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Cross-Reactivity between Immunoglobulin G Antibodies of Whales and Dolphins Correlates with Evolutionary Distance▽

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Growing morphological and molecular evidence indicates that the porpoises, dolphins, and whales evolved within the even-toed ungulates, formerly known as Artiodactyla. These animals are now grouped in the Cetartiodactyla. We evaluated the antigenic similarity of the immunoglobulin G (IgG) molecules of 15 cetacean species and the domestic cow. The similarity was scored using three distinct antibodies raised against bottlenose dolphin (*Tursiops truncatus*) IgG in a Western blot, an indirect enzyme-linked immunosorbent assay (ELISA), and a competitive ELISA format. A score was generated for the genetic distance between each species and *T. truncatus* using the cytochrome *b* sequence. Each antibody displayed a distinct pattern of reactivity with the IgG antibodies of the various species. The monoclonal antibody (MAb) specific for the γ heavy chain of *T. truncatus* was reactive with all monodontids, delphinids, and phocoenids. The light-chain-specific MAb reacted with IgG of delphinoid and phocoenid species and one of the two mysticete species tested. The polyclonal antibody was broadly cross-reactive across all cetaceans and the domestic cow. Using the MAb specific for the γ heavy chain, the degree of IgG cross-reactivity ranged from less than 17% for the mysticetes to 106% for killer whale *Orcinus orca*. The IgG in beaked whale and baleen whale sera was significantly less cross-reactive with bottlenose dolphin IgG than sera from other toothed whales. A strong negative correlation was demonstrated between antigenic cross-reactivity of IgG molecules and the genetic distance of their hosts. The data generated will be useful for the development of clinical serodiagnostics in diverse cetacean species.

Marine mammal medicine is a rapidly developing field, and the number of infectious agents known in whales and dolphins is expanding rapidly (22, 28). Some cetacean populations are declining, and while human influence may play a significant role in the decline (31, 36), infectious disease also has significant potential to cause population declines (15, 39). Serodiagnostic testing is a valuable tool for understanding the dynamics of infectious agents in animal populations. There are approximately 80 species of porpoises, dolphins, and whales, many of which are endangered (32). One difficulty with many serodiagnostic tests in diverse groups of wildlife is the relative lack of reagents, such as specific secondary antibodies. While anti-bottlenose dolphin immunoglobulin G (IgG) antibodies have been developed, there are limited options in other cetacean species (27). Protein A and protein G are bacterial proteins that bind to IgG and have been used in immunoassays to detect antibodies in various cetacean species (18, 20, 35, 38). However, binding of these proteins to IgG varies significantly according to host species, with protein G showing less than 1/6 the affinity for binding rat IgG compared to human IgG and protein A showing less than 1/120 the affinity for goat IgG compared to bovine IgG (1). Data on the comparative affinity of protein A for cetacean IgG are available for only two species and show that affinity for bowhead whale (*Balaena mystecetus*) IgG is low compared to that for Atlantic bottlenose dolphin (*Tursiops truncatus*) IgG (19). Although protein G is frequently applied as a detection reagent, we are not aware of any data on the comparative affinity of protein G for IgG of the different cetacean species. There is a need for validated reagents for use in serologic assays in diverse species of cetaceans.

The cetaceans, hippopotami, ruminants, suids, and camelids form a monophyletic group known as the Cetartiodactyla. The closest extant terrestrial relatives of the cetaceans are the hippopotami, and together they are referred to as the Cetancodonta (3). The assembly of Ruminantia and Cetancodonta is referred to as the Cetruminantia (37). The divergences of the camelids and suids from the Cetruminantia represent the earliest branchings within the Cetartiodactyla. Cetancodonta and Ruminantia are estimated to have diverged near the K/T boundary (65.5 million years ago), and Cetacea and Hippopotamidae are estimated to have diverged 53.5 million years ago (4, 7). The earliest known well-preserved cetacean is *Pakicetus inachus*, a terrestrial species; a specimen found in Pakistan has been dated back to the middle Eocene (23). Modern whales are subdivided into two subgroups: the odontocetes (toothed whales) and the mysticetes (filter-feeding baleen whales). Odontocetes and mysticetes are estimated to have diverged approximately 35 million years ago (4). There

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TABLE 1. Phylogenetic lineages, species, common names, and sample sizes for samples of the clade Cetartiodactyla analyzed in this study

Order	Suborder	Superfamily	Family	Scientific name	Common name	n^a
Artiodactyla	Ruminantia		Bovidae	*Bos taurus*	Domestic cow	1
Cetacea	Odontoceti	Ziphioidea	Ziphiidae	*Mesoplodon europaeus*	Gervais' beaked whale	3
		Physeteroidea	Kogiidae	*Kogia breviceps*	Pygmy sperm whale	3
		Delphinoidea	Monodontidae	*Delphinapterus leucas*	Beluga	2
			Delphinidae	*Steno bredanensis*	Rough-toothed dolphin	3
				Lissodelphis borealis	Northern right-whale dolphin	2
				Globicephala macrorhynchus	Short-finned pilot whale	2
				Pseudorca crassidens	Pseudorca whale	2
				Orcinus orca	Killer whale	3
				Tursiops truncatus	Bottlenose dolphin	3
				Delphinus capensis	Common dolphin	3
				Lagenorhynchus obliquidens	Pacific white-sided dolphin	3
			Phocoenidae	*Phocoena phocoena*	Harbor porpoise	2
				Phocoenoides dalli	Dall's porpoise	3
	Mysticeti		Eschrichtiidae	*Eschrichtius robustus*	Gray whale	3
			Balaenopteridae	*Megaptera novaeangliae*	Humpback whale	2

[a] Sample size.

are five primary extant odontocete lineages: Physeteroidea (sperm whales and pygmy sperm whales), Platanistidoidea (Indian river dolphins), Ziphioidea (beaked whales), Inoidea (Amazon river dolphins and franciscana), and Delphinoidea (porpoises, narwhals, and dolphins) (21) (Table 1). There are currently four recognized families of mysticetes: the right whales (Balaenidae), the pygmy right whale (Neobalaenidae), the gray whale (Eschrichtiidae), and the rorquals and humpback whale (Balaenopteridae) (33).

