Locus	Total bps	Ts/Tv	% GC	λ (%)	Species	*n*	*k*	*S*	Indels	π/bp (× 100)	Δ*K* (× 100)	Tajima's *D*
Cyt b	496	7.8	44	0.581	LOS	5	4	8		0.74 ± 0.16		0.08
					LOB	6	3	3		0.25 ± 0.06	−3.684	−0.45
					LAC	3	3	5		0.70 ± 0.22		
					total	14	10	52	0			
ACT	1130	2.3	36	0.098	LOS	6	5	6		0.22 ± 0.06		−0.35
					LOB	12	3	2		0.05 ± 0.03	0.067	−0.25
					LAC	8	3	2		0.09 ± 0.02		0.93
					total	26	11	13	3			
BTM	1899	1.8	42	0.015	LOS	10	6	8		0.13 ± 0.02		0.14
					LOB	10	3	6		0.15 ± 0.03	0.021	1.32
					LAC	2	1	0		0		
					total	22	10	33	4			
CAMK	2144	3.7	34	0.035	LOS	10	5	5		0.11 ± 0.02		1.28
					LOB	10	6	7		0.10 ± 0.03	0.096	−0.77
					LAC	2	1	0		0		
					total	22	12	36	4			
HEXB	1945	4.3	37	0.016	LOS	10	9	9		0.17 ± 0.03		0.09
					LOB	10	8	9		0.15 ± 0.04	0	−0.67
					LAC	2	2	1		0.05 ± 0.03		
					total	22	19	32	4			

In tests of the relative substitution rate in *L. obliquidens* versus *L. obscurus* using *L. acutus* as an outgroup, topological weighting was employed to correct for biases that potentially result from unbalanced sampling (Robinson et al. 1998). Synonymous substitution rates were compared between lineages for *cyt b* using weights from the maximum-likelihood gene tree topology. Kimura two-parameter substitution rates were compared for introns using topology weights from neighbor-joining gene trees.

RESULTS

DNA Diversity and Divergence

Intraspecific nucleotide and insertion/deletion (indel) variation was low in intron sequences from all four nuclear loci (Table 1). Within species, the nucleotide diversity of nuclear introns varied from 0.05% to 0.22%, with an overall average across loci of 0.13 substitutions per 100 bp. Mitochondrial *cyt b* had average intraspecific nucleotide diversities ranging from 0.25% to 0.75% (Table 1), levels two to eight times higher than at nuclear introns. Low polymorphism precluded strong tests of selection and relative rates of evolution, but no dramatic deviations from neutrality or a molecular clock were apparent (Table 1). The numbers of nucleotide differences per site at nuclear introns between *L. acutus* and the two Pacific species, corrected for intraspecific variation (Nei and Li 1979), ranged from 0.45% to 1.27% for *L. obscurus* and 0.67% to 1.28% for *L. obliquidens*. The number of nucleotide differences per site at *cyt b* for the same comparisons were 9.31% and 8.29%, respectively. Between the two Pacific species, *L. obliquidens* and *L. obscurus*, net difference per site ranged from 0.03% to 0.21% at nuclear introns (average = 0.086 ± 0.082%) and was 1.22 ± 0.4% at *cyt b*.

In a preliminary study (Cipriano 1997), molecular clock calibrations for *cyt b* in cetaceans were based on the rate of accumulation of transversions over time (a single transversion was found at one end of the *cyt b* fragment analyzed). Resequencing and additional specimens (this study) found no transversion differences between *L. obliquidens* and *L. obscurus*. Because there are no reliable fossil cetacean calibration points more recent than the radiation of delphinid families 11 million years ago (Barnes 1990; Fordyce and Barnes 1994), more recent artiodactyl calibrations also were used. In addition, a calibration utilizing noncetacean data must be based on third codon positions because *cyt b* substitution rates vary dramatically among lineages at first and second codon sites (Irwin et al. 1991). For the 496-bp segment of *cyt b* studied here, third codon position transition differences in cetacean and artiodactyl *cyt b* (Table 2) show marked saturation by 11 million years ago, but transversion differences accumulate at a linear rate for as long as 25 million years (Fig. 2). Third position *cyt b* transversion differences between delphinid families and between several artiodactyl taxa indicate a divergence rate of 0.35% per site per million years. Using a *Lagenorhynchus* transition/tranversion ratio of 7.8 (Table 1), we calculated the total rate of divergence at *cyt b* third positions as $r_3 = r_{tv}(1 + R)$, where r_{tv} is the transversion rate and R is the transition/transversion ratio (Nei 1992). The overall divergence rate at *cyt b* third positions was $r_3 = 3.08\%$ per site per million years, or 3.15% after Jukes-Cantor correction for multiple hits.

Based on a Jukes-Cantor corrected net divergence at third codon sites of 3.3 ± 0.97% between *L. obliquidens* and *L. obscurus*, we estimated a divergence time of 1.05 (0.74–1.36) million years. Overall substitution rate per site (including all three codon positions) was estimated for the 496-bp segment

TABLE 2. Uncorrected number of transition (Ti) and transversion (Tv) differences per site at third codon positions between cytochrome *b* sequences and the approximate divergence time for the taxa compared. Cow, sheep, goat, pronghorn, and giraffe are represented by one sequence each. Values for other taxa are means of multiple comparisons using representative species. All comparisons use cytochrome *b* sequence segments from GenBank that correspond to the cytochrome *b* positions examined in *Lagenorhynchus.* Artiodactyl fossil dates in Irwin et al. (1991) were derived from Savage and Russell (1983).