This phylogenetic branching order of the cetartiodactyls has been extensively explored using a range of markers. A number of earlier studies using morphological and biochemical markers, such as precipitation assays, karyotyping, amino acid sequencing, and isotope analysis, indicated a close relationship between cetaceans and artiodactyls (2, 9). Analyses of full-length mitochondrial and cytochrome *b* genes (13, 17, 21), Y chromosome sequences (26), and retroposon analyses (24, 25) have since provided additional evidence for the monophyly of the Cetacea and placement within Cetartiodactyla and for the phylogenetic topology of the cetacean species.

Although less commonly used, the comparison of serum protein epitopes is a reliable alternative indicator of evolutionary relatedness among species (6, 14, 16, 30). Ig antibodies are often the target protein of choice for these cross-reactivity based relatedness studies, because Ig antibodies are large protein molecules carrying multiple antigenic epitopes. Moreover, Ig antibodies are found in sera from all vertebrate species (10). They form an essential component of the humoral immune system and are therefore sufficiently conserved to allow for comparison across a broad range of species (5, 8, 30). However, high-quality blood and serum samples are needed to allow for the evaluation of Ig cross-reactivity. The relative lack of Ig cross-reactivity data in support of molecular phylogenetic studies of less easily accessible species may be due in part to limited availability of good-quality samples.

The cross-reactivity of serum protein epitopes is usually assayed using either polyclonal antibodies (polyAbs) or monoclonal antibodies (MAbs). PolyAbs consist of a heterogeneous mixture of antibodies that bind to various epitopes of their target protein. In contrast, a MAb binds one specific epitope on the target protein. The degree of cross-reactivity of either type of antibody with the target protein among species reflects the degree of conservation and therefore genetic proximity of the various species (8, 30). However, since the polyAb mixture binds multiple epitopes, a broader spectrum of cross-reactivity would be expected with polyAb than with a MAb. We previously purified IgG from bottlenose dolphins and developed MAbs and polyAbs with specificities for either bottlenose dolphin IgG heavy chain, IgG light chain, or the whole IgG molecule (27). Using these reagents, we then developed and validated a competitive enzyme-linked immunosorbent assay (cELISA) for the quantification of circulating dolphin IgG concentrations, using the MAb specific for the IgG γ heavy chain (32a). These MAbs, the polyAb, and the cELISA were utilized in this study to quantify the degree of cross-reactivity between Ig antibodies of a diverse sampling of cetacean species. We then evaluated the correlation between cross-reactivity of Ig and genetic distance using the cytochrome *b* gene among species.

MATERIALS AND METHODS

Animals and sera. Serum samples were collected from 1 domestic cow (*Bos taurus*) and 39 cetaceans belonging to 15 species. The families and representative species used in this study are listed in Table 1. Samples from at least two individuals were available for each cetacean species. Blood samples were collected from collection animals or opportunistically during stranding events from the ventral tail vein, the ventral fluke vein, or via cardiocentesis on postmortem examination. Samples were typically collected using a 20- or 21-gauge 1.5-in. Vacutainer needle (Becton Dickinson Vacutainer Systems, Rutherford, NJ), and blood was collected into a Vacutainer serum separator tube or a Vacutainer without anticoagulant. The blood samples were centrifuged at 3,000 rpm at 21°C for 10 min. Fibrin clots were removed, and serum was transferred to cryovials. Archived sera were stored at −80°C until shipment to the laboratory for IgG analysis. The total protein concentrations of the samples were determined spectrometrically in a standard reference laboratory using an automated Hitachi 912 chemistry analyzer and ranged between 5.7 and 9.6 g/dl (mean, 7.4 g/dl; median, 6.8 g/dl; and standard deviation [SD], 1.3 g/dl).

MAbs and polyAbs. Two MAbs and one polyAb raised against IgG of the bottlenose dolphin (*Tursiops truncatus*) were used. Their derivation, evaluation, and the validation of their specificities for *T. truncatus* IgG molecules have been described in detail previously (27). The biotinylated MAb HL1912 is specific for the γ heavy chain of *T. truncatus*. The biotinylated MAb HL1914 has specificity