Comparison	Tv at 3rd position	Ti at 3rd position	Age estimate (million years)	Reference
Phocoenid × monodontid	0.033	0.279	11	Barnes 1990; Fordyce and Barnes 1999
Delphinid × monodontid	0.031	0.253	11	Barnes 1990; Fordyce and Barnes 1999
Delphinid × phocoenid	0.043	0.272	11	Barnes 1990; Fordyce and Barnes 1994
Sheep × goat	0.017	0.245	5	Irwin et al. 1991
Cow × goat	0.063	0.261	20	Irwin et al. 1991
Cow × sheep	0.057	0.307	20	Irwin et al. 1991
Cow × pronghorn	0.102	0.205	20	Irwin et al. 1991
Bovid × giraffe	0.080	0.256	25	Irwin et al. 1991

of *cyt b* and each intron locus by calculating $\lambda = d/2T$ (Table 1), where d is the net Jukes-Cantor corrected divergence between *L. obliquidens* and *L. obscurus* and T is 1.05 million years.

Genealogical Patterns

A maximum-likelihood gene tree based on *cyt b* sequences showed that the Pacific species formed a clade that was well diverged from and reciprocally monophyletic with *L. acutus* (Fig. 3). In the South Pacific, *L. obscurus* was monophyletic, but the North Pacific *L. obliquidens* was paraphyletic with respect to its antitropical sister species. A log likelihood-ratio test did not reject reciprocal monophyly of the sister species ($P > 0.1$). Nonetheless, four of five fixed differences between the sister species diagnose *L. obscurus* as a derived monophyletic clade with 81% maximum-parsimony bootstrap support (Fig. 3). Bootstrap support for a monophyletic *L. obliquidens* clade was only 60% in parsimony analyses of *cyt b* because two haplotypes from this species contained ancestral character states.

Haplotypes were determined at two of the four nuclear loci using allele-specific sequencing. The cis/trans phase of multiple heterozygous sites was resolved for all but three individuals at both the *BTM* and *CAMK* loci, and each of these six diploid sequences had only one heterozygous site that remained ambiguous with respect to phase (Hare and Palumbi 1999). Once haplotypes were resolved, recombination was inferred if an alignment contained four haplotypes with every combination of nucleotides at two polymorphic sites (Hudson and Kaplan 1985). This was observed for *CAMK* in both *L. obscurus* and *L. obliquidens*, but not for *BTM.*

Maximum-parsimony networks for the *BTM* and *CAMK* loci showed long branches connecting the *L. acutus* sequences to a monophyletic clade containing both *L. obliquidens* and *L. obscurus* (Fig. 4), similar to the mtDNA tree. However, neither Pacific species was monophyletic at a nuclear locus, and this was also true for neighbor joining and maximum-likelihood results (not shown). In all analyses, nuclear polyphyly was evident from clades that contained haplotypes of both *L. obliquidens* and *L. obscurus*, although the two species never shared identical haplotypes. The polyphyletic clades had strong bootstrap support only in the *BTM* gene tree (86%, consistency index of 1.0, Fig. 4), despite the fact

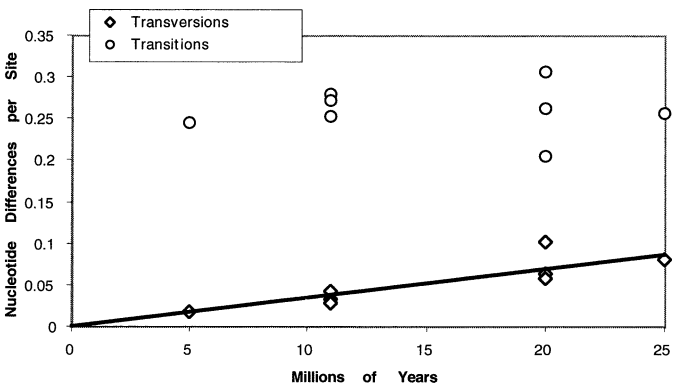

FIG. 2. Plot of cytochrome *b* transition and transversion differences per site against divergence dates estimated from the fossil record for cetacean and artiodactyl comparisons (see Table 2). The segment of cytochrome *b* used for this molecular clock calibration is the same as that examined here in *Lagenorhynchus*.

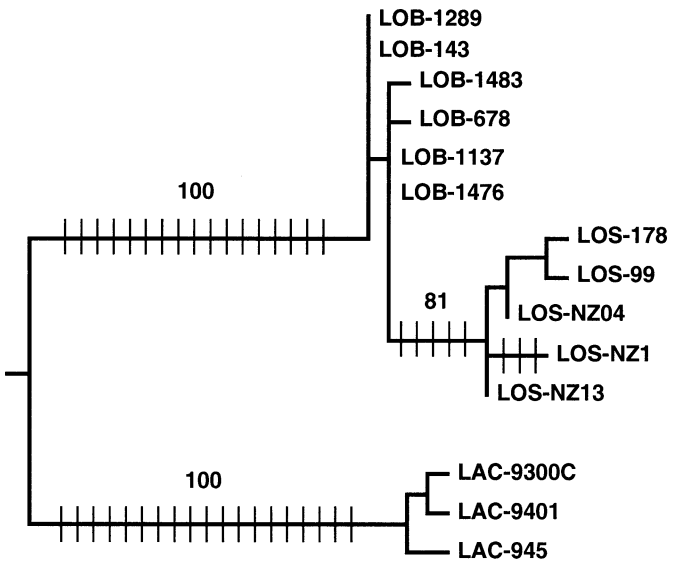

FIG. 3. Maximum-likelihood tree of mitochondrial cytochrome *b* sequences for three *Lagenorhynchus* species, with species names abbreviated as in Table 1. Outgroups used (but not shown) were *Phocoena dalli* and *P. sinus.* Hatch marks represent nucleotide changes on branches with more than one change. Maximum-parsimony bootstrap percentages for clades are shown above the branches.

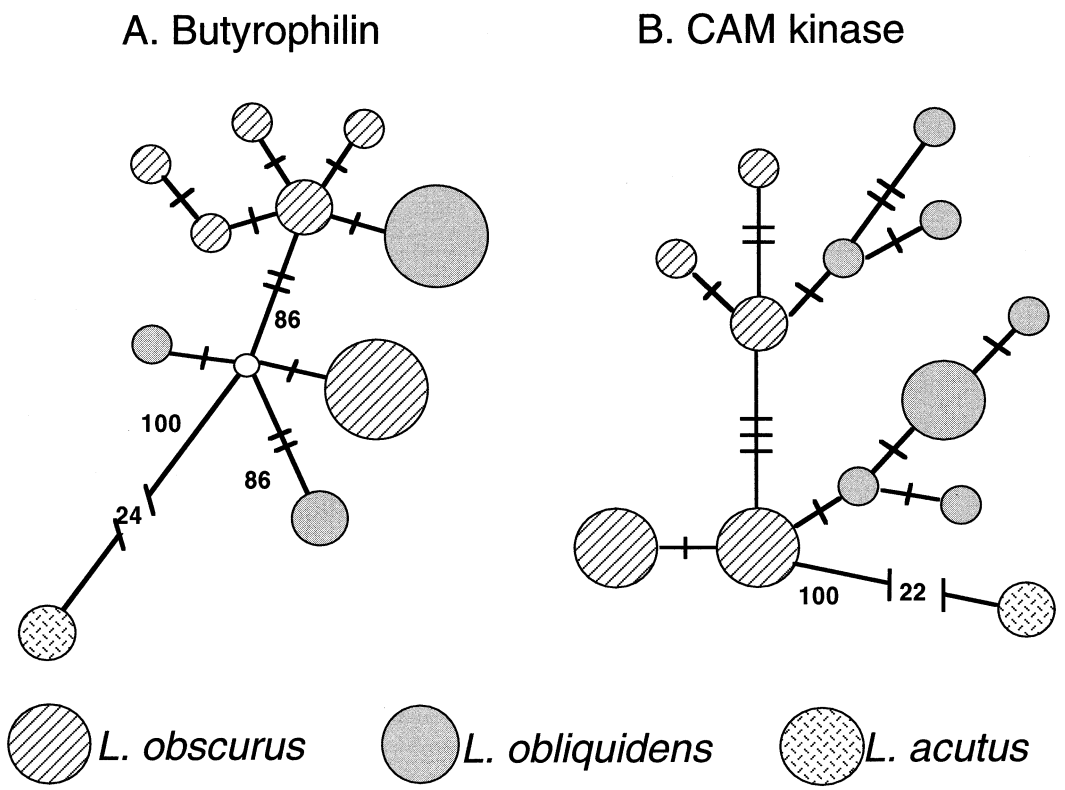

FIG. 4. Unrooted parsimony networks of the intron sequences observed at butyrophilin (A) and CAM kinase (B). Hatch marks or numbers on branches represent nucleotide or indel changes, and the size of circles is proportional to the haplotype frequency. The open circle represents a hypothesized but unobserved haplotype. Bootstrap values are next to branches with >70% support for the adjoining clade. The butyrophilin data had 34 variable characters including four indels, and seven of these were phylogenetically informative (consistency index, CI = 1.0). The CAM kinase data had 40 variable characters including four indels, and seven of them were informative (CI = 0.87). The *CAMK* network shown was one of 18 most parsimonious trees, all of which showed *Lagenorhynchus obscurus/obliquidens* polyphyly.

that both *BTM* and *CAMK* had multiple shared polymorphisms and no fixed differences between the two Pacific species (Table 3). At *CAMK*, bootstrap support was low for transspecific clades because numerous equally parsimonious trees each had four to five homoplasies (consistency index of 0.87, Fig. 4).

If the shared polymorphisms at *CAMK* are due to back or parallel mutations, then they do not support an inference of polyphyly. However, if the polymorphisms are shared because of shared ancestry and intragenic recombination has shuffled the variation, then homoplasy is expected to result at the haplotype level in a pattern that mimics back and parallel mutations. Therefore, if *CAMK* homoplasy can be shown to result from recombination rather than back mutations, polyphyly remains the most parsimonious inference even in the absence of strong bootstrap support for mixed clades.

TABLE 3. Number of shared (S), fixed (F), and exclusive (X) polymorphisms in or between *Lagenorhyncus obscurus* (LOS), *L. obliquidens* (LOB), and *L. acutus* (LAC) at mitochondrial cytochrome *b* and at introns in four nuclear loci. Samples sizes as in Table 1.