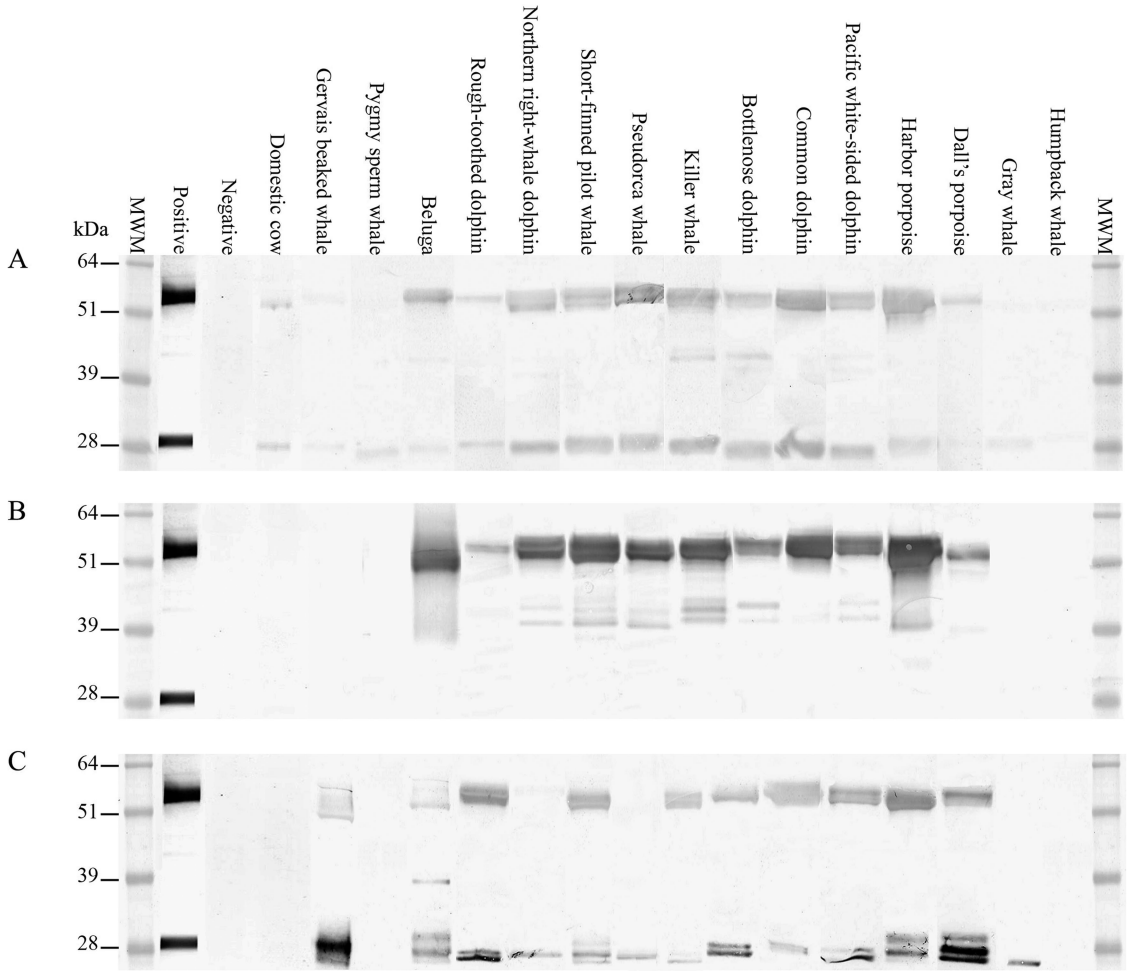

FIG. 1. Specificity and cross-reactivity on Western blot of three anti-bottlenose dolphin IgG antibodies with Ig of other species. The antibodies were raised against bottlenose dolphin IgG whole molecule (A), IgG γ heavy chain (B), or IgG κ or λ light chains (C). Purified dolphin IgG (lanes 2), probed with the polyAb was included as a positive control on each blot (A, B, and C). Duplicate *T. truncatus* lanes (lanes 3) were probed with PBS to serve as negative controls. MWM, molecular size markers.

for the κ or λ IgG light chains of *T. truncatus*, while the polyAb SB-A9713 has specificity for both heavy- and light-chain epitopes of *T. truncatus* IgG.

iELISA. The ability of both MAbs and the polyAb to react with bovine and cetacean Igs was first evaluated using an indirect ELISA (iELISA). Six wells of a high-protein-binding microplate (Nunc Maxisorp, Fisher Scientific, Pittsburgh, PA) were coated with 50 μl of whole serum of each sample ($n = 40$) diluted 1:1,000 in 1× phosphate-buffered saline (PBS) and were left to adsorb overnight at 4°C. Six wells were coated using 1% bovine serum albumin (BSA [Roche, Indianapolis, IN]) in PBS to serve as duplicate negative controls for each antibody. After this and each subsequent step, all wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T) using an automated microplate washer (Biotek Instruments, Winooski, VT). After washing, the wells were blocked with 1% BSA (Roche Diagnostics, Indianapolis, IN), after which one of the antibodies was applied to each of two wells (MAbs at 5 μg/ml in 1% BSA in PBS containing 0.02% sodium azide [PBS/Az] and polyAb diluted 1:1,000 in 1% BSA in PBS/Az). Alkaline phosphatase-labeled goat anti-rabbit polyAb (1:2,000 in PBS/Az) was used to detect bound polyAb. Alkaline phosphatase-labeled streptavidin (1:2,000 in PBS/Az) was used to detect bound MAbs HL1912 and HL1914. Each step of the ELISA was left to incubate with gentle agitation (Nutator; Adams, Fisher Scientific, Pittsburgh, PA) for 1 h at room temperature. Finally, 1.0 mg ml^{-1} *p*-nitrophenyl phosphate (PNPP [Sigma, St. Louis, MO]) substrate was added and the optical density at 405 nm (OD_{405}) was recorded after 30 min using a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA). Duplicate OD_{405} readings for each serum sample-antibody combination were averaged. For analysis, the average OD_{405} of the 1% BSA-negative control was subtracted from the average OD_{405} of all other samples.

Western blotting. The sample with the highest reactivity on the iELISA for each species ($n = 16$) was selected for the Western blot analysis. All serum samples were electrophoretically separated on triplicate blots and probed with the polyAb or MAbs HL1912 and HL1914 (Fig. 1A, B, and C). The sera were separated at a 1:2,000 dilution under reducing conditions on a precast 10% (wt/vol) polyacrylamide Nupage Novex BisTris gel with morpholinepropanesulfonic acid running buffer. Purified dolphin IgG (lane 2 [0.1 μg]), probed with the polyAb was included as blotting control on each blot (Fig. 1A, B, and C). The IgG-positive control and reduced serum Ig antibodies, including *T. truncatus* serum (lane 13), were electrophoretically transferred to a nitrocellulose sheet using a Novex Western transfer apparatus. The blotting time was 60 min at a constant voltage of 30 V. The nitrocellulose sheet was placed in Superblock (Pierce, Rockford, IL) to block overnight at room temperature with gentle agitation. The blocked nitrocellulose sheets were washed four times for 5 min each in 0.05% PBS-T. Lanes 2 and 4 to 19 of each of the three blots were incubated with polyAb (1:70,000 in 1% BSA [Fig. 1A]), MAb HL1912 (0.05 μg/ml in 1% BSA [Fig. 1B]), or MAb HL1914 (0.05 μg/ml in 1% BSA [Fig. 1C]). The cow serum (lane 2) was probed with a goat anti-cow polyAb, and duplicate *T. truncatus* lanes (lane 3) were incubated with PBS/Az to serve as negative controls. The nitrocellulose sheets were incubated with gentle rocking at room temperature for 1 h, after which the blot was washed four times for 5 min each with 0.05% PBS-T. Alkaline phosphatase-labeled goat anti-rabbit polyAb (1:

TABLE 2. Genetic distance and results from the iELISA, cELISA, and Western blotting

Species	Genetic distance from *T. truncatus*	Result by[a]: Anti-whole-*Tursiops*-IgG molecule polyAb		Anti-*Tursiops* γ MAb HL1912			Anti-*Tursiops* κ/λ MAb HL1914	
		iELISA OD_{405}	Western blot	iELISA OD_{405}	cELISA % inhibition	Western blot	iELISA OD_{405}	Western blot
Domestic cow	0.133	2.477 ± 0.098	+	0.029 ± 0.096	0.0 ± 0.0	−	0.029 ± 0.037	−
Gervais' beaked whale	0.090[b]	2.839 ± 0.182	+	0.290 ± 0.152	18.1 ± 9.3	−	0.032 ± 0.012	−
Pygmy sperm whale	0.091	2.910 ± 0.128	+	1.344 ± 1.741	51.1 ± 27.2	−	0.028 ± 0.004	−
Beluga	0.065	2.558	+	3.557	102.5 ± 0.7	+	0.408	−
Rough-toothed dolphin	0.015	2.776 ± 0.071	+	3.321 ± 0.268	94.0 ± 5.6	+	0.392 ± 0.013	+
Northern right-whale dolphin	0.015	2.866	+	3.628	103.9 ± 1.2	+	0.593	+
Short-finned pilot whale	0.025	3.048	+	3.246	103.5 ± 1.3	+	0.558	+
Pseudorca whale	0.041	2.826	+	3.597	91.1 ± 5.6	+	0.399	+
Killer whale	0.038	2.900 ± 0.243	+	3.359 ± 0.278	106.0 ± 0.8	+	0.444 ± 0.120	+
Bottlenose dolphin	0.000	3.016 ± 0.101	+	2.848 ± 0.320	100.0 ± 1.2	+	0.428 ± 0.203	+
Common dolphin	0.005[c]	2.975 ± 0.105	+	3.280 ± 0.401	101.9 ± 4.1	+	0.884 ± 0.514	+
Pacific white-sided dolphin	0.020	3.049 ± 0.198	+	3.464 ± 0.471	100.6 ± 5.0	+	0.423 ± 0.225	+
Harbor porpoise	0.060	2.962	+	3.930	102.4 ± 0.7	+	0.424	+
Dall's porpoise	NA[d]	2.807 ± 0.093	+	3.544 ± 0.033	101.5 ± 2.3	+	0.429 ± 0.047	+
Gray whale	0.088	2.789 ± 0.090	+	0.081 ± 0.102	16.3 ± 8.9	−	0.277 ± 0.346	+
Humpback whale	0.074	2.270	+	1.178	10.8 ± 1.8	−	0.109	+

[a] iELISA and cELISA results are means ± SD.
[b] *Mesoplodon peruvianus* substituted for *M. europaeus*.
[c] *Delphinus delphis* substituted for *D. capensis*.
[d] NA, no sequence available.

2,000 in PBS/Az) was used to detect bound polyAb (Fig. 1A). Alkaline phosphatase-labeled streptavidin (1:2,000 in PBS/Az) was used to detect bound MAbs HL1912 and HL1914. The blot was incubated with gentle rocking for 60 min, and after four more washes the blot was incubated with substrate buffer (0.1 M Tris HCl, 1 mM $MgCl_2$ [pH 8.8]) containing 4.4 μl/ml nitroblue tetrazolium chloride and 3.3 μl/ml 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt.

cELISA. The cELISA was developed and optimized for use with the γ heavy-chain-specific MAb (MAb HL1912) only. The conditions and protocol for analysis of serum samples will be described elsewhere (32a). Briefly, wells of a high-protein-binding ELISA microplate were coated with 100 μl of 0.1 μg/ml purified dolphin IgG and incubated overnight at 4°C. After this and each subsequent step, the plate was washed three times with PBS-T using an automated microplate washer. A round-bottom assay plate was blocked with 300 μl of 1% BSA and allowed to incubate at 4°C for 1 h. The round-bottom assay plate was washed three times with PBS-T. Sixty microliters of 0.06 μg/ml biotinylated MAb HL1912 (0.03 μg/ml in PBS) and 60 μl of a serum sample diluted 1:256,000 in PBS/Az were allowed to compete in duplicate reactions in the round-bottom assay plate at 4°C overnight. The high-protein-binding ELISA microplate was blocked with 300 μl of 1% BSA. This plate and those used in each subsequent step were incubated for 1 h at room temperature. A total of 100 μl of the competition reaction was transferred, in duplicate, from the round-bottom assay plate to the ELISA plate. Duplicate wells of biotinylated MAb HL1912 (12.5 μg/ml) and fetal dolphin serum were used as the respective positive and negative controls on each plate. One hundred microliters of alkaline phosphatase-labeled streptavidin (1:1,500 in 1% BSA) was used as a secondary detection reagent. Finally, 100 μl of PNPP substrate (1 mg/ml) was added. The OD_{405} was recorded after 60 min of incubation, and the duplicate OD_{405} readings for all serum samples were averaged. For each species, the percentage of inhibition was calculated as (OD in sample serum − OD in fetal dolphin serum)/(OD in *T. truncatus* serum − OD in fetal dolphin serum) × 100.

Genetic distance of host species. Since the polyAb and MAbs are specific for IgG of *T. truncatus*, genetic distance for each species was estimated using distance of cytochrome *b* peptide sequences from *T. truncatus*. Cetartiodactyl cytochrome *b* sequences were downloaded from GenBank under the following accession numbers: *Tursiops truncatus*, ABK63391; *Bos taurus*, BAA07016; *Kogia breviceps*, CAD87948; *Delphinapterus leucas*, AAC31654; *Steno bredanensis*, AAD54454; *Lissodelphis borealis*, ABK63390; *Globicephala macrorhynchus*, AAD54432; *Pseudorca crassidens*, AAD54434; *Orcinus orca*, AAD54438; *Lagenorhynchus obliquidens*, ABK63403; *Phocoena phocoena*, ABK63372, *Eschrichtius robustus*, BAD91745, and *Megaptera novaeangliae*, BAD91693. Cytochrome *b* sequence was not available for *Delphinus capensis*, so data from *Delphinus delphis* were substituted (GenBank accession no. ABK63394). The available complete cytochrome *b* sequence for *Mesoplodon europaeus* contained numerous errors, as evidenced by the presence of multiple stop codons, so data from *Mesoplodon peruvianus* were substituted (GenBank accession no. AAC48451). Complete cytochrome *b* sequence was not available for *Phocoenoides dalli*, and this data point was dropped.

Complete cytochrome *b* peptide sequences, comprising 379 amino acids, were aligned using three methods: ClustalX (34), T-COFFEE (29), and MUSCLE (11). Distance calculations were made using the protdist program from PHYLIP (Phylogeny Inference Package, version 3.66 [12]), with amino acid substitution model PMB (40).

Interassay agreement. The results from the iELISA (OD_{405}) and cELISA (percentage of inhibition) assays were compared by Spearman's nonparametric correlation test using inStat (GraphPad Software, San Diego, CA). For relationships that were significant, a linear regression was calculated using inStat.