	Polymorphisms between LOS and LOB				Polymorphisms between LOS-LOB and LAC	
Locus	S	F	X_{LOS}	X_{LOB}	S	F
MtDNA	0	5	9	3	0	32
ACT	2	1	6	0	0	4
BTM	2	0	5	4	0	23
CAMK	3	0	3	3	0	22
HEXB	2	0	8	8	0	17

To test whether the *CAMK* homoplasy was likely a result of parallel and back mutations, we applied the homoplasy test (Maynard Smith and Smith 1998). Using *L. acutus* as an outgroup to estimate the effective number of sites free to change at *CAMK*, we tested for an excess of homoplasies in the observed tree compared to the mutational homoplasies expected without recombination. Assuming equal rates of substitution at all sites, we can reject parallel and back mutations as the cause of the *CAMK* homoplasies ($P = 0.03$). Thus, recombination is the most parsimonious explanation for the phylogenetic homoplasies in *CAMK*, consistent with the observation of recombination intervals at this locus based on the four-gamete test of Hudson and Kaplan (1985). Polyphyly at *BTM* and *CAMK* is therefore indicative of shared ancestral polymorphisms, rather than an artifact of mutational homoplasy.

Gene tree reconstruction was not attempted with the *ACT* and *HEXB* data because haplotypes were not resolved from

TABLE 4. Estimated population parameters for two antitropical sister species of *Lagenorhynchus* and their most recent common ancestor based on the Wang et al. (1997) method. Effective population size (N) was calculated from θ, with overall weighted $\mu = 0.69 \times 10^{-9}$ per bp per year and a generation time of 10 years. Divergence time estimates also assume a 10-year generation time.

Population	θ	N ($\times 10^4$)	Divergence time (million years ago)
L. obliquidens	0.0014	5.09	0.74
L. obscurus	0.0030	10.9	0.74
Common ancestor	0.0044	15.9	

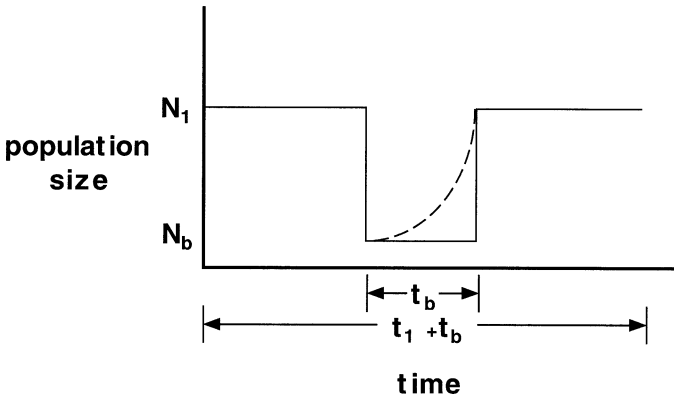

FIG. 5. Theoretical model defining the time and population size parameters of a historical bottleneck. Timing of the bottleneck is arbitrarily drawn; the theory does not stipulate when the bottleneck occurred. The solid line denotes a simple bottleneck history assumed by the theory, and the dashed line represents an alternate history (see Discussion).

diploid sequences. However, genealogical patterns were evaluated on a site-by-site basis in all five loci. A single nucleotide polymorphism observed in two taxa (a shared polymorphism) demonstrates polyphyly for that site, whereas a site that is monomorphic within taxa but different between them (a fixed difference) contributes to a reciprocally monophyletic genealogy. In addition, a polymorphism may be observed exclusively in one or the other taxon. There were no mtDNA polymorphisms shared between any of the *Lagenorhynchus* species (Table 3), but 32 *cyt b* differences were fixed between dolphins in the Atlantic and Pacific, and five fixed differences distinguished the antitropical species pair.

Comparing all four nuclear loci on a site-by-site basis, fixed differences were always abundant and shared polymorphisms completely absent between *L. acutus* and the clade of Pacific species (Table 3). In contrast, the antitropical species pair had two or three shared polymorphisms at every nuclear locus examined, whereas only one fixed difference was observed overall in nuclear DNA (Table 3). A single fixed difference was observed at the *ACT* locus, for which *L. obscurus* was poorly sampled (six instead of the usual 10 alleles analyzed). Thus, on the cumulative basis of single-site genealogies, all four nuclear loci were reciprocally monophyletic between the Atlantic and Pacific dolphin species but showed polyphyletic variation between the two antitropical Pacific species.

Historical Demography

Under a strict isolation model of divergence, the expected numbers of shared, fixed, and exclusive polymorphisms in two daughter populations depend on their divergence time (T) and the effective sizes of the daughter populations (N_1, N_2) relative to their common ancestor (N_A; Wakeley and Hey 1997). Assuming a constant mutation rate per locus under an infinite-sites model, these four population parameters can be estimated by choosing values that most closely equate observed and expected numbers of shared, fixed, and exclusive polymorphisms at independent loci (Wakeley and Hey 1997). The analysis assumes that all assayed variation is neutral, populations are randomly mating and generations are nonoverlapping. In addition, the ancestral and two descendent populations are each assumed to be constant in size, although they may differ from each other because of an instantaneous size change at the time of divergence (Wakeley and Hey 1997). We used this framework, modified to account for differences in ploidy among loci (WWH method; Wang et al. 1997), to estimate parameters from the mitochondrial and nuclear polymorphism data in *Lagenorhynchus* sister species (Table 3).