Correlation between IgG cross-reactivity and evolutionary distance. Distances from *T. truncatus*, as calculated using cytochrome *b* sequences, were compared to OD_{405} values from the ELISAs by Spearman's nonparametric correlation test using inStat (GraphPad Software, San Diego, CA). For relationships that were significant, a linear regression was calculated.

RESULTS

iELISA. The measured OD_{405} readings ranged from 0.012 to 3.930 (Table 1). Overall, the anti-whole-molecule polyAb and anti-heavy-chain MAb HL1912 showed a stronger reactivity than the anti-light-chain MAb HL1914 (two-tailed Student's *t* test, $P < 0.001$ [Table 2 and Fig. 2]). The polyAb was broadly cross-reactive with all species tested, including the domestic cow, whereas the reactivities of both MAbs varied markedly between animals (ratios of SD to mean of 0.094, 0.617, and 0.823 for polyAb, MAb HL1912, and MAb HL1914, respectively). In the iELISA format, the sera from the bottlenose dolphin and all other members of the Delphinoidea (monodontids, delphinids, and phocoenids) were equally reactive using both MAbs. However, the reactivities of both MAbs with the nondelphinoid (domestic cow, ziphioid, physeteroid, and mysticete) samples were significantly reduced (two-tailed Student's *t* test, $P < 0.05$).

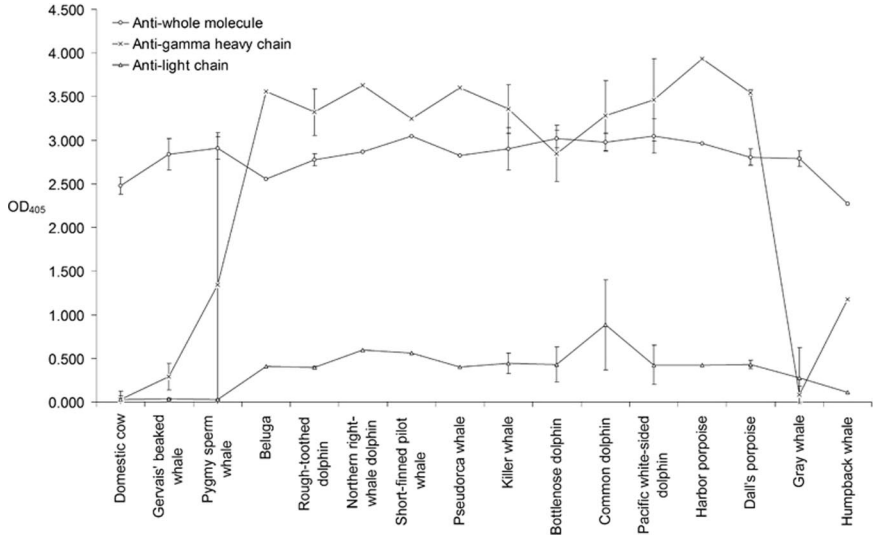

FIG. 2. Cross-reactivity (± SD) of the IgG whole molecule, γ heavy chain, and κ/λ light chain of various cetacean species and the domestic cow as detected using an iELISA. A broad cross-reactivity was observed when targeting the whole molecule, whereas the reactivities of the heavy and light chains varied between species.

Western blotting. The specificity of the antibodies for the whole molecule, γ chain, or κ or λ chains of the IgG of the cetartiodactyl species was confirmed via Western blotting (Fig. 1). Bands consistent in size with the γ chain (55 kDa) and κ or λ chains (25 kDa) of *T. truncatus* were detected in the bovine sample and in all 15 cetacean samples using the polyAb (Fig. 1A). Bands consistent in size with the γ chain (Fig. 1B) and the κ or λ chains (Fig. 1C) were detected in all delphinoid species tested ($n = 11$) using, respectively, the γ-chain-specific MAb and the light-chain-specific MAb (Fig. 1C) in all delphinid and phocoenid species tested. The heavy-chain-specific MAb detected no bands in nondelphinoid species, whereas the light-chain-specific MAb did detect a band of the appropriate size in the gray whale and beaked whale samples.

cELISA. The percent cross-reactivity of the IgG γ heavy chain ranged from less than 17% for the mysticetes to 106% for killer whale *Orcinus orca* (Table 1 and Fig. 3). No cross-reactivity was detected between the bovid and bottlenose dolphin IgG using the cELISA ($\bar{x} = 0.0\% \pm 0.1\%$). The IgG of baleen whales ($\bar{x} = 11.7\% \pm 8.8\%$) was significantly less cross-reactive than sera from all other cetacean lineages combined ($\bar{x} = 87.8\% \pm 28.1\%$). The Ziphioidea were the least cross-reactive odontocetes ($\bar{x} = 18.1\% \pm 9.3\%$), followed by the Physeteroidea ($\bar{x} = 51.1\% \pm 27.2\%$), and Delphinoidea ($\bar{x} = 99.1\% \pm 6.6\%$). The assay was unable to detect differences among the cross-reactivities of the delphinoid lineages (Monodontidae, $\bar{x} = 102.5\% \pm 0.7\%$; Delphinidae, $\bar{x} = 98.9\% \pm 7.3\%$; and Phocoenidae, $\bar{x} = 100.9\% \pm 2.0\%$).

Interassay agreement. A significant correlation was found between the anti-*Tursiops* γ MAb cELISA percent inhibition values and iELISA OD_{405} values, with a Spearman's test r^2 value of 0.5067 and a two-tailed P value of 0.0020. A linear regression was calculated, with a slope ± standard error (SE) of 27.419 ± 2.003 and a y intercept ± SE of 5.491 ± 5.793. A significant correlation was found between the anti-*Tursiops* γ MAb iELISA OD_{405} values and the anti-*Tursiops* κ/λ MAb iELISA OD_{405} values, with a Spearman's test r^2 value of 0.3189 and a two-tailed P value of 0.0227. A linear regression was calculated, with a slope ± SE of 0.1228 ± 0.02825 and a y intercept ± SE of 0.05377 ± 0.08169. The correlation between the anti-*Tursiops* γ MAb iELISA OD_{405} values and polyAb