The effects of population size and mutation rate, μ, are combined in the WWH estimate of a population-mutation parameter, θ ($= 4N\mu$ per nucleotide per generation), based on the polymorphism patterns at all loci examined. To estimate effective population size from θ, a generation time of 10 years was assumed (Ferraro and Walker 1996) and an overall weighted mean substitution rate per nucleotide site was calculated from all locus specific rates (Table 1). The substitution rate for every locus was calibrated using the 1.05 million years divergence time between *L. obliquidens* and *L. obscurus* estimated from *cyt b* third codon positions (Fig. 2). A substitution rate for the entire 496-bp segment of *cyt b* was used in the weighted among-locus average because all *cyt b* sites were included in the WWH analysis (not only third codon sites). By using this procedure, an overall mean substitution rate of 0.069% per site per million years was calculated for the loci examined in this study.

Considering all 64 variable sites observed among the five loci, the effective population sizes of extant populations estimated using the WWH method were 5.09×10^4 for *L. obliquidens* and 10.9×10^4 for *L. obscurus*. Population size for the common ancestor was large, 15.9×10^4 (Table 4). The fact that estimated extant population sizes sum approximately to the ancestral size is a feature of these data, not a function of the model assumptions (Wakeley and Hey 1997). The divergence time of 7.43×10^4 generations estimated for these species by WWH (Table 4) translates to 0.74 million years ago based on a generation time of 10 years (Ferraro and Walker 1996). Results were similar if mtDNA was excluded from the WWH analysis (data not shown).

The absence of monophyletic nuclear loci in the two antitropical dolphin species suggests that each species has evolved without experiencing a severe bottleneck. Exactly how small a bottleneck (N_b) can be rejected based on an allelic genealogy depends on the ratio of N_b and bottleneck duration, t_b (Fig. 5). The probability of monophyly in a population of constant size N can be described by coalescence theory as the time-backward probability of i sampled alleles coalescing

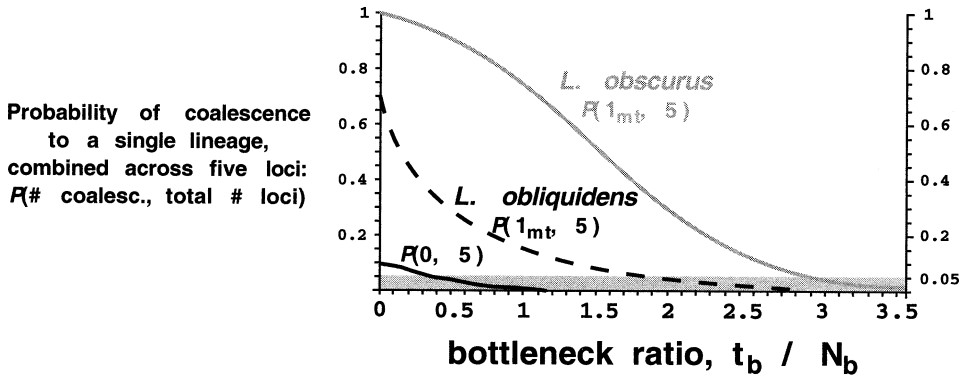

FIG. 6. The probability of coalescence to a single lineage (monophyly) for 10 sampled alleles through a bottleneck history as depicted in Figure 5. Combined probabilities, *P*(number of coalesced loci, total number of loci examined), were calculated assuming independence of loci and a ratio of four for nuclear/mitochondrial effective population size. Probabilities were calculated over $t_1 = 7.4 \times 10^4$ generations at a population size N_1 as estimated for each species (Table 3), but with various ratios of bottleneck size, N_b, and duration, t_b. The bottleneck ratio is an index of severity for a population size reduction in terms of genetic drift because the ratio increases for bottlenecks of longer duration and smaller size, both of which accelerate the approach to monophyly. The $P \leq 0.05$ rejection region is gray. The absence of monophyly in *Lagenorhynchus obliquidens* (solid black curve) makes most bottleneck histories improbable, whereas a moderate bottleneck is compatible with the *L. obscurus* data (gray curve). Phylogenetic support for paraphyly was weak in *L. obliquidens*, so the probability of bottleneck histories is also given as a dashed curve assuming mitochondrial DNA monophyly.

into a single lineage over t generations (Tavaré 1984, eq. 6.2). In a population of constant size, the probability of coalescence to a single lineage increases as longer time periods are considered. During a total time period t, a temporary reduction of population size for t_b generations would increase the overall probability of coalescence above the constant population size expectation. Takahata (1989) described this increased probability of coalescence by recalculating the effective number of generations as $t_e = t_1 + (N_1/N_b)t_b$, where t_1 is the number of generations at population size N_1 and t_b is the number of generations at reduced population size N_b, with abrupt transitions between the two population sizes (Fig. 5). If, for example, there was a 10-fold reduction in population size ($N_1/N_b = 10$), then the number of effective generations is 10 times the actual duration of low population size, plus the time period spent at the nonbottleneck population size ($t_e = t_1 + 10t_b$).