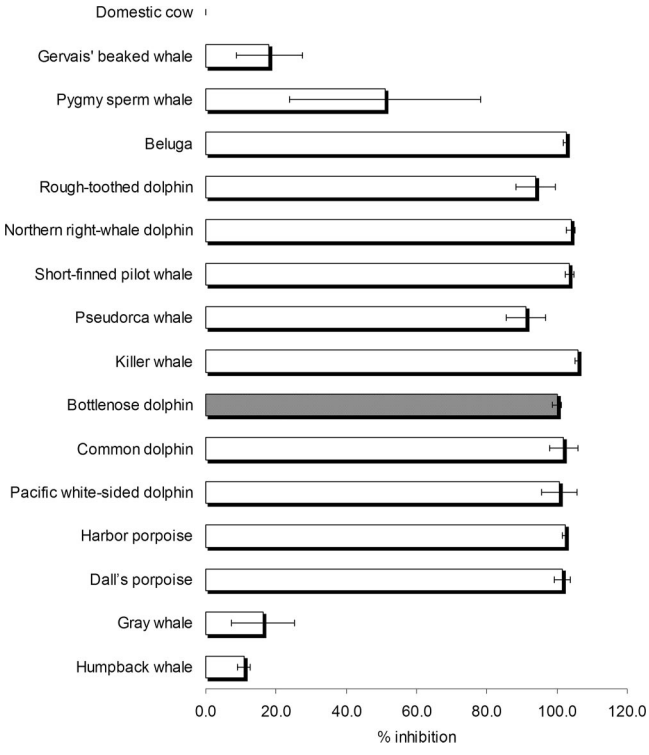

FIG. 3. Percent cross-reactivity (± SD) of the IgG γ heavy chain of various cetacean species and the domestic cow as detected on a cELISA. The percent cross-reactivity was highest for all delphinoids and lowest for the mysticetes. No cross-reactivity was detected with IgG of the domestic cow.

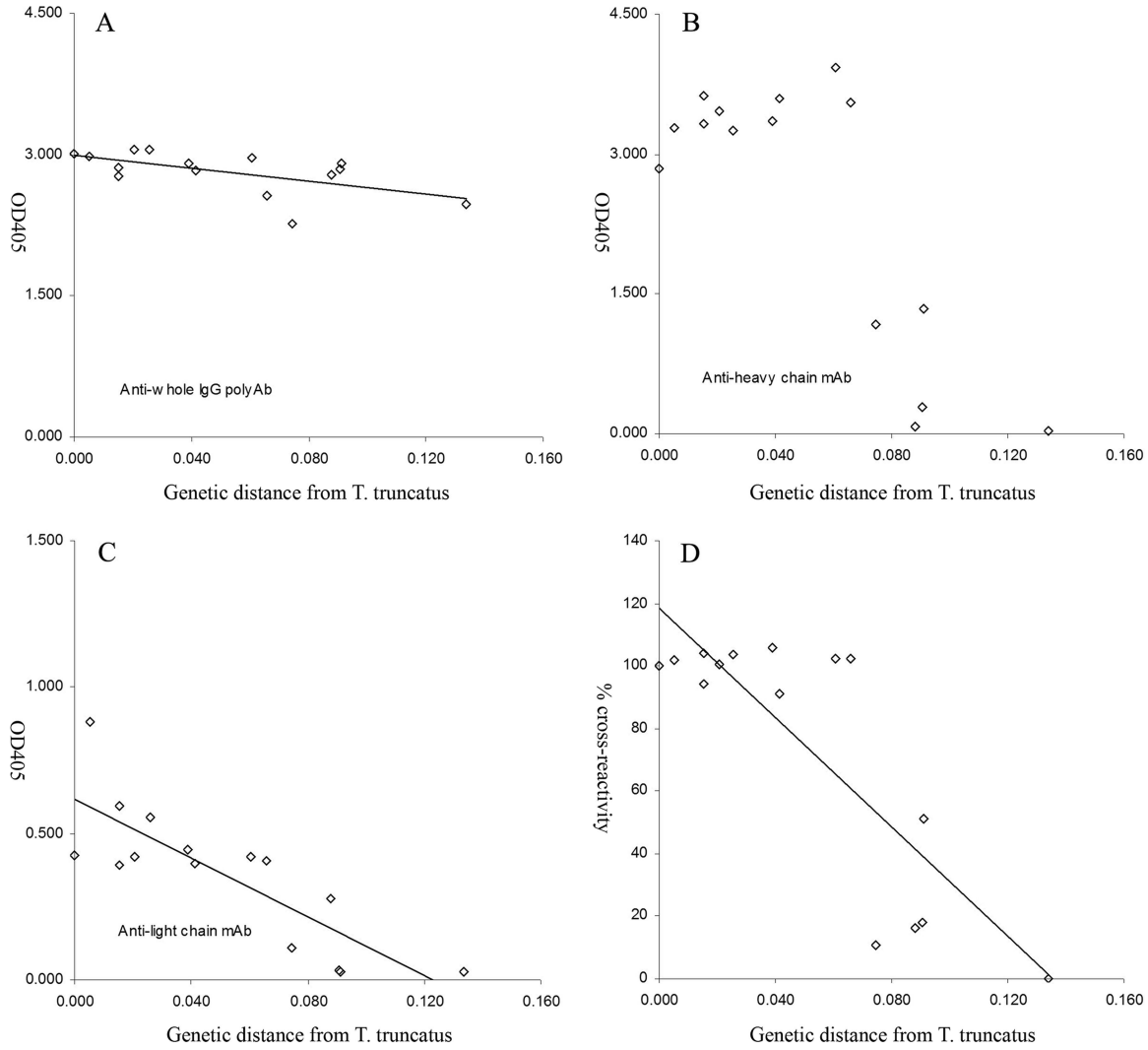

FIG. 4. Correlation between IgG cross-reactivity and evolutionary distance from the bottlenose dolphin, *Tursiops truncatus*. The reactivity of the anti-whole-IgG polyAb (A), anti-γ heavy-chain MAb (B), anti-κ/λ light-chain MAb (C) in an indirect ELISA and percent cross-reactivity as determined via a competitive ELISA (D) decreased with increasing genetic distance of the host species from *T. truncatus*. Linear correlations, where statistically significant ($P < 0.05$), are plotted.

iELISA OD_{405} values was not statistically significant, with a Spearman's test r^2 value of 0.0553 and a two-tailed *P* value of 0.3804. A significant correlation was found between the anti-*Tursiops* κ/λ MAb iELISA OD_{405} values and polyAb iELISA OD_{405} values, with a Spearman's test r^2 value of 0.2865 and a two-tailed *P* value of 0.0326. A linear regression was calculated, with a slope ± SE of 0.4840 ± 0.2141 and a *y* intercept ± SE of 2.640 ± 0.09176.

Correlation between IgG cross-reactivity and evolutionary distance. Alignment of cetartiodactyl cytochrome *b* sequences showed identical results using all three alignment methods, with no gaps present in any sequences. Calculated genetic distances are shown in Table 2. A significant correlation was found between the anti-*Tursiops* IgG polyclonal Ab iELISA OD_{405} values and the genetic distance of cytochrome *b* peptide sequences, with a Spearman's test r^2 value of 0.2794 and a two-tailed *P* value of 0.0428. A linear regression is shown, with a slope ± SE of −0.1029 ± 0.03928 and a *y* intercept ± SE of 0.3411 ± 0.1110 (Fig. 4A). The correlation between the anti-*Tursiops* γ MAb iELISA OD_{405} values and genetic distance of cytochrome *b* peptide sequences approached statistical significance, with a Spearman's test r^2 of 0.2394 and a two-tailed *P* value of 0.0642 (Fig. 4B). A significant correlation was found between the anti-*Tursiops* γ MAb competitive ELISA percent inhibition values and genetic distance of cytochrome *b* peptide sequences, with a Spearman's test r^2 of 0.3473 and a two-tailed *P* value of 0.0208. A linear regression is shown, with a slope ± SE of −0.0007875 ± 0.0001465 and a *y* intercept ± SE of 0.1090 ± 0.01224 (Fig. 4C). A significant correlation was found between the anti-*Tursiops* κ/λ MAb iELISA OD_{405} values and genetic distance of cytochrome *b* peptide sequences, with a Spearman's test r^2 of 0.6458 and a two-tailed *P* value of 0.0003. A linear regression is shown, with a slope ± SE of −0.1350 ± 0.02596 and a *y* intercept ± SE of 0.1000 ± 0.01112 (Fig. 4D).

DISCUSSION

In this study, the cross-reactivity IgG components of a phylogenetically diverse selection of cetaceans and a representative of the Ruminantia with IgG of *Tursiops truncatus* was assayed using three distinct antibodies. Western blotting was used to support the specificity of these antibodies for the IgG molecules of the various species, by demonstrating bands consistent in size with the γ chain (55 kDa) and κ or λ chains (25 kDa). The presence of heavier bands using the light-chain antibody is likely due to incomplete denaturation of the serum IgG. Once the specificity of the antibodies was confirmed, the degree of cross-reactivity was quantified using both an iELISA and a cELISA format. Since cELISA does not depend on host species-specific reagents, it may be considered as a "gold standard" for detection of antibodies in novel host species. However, the high correlation between cELISA and iELISA results does validate the use of the iELISA in the species tested. Overall, there was good agreement between the assays using all antibodies.

Each antibody provided a different level of phylogenetic resolution. Overall, the observed cross-reactivity patterns mirrored the phylogenetic branching orders for the Cetartiodactyla that have been proposed based on mitochondrial and nuclear sequence data. The polyAb, which has the broadest predicted range of reactivity, was able to cross-react with IgG from all cetacean and the bovid samples. The anti-Tursiops γ-chain MAb, which has a more narrow predicted phylogenetic range of reactivity, reacted equally well with the IgG antibodies of all delphinoid relatives of *Tursiops truncatus*, including the Phocoenidae, whereas it reacted much less strongly with those of the much more distantly related Physeteroidea, Ziphioidea, and Mysticeti. The anti-Tursiops γ-chain MAb did not show any reactivity with the IgG from the bovid sample. We detected a strong negative correlation between the antigenic cross-reactivity of IgG γ-chain molecules and the genetic distance of their hosts using three out of four assay formats (Fig. 4). This, again, supports the close relationship of the members of the Delphinoidea and phylogenetic branching order of the cetaceans that has been proposed based on sequence data. The anti-Tursiops κ/λ-chain MAb appeared to have a less predictable range of reactivity. Like the γ chain, the Tursiops κ/λ chain reacted equally well with the IgG antibodies of all delphinoid relatives of *Tursiops truncatus* and no reactivity was observed with the IgG from the artiodactyl sample. However, we did not detect any reactivity with physeteroid and ziphioid IgG, whereas partial cross-reactivity with the κ/λ light chains of one of the mysticete species (humpback whale) was observed. We were unable to find data on the patterns and relative rates of evolutionary change of the γ heavy chain and the κ/λ light chains in mammals. We are therefore unable to explain the observed differences in the γ and κ/λ cross-reactivity patterns in the more distant relatives of *Tursiops truncatus*.

The lack of validated reagents for use in serologic assays in diverse cetacean species has limited our ability to diagnose and understand infectious diseases in these animals. The data presented here represent the first validation of serodiagnostic reagents for iELISA in cetacean species beyond *Tursiops truncatus*. Fifteen of approximately 80 cetacean species are represented, including several uncommon species. Furthermore, the relationship seen with these reagents between genetic distance and antigenic cross-reactivity provides a predictive framework for how they may be expected to react to IgG antibodies of additional cetacean species. The polyAb appears to react well with diverse cetartiodactyl IgG antibodies. The MAbs appear to react well with IgG antibodies of diverse Delphinoidea. The reactivity of the light-chain-specific MAb, although significantly influenced by evolutionary distance, did appear to be least predictable.

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REFERENCES

1. **Åkerstrom, B., T. Brodin, K. Reis, and L. Björck.** 1985. Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. J. Immunol. **135:**2589–2592.
2. **Arnason, U.** 1969. The karyotype of the fin whale. Hereditas **62:**273–284.
3. **Arnason, U., A. Gullberg, S. Gretarsdottir, B. Ursing, and A. Janke.** 2000. The complete mitochondrial genome of the sperm whale and the establishment of a new molecular reference for estimating eutherian divergence dates. J. Mol. Evol. **50:**569–578.
4. **Arnason, U., A. Gullberg, and A. Janke.** 2004. Mitogenomic analyses provide new insights into cetacean origin and evolution. Gene **333:**27–34.
5. **Asada, Y., Y. Kawamoto, T. Shotake, and K. Terao.** 2002. Molecular evolution of IgG subclasses among nonhuman primates: implication of difference in antigenic determinants among apes. Primates **43:**343–349.
6. **Ben-Barak, Y., J. T. Russell, M. Whitnall, K. Ozato, and H. Gainer.** 1984. Phylogenetic cross-reactivities of monoclonal antibodies produced against rat neurophysin. Cell. Mol. Neurobiol. **4:**339–349.
7. **Bininda-Emonds, O. R., M. Cardillo, K. E. Jones, R. D. MacPhee, R. M. Beck, R. Grenyer, S. A. Price, R. A. Vos, J. L. Gittleman, and A. Purvis.** 2007. The delayed rise of present-day mammals. Nature **446:**507–512.
8. **Black, C. M., J. S. McDougal, R. C. Holman, B. L. Evatt, and C. B. Reimer.** 1993. Cross-reactivity of 75 monoclonal antibodies to human immunoglobulin to sera of non-human primates. Immunol. Lett. **37:**207–213.
9. **Boyden, A., and D. Gemeroy.** 1950. The relative position of the Cetacea among the orders of Mammalia as indicated by precipitin tests. Zoologica **35:**145–151.
10. **Criscitiello, M. F., and M. F. Flajnik.** 2007. Four primordial immunoglobulin light chain isotypes, including k and j, identified in the most primitive living jawed vertebrates. Eur. J. Immunol. **37:**2683–2694.
11. **Edgar, R. C.** 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32:**1792–1797.
12. **Felsenstein, J.** 1989. PHYLIP—phylogeny inference package. Cladistics **5:**164–166.
13. **Gatesy, J., M. Milinkovitch, V. Waddell, and M. Stanhope.** 1999. Stability of cladistic relationships between Cetacea and higher-level artiodactyl taxa. Syst. Biol. **48:**6–20.
14. **Goodman, M.** 1962. Evolution of the immunologic species specificity of human serum proteins. Hum. Biol. **34:**104–150.
15. **Guimarães, P. R., Jr., M. A. de Menezes, R. W. Baird, D. Lusseau, P. Guimarães, and S. F. dos Reis.** 2007. Vulnerability of a killer whale social network to disease outbreaks. Phys. Rev. E Stat. Nonlin. Soft Matter Phys. **76:**042901.
16. **Hafleigh, A. S., and C. A. Williams, Jr.** 1966. Antigenic correspondence of serum albumins among the primates. Science **151:**1530–1535.
17. **Irwin, D. M., and U. Arnason.** 1994. Cytochrome b gene of marine mammals: phylogeny and evolution. J. Mamm. Evol. **2:**37–55.
18. **Jepson, P. D., S. Brew, A. P. MacMillan, J. R. Baker, J. Barnett, J. K. Kirkwood, T. Kuiken, I. R. Robinson, and V. R. Simpson.** 1997. Antibodies to *Brucella* in marine mammals around the coast of England and Wales. Vet. Rec. **141:**513–515.
19. **Kronvall, G., U. S. Seal, J. Finstad, and R. C. Williams, Jr.** 1970. Phyloge-

netic insight into evolution of mammalian Fc fragment of gamma G globulin using staphylococcal protein A. J. Immunol. **104:**140–147.
20. **Lindmark, R., K. Thorén-Tolling, and J. Sjöquist.** 1983. Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera. J. Immunol. Methods **62:**1–13.
21. **May-Collado, L., and I. Agnarsson.** 2006. Cytochrome b and Bayesian inference of whale phylogeny. Mol. Phylogenet. Evol. **38:**344–354.
22. **Mihindukulasuriya, K. A., G. Wu, J. St. Leger, R. W. Nordhausen, and D. Wang.** 2008. Identification of a novel coronavirus from a beluga whale by using a panviral microarray. J. Virol. **82:**5084–5088.
23. **Milinkovitch, M. C.** 1992. DNA-DNA hybridizations support ungulate ancestry of Cetacea. J. Evol. Biol. **5:**149–160.
24. **Nikaido, M., A. P. Rooney, and N. Okada.** 1999. Phylogenetic relationships among cetartiodactyls based on insertions of short and long interspersed elements: hippopotamuses are the closest extant relatives of whales. Proc. Natl. Acad. Sci. USA **96:**10261–10266.
25. **Nikaido, M., F. Matsuno, H. Hamilton, R. L. Brownell, Y. Cao, W. Ding, Z. Zuoyan, A. M. Shedlock, R. E. Fordyce, M. Hasegawa, and N. Okada.** 2001. Retroposon analysis of major cetacean lineages: the monophyly of toothed whales and the paraphyly of river dolphins. Proc. Natl. Acad. Sci. USA **98:**7384–7389.
26. **Nishida, S., M. Goto, L. A. Pastene, N. Kanda, and H. Koike.** 2007. Phylogenetic relationships among cetaceans revealed by Y-chromosome sequences. Zoolog. Sci. **24:**723–732.
27. **Nollens, H. H., L. G. Green, D. Duke, M. T. Walsh, B. Chittick, S. Gearhart, P. A. Klein, and E. R. Jacobson.** 2007. Development and validation of monoclonal and polyclonal antibodies for the detection of immunoglobulin G of bottlenose dolphins (*Tursiops truncatus*). J. Vet. Diagn. Investig. **19:**465–470.
28. **Nollens, H. H., J. F. X. Wellehan, J. T. Saliki, S. L. Caseltine, E. D. Jensen, W. Van Bonn, and S. Venn-Watson.** 2008. Characterization of a parainfluenza virus isolated from a bottlenose dolphin (*Tursiops truncatus*). Vet. Microbiol. **128:**231–242.
29. **Notredame, C., D. G. Higgins, and J. Heringa.** 2000. T-COFFEE: a novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. **302:**205–217.
30. **Omatsu, T., Y. Ishii, S. Kyuwa, E. G. Milanda, K. Terao, and Y. Yoshikawa.** 2003. Molecular evolution inferred from immunological cross-reactivity of immunoglobulin G among Chiroptera and closely related species. Exp. Anim. **52:**425–428.
31. **Pichler, F. B., and C. S. Baker.** 2000. Loss of genetic diversity in the endemic Hector's dolphin due to fisheries-related mortality. Proc. Biol. Sci. **267:**97–102.
32. **Reeves, R. A., B. S. Stewart, and P. J. Clapham (ed.).** 