Based on this theory, we estimated probabilities for coalescence to a single ancestral lineage in dolphin populations with different hypothetical bottlenecks. Bottlenecks of long duration or great severity have the same effect on coalescence (Nei et al. 1975), so we calculated coalescence probabilities relative to the bottleneck ratio, t_b/N_b, which is higher for a small bottleneck size or a longer bottleneck duration. A bottleneck ratio of 2.0, for example, represents a bottleneck of 10 individuals for 20 generations or 100 individuals for 200 generations—both have the same impact on coalescence. With a sample of 10 nuclear alleles in *L. obliquidens*, if a bottleneck of $N_b = 10$ individuals lasted for $t_b = 20$ generations, and the remainder of the species history, $t_1 = 7.43 \times 10^4$ generations was spent at constant size $N_1 = 5.09 \times 10^4$ (WWH estimates, Table 4), then monophyly at a single nuclear locus would be observed with a 0.58 probability and nonmonophyly with a 0.42 probability. The probability of monophyly for mtDNA, 0.95, is relatively high for this scenario because of the smaller effective size of mtDNA. To determine the spectrum of bottleneck histories that are too extreme to be consistent with the extensive polyphyly documented here, we assumed locus independence to estimate the combined probability of the patterns observed at one mitochondrial and four nuclear loci in the two dolphin species for various bottleneck ratios (Fig. 6). Given a 0.74 million year divergence time (Table 4), bottleneck ratios of 0.36 and greater can be rejected for *L. obliquidens* because the probability of observing nonmonophyly at all five loci is ≤ 0.05 under those conditions (Fig. 6, solid black curve). Mitochondrial variation was monophyletic in *L. obscurus*, making it difficult to reject anything but extreme bottleneck ratios with the current data. The mtDNA monophyly, in combination with four nonmonophyletic nuclear loci in *L. obscurus*, generates coalescent probabilities ≤ 0.05 only for bottleneck ratios more extreme than 2.9 (Fig. 6, solid gray curve).

DISCUSSION

We examined patterns of DNA variation at multiple independent loci to examine genealogical differentiation and estimate historical population size changes accompanying the founder-speciation of antitropical dolphin sister species. Our empirical approach is based on the general expectation that recently diverged sister species will show mixed support for monophyly among independent neutral loci (Tajima 1983; Wakeley and Hey 1997; Palumbi et al. 2001). At the level of nucleotide sites, this stage of divergence is characterized by the presence of both shared polymorphisms and fixed differences. At the locus level, haplotype-based analyses will show some loci to be reciprocally monophyletic whereas others have a genealogy with intermingled alleles from the two species. Because of the four-fold smaller effective population size of mtDNA relative to autosomal nuclear loci, mixed-monophyly within diverging genomes is initially expected to entail monophyly at mtDNA, whereas most nuclear loci continue to sort shared ancestral polymorphisms (Hare 2001; Palumbi et al. 2001). The mtDNA-nDNA contrast predictably occurs under most models of historical population size change and is exemplified here by the genealogical data from

L. obscurus (Figs. 3, 4). After an extended period of divergence, lineage sorting is expected to go to completion at most loci, mitochondrial and nuclear, as demonstrated here by the fixed differences observed between Atlantic and Pacific dolphin species (Table 3).

The mixed-monophyly stage of divergence affords an opportunity to empirically evaluate the demography of speciation. For a given divergence time and effective population size, the degree of lineage sorting observed among independent loci (or sites) can be compared to expectations under different population size histories to test for historical bottlenecks. Evidence for a history of small population size does not demonstrate that the bottleneck was coincident with speciation, but genetic patterns incompatible with small population size can provide an indirect assessment of the minimum population size at speciation.

Among the five loci examined, support for reciprocal monophyly between the antitropical *L. obliquidens* and *L. obscurus* was nearly absent. The overwhelming abundance of shared nucleotide polymorphisms relative to fixed differences could not be attributed to mutational homoplasy because rates of substitution were generally low. These shared polymorphisms must therefore result either from gene flow or incomplete lineage sorting of ancestral variation. Recent gene flow through the tropics is unlikely for temperate-adapted dolphins and was not supported by the genetic data—no alleles were shared between the two species at any locus based on sample sizes of five to 12 alleles from each species. Instead, we interpret the sharing of single nucleotide polymorphisms between species as evidence of ancestral variation, indicating the continued intermingling of genealogies despite divergence of phenotypic traits. These results support a Pleistocene origin for the antitropical distribution of the dolphin sister species and indicate that colonization across the warm tropics probably occurred without a severe reduction in effective population size. We proceed by exploring the minimum population size statistically compatible with these data, then discuss implications of these results for the origin of antitropical distributions in the Pacific and examine the broader implications of genealogical polyphyly.

Speciation without a Genetic Bottleneck?

Controversy continues to surround experimental tests of founder speciation models (Rice and Hostert 1993; Templeton 1996, 1999; Rundle et al. 1998, 1999) as well as the likelihood of the model conditions in nature (Slatkin 1996, 1997; Charlesworth 1997; Whitlock 1997). In light of this controversy, it remains important to strengthen empirical testing of historical bottlenecks associated with founder events in natural populations. The transilience model makes explicit predictions about how extreme a founder bottleneck must be to severely alter allele frequencies without losing too much genetic variation (Templeton 1980, 1996). Peripatric speciation models make the prediction that sister species should have very disparate historical population sizes (Bush 1975; Mayr 1982; Lynch 1989). Lynch (1989) used geographic distribution as a proxy for the historical population size of species and concluded from a literature review that peripatric speciation of peripheral isolates is rare. However, the assumption is tenuous that species range distributions are associated with historical N (Chesser and Zink 1994). In sister species that are still sorting ancestral polymorphisms in a portion of their genomes, measuring historical population size provides a more direct method of testing these speciation models.

Genealogical patterns have provided evidence against speciation bottlenecks in the island endemic *Drosophila mauritiana* (Hey and Kliman 1993) and in Lake Victoria cichlids (Nagl et al. 1998). Although the highly polymorphic MHC locus in Darwin's finches was incompatible with a founding population smaller than 26 individuals (rejecting more extreme founder events; Vincek et al. 1997), very small bottlenecks could not be statistically rejected in the history of maize domestication (Hilton and Gaut 1998) or during peripheral speciation of the beetle *Ophraella bilineata* (Knowles et al. 1999).

If *L. obliquidens* has been isolated from *L. obscurus* for 0.74 million years, the abundant sharing of polymorphisms by these species at multiple loci is expected with high probability only if both species have had continuously large population sizes during and since speciation. Mild bottlenecks, however, could have occurred without fixation of most neutral nuclear polymorphisms (Nei et al. 1975), so we used gene tree coalescent probabilities to explore the range of bottlenecks consistent with our data. We calculated the severity of hypothetical bottlenecks as a ratio of bottleneck duration over bottleneck size and found that the combined mtDNA and nuclear data statistically rejected bottleneck ratios greater than 0.36 or 2.9, depending on the dolphin species examined (Fig. 6).

To interpret hypothesized bottleneck ratios in a biologically meaningful way and to estimate the minimum population size consistent with these data, assumptions must be made about bottleneck duration. Our simple model defines bottleneck duration as the time interval between an instantaneous reduction to size N_b and an instantaneous return to the original size (Fig. 5). In reality, population growth is expected during the bottleneck interval and the growth rate defines a minimum bottleneck duration (Fig. 5, dashed line).

At a 1.8% annual growth rate (Perrin and Reilly 1984; Slooten and Lad 1991) a dolphin population would have a doubling time of 39 years and 20 individuals would grow to a population size of 5.09×10^4 (Table 4; *L. obliquidens*) in 443 years, or about 44 generations. With linear growth over this period, the harmonic mean of population sizes would be 156 individuals, so the bottleneck ratio for this scenario is $44/156 = 0.3$. Bottleneck ratios for similar demographic recoveries from a minimum population of 10, five, or two individuals are 0.6, 1.1, and 2.9, respectively. Therefore, in *L. obliquidens* historical bottlenecks involving 10 or fewer individuals can be rejected, assuming this population growth model, because such histories would have likely ($P > 0.95$) resulted in monophyly for at least one out of the five loci (Fig. 6, bottleneck ratios ≥ 0.6). The minimum founder size of $N > 10$ corresponds to a census size of more than 100 individuals if the ratio of effective to census size is 0.1 (Frankham 1995).

By contrast, it is more difficult to reject small bottlenecks for the southern species, *L. obscurus*. The southern species

was monophyletic for mtDNA. In combination with four nuclear loci found to be polyphyletic, only very long population size reductions (e.g., a population size of 10 individuals for 30 generations) can be rejected as statistically improbable (Fig. 6). This is despite a best estimate of N in *L. obscurus* of 5.09×10^4, a WWH result that stems from the relatively large number of exclusive polymorphisms in *L. obscurus* as well as the many polymorphisms shared with *L. obliquidens* (Table 3). Thus, the neutral stochasticity of lineage sorting ensures a wide gap between the theoretically best estimates of N indicating consistently large populations based on these data (WWH results in Table 4) and rejectable alternative population size histories (Fig. 6). Only with data from many more nuclear loci can historical bottlenecks be inferred with any certainty. For example, a bottleneck ratio of 1.1 could be rejected for *L. obscurus* if, in addition to mtDNA monophyly, 21 or more independent nuclear loci all had variation that was polyphyletic with respect to *L. obliquidens*. Similar conclusions would hold for *L. obliquidens* if, instead of the mtDNA paraphyly inferred here, additional mitochondrial data provided evidence of monophyly (Fig. 6, dashed curve).

These conclusions rest on several assumptions and approximations. Our use of coalescent probabilities to explore implausible bottleneck histories assumes that the WWH estimates of effective population size and divergence time are accurate. Confidence limits around our WWH parameter estimates are expected to be very large, but their estimation by simulation is sensitive to locus-specific recombination rates that cannot be accurately determined from the present data (Kliman et al. 2000). In addition, the WWH method assumes panmictic populations, yet *L. obscurus* contains three disjunct populations of which two were sampled, Peru and New Zealand. Simulation studies of an isolation model with population structure (Wakeley 2000) indicated that by not taking population structure into account, our estimate of *L. obscurus* population size was inflated and species divergence time was underestimated. Large population size and short divergence time both make gene polyphyly more likely, so this bias increased the calculated probability of the observed data given various bottleneck histories (Fig. 6). In other words, curves in Figure 6 shifted up as a result of this bias, making it more difficult to reject moderate bottleneck histories. Estimates of ancestral population size using the WWH method are probably also affected by population structure in the descendent populations (Wakeley 2000), but the direction of bias is difficult to predict because it depends on whether population structure existed in the ancestor, among other things. Finally, any bias in the calibration of *cyt b* substitution rate is expected to bias estimates of population size and divergence time in the same direction. A calibration bias does not change the ratio of N to divergence time estimated by WWH. Because this ratio determines the coalescent probabilities in Figure 6, our conclusions about the probability of historical bottlenecks are insensitive to the molecular clock calibration.

The Timing and Demography of Antitropical Speciation

A strict vicariant origin for antitropical sister species requires that a broadly distributed ancestral population inhabited both hemispheres before equatorial waters warmed to isolate them. Because warm (20–28°C) equatorial surface waters date back to the mid-Miocene (15 million years ago; White 1986; Adams et al. 1990), this ancient vicariance model is rejected for the antitropical dolphins based on a 0.74–1.05 million years divergence time estimated with all loci and *cyt b*, respectively. Ancient vicariance has also been rejected for several pelagic fish species (Stepien and Rosenblatt 1996; Bowen and Grant 1997) and various invertebrates with antitropical distributions (Lindberg 1991).

Historical models of vicariance and dispersal lose their distinction if a dispersal barrier was intermittently weakened because both processes would contribute to colonization and speciation. In the Pacific Ocean, opportunities for antitropical speciation hinge on dispersal abilities and the details of barrier disruption (Davies 1963; Lindberg 1991). Assuming that colonization across a formidable and stable barrier generally involves fewer individuals than if an ephemeral barrier is involved, empirical tests of historical population size can illuminate the environmental conditions that led to antitropical sister species.

This is the first study to estimate the size of antitropical founding populations. Given the uncertainties of our effective population size estimation with WWH, the trend of diminishing population sizes in *Lagenorhynchus* dolphins does not reliably indicate a larger population size (or broader geographic distribution) in the common ancestor than in the contemporary species. Nonetheless, these data are most consistent with historically large populations that never experienced an extended population bottleneck. Pod sizes average 100 individuals in *Lagenorhynchus* species (Brownell and Cipriano 1999; Brownell et al. 1999), so gregariousness might have reduced the likelihood of a founder effect in this group regardless of dispersal barrier strength. If mid-Pleistocene disruptions of the warm equatorial dispersal barrier were common or extreme (CLIMAP 1976; Haslett 1992; Mix et al. 1999), however, large founder population sizes are also expected in other antitropical sister species, including poikilotherms.

The direction of dispersal across the tropics is an important aspect of antitropical origins that can sometimes be inferred with phylogenetic methods (e.g., Bowen and Grant 1997; Hilbish et al. 2000). The *cyt b* paraphyly between *Lagenorhynchus* sister species (Fig. 3) implies that South Pacific *L. obscurus* populations were derived from the North Pacific *L. obliquidens*. This is a surprising result given that the geographic distribution of close relatives suggests a Southern Hemisphere center of origin. In a larger phylogenetic analysis of delphinids, *L. obliquidens* was one of only two Northern Hemisphere species among eight species contained in a well-supported *cyt b* clade (LeDuc et al. 1999). Unfortunately, ancestor-descendent relationships were not resolved within the clade (LeDuc et al. 1999), and this study provides no phylogenetic data bearing on the most recent common ancestor of *L. obscurus* and *L. obliquidens*. Relative population sizes in antitropical species pairs can provide a clue about directionality under a peripatric speciation model, but in this study population structure in *L. obscurus* complicates the necessary comparison. Strong conclusions about the directionality of *Lagenorhynchus* colonization will depend on re-

solving the most recent common ancestor of *L. obscurus* and *L. obliquidens*.

Implications of Polyphyly

Although phylogenetic methods have become increasingly refined for inferring the branching order of taxa and their divergence times, genetic inferences of historical demography have often been crude. For example, ascribing low genetic variation to a historical bottleneck, or normal levels of variation to a history free of bottlenecks, has been criticized as an overly simplistic assumption (Amos and Harwood 1998). The need for data from many loci has been stressed both for phylogenetics (Moore 1995) and for historical demography (Harpending et al. 1998; Edwards and Beerli 2000; Wall 2000). The analyses presented here underscore the risks associated with casual statements about the meaning of polyphyly and ancestral polymorphisms at one or a few loci.

This caution should also extend to inferences about past hybridization as a cause of polyphyletic gene genealogies (Neigel and Avise 1986). Particularly in species with large population sizes, the results presented here suggest that intermixed patterns of genetic variation will persist in nonhybridizing species for hundreds of thousands or millions of years. The polyphyletic variation documented here for *Lagenorhynchus* species may be typical of marine species with large populations. Thus, attempts to use polyphyletic patterns to infer reticulate evolution in corals (Hatta et al. 1999) and plants (Ungerer et al. 1998) must consider the long maintenance of ancestral polymorphism as a likely alternative hypothesis.

Finally, our data on *L. obliquidens* provide a clear empirical demonstration that large stable population sizes can make neutral gene monophyly too conservative a criterion of evolutionary distinctiveness for the purposes of taxonomy or conservation, even when applied to mtDNA (Cronin 1993; Moritz 1994; Paetkau 1999). Clearly, because of its dependence on historical population size, monophyly means different things in different taxa. Interpretations of gene monophyly, therefore, are best made in the context of divergence time, demographic history, and patterns of variation in other heritable characters, such as morphology and behavior (Avise and Ball 1990; Moritz 1994; Crandall et al. 2000).

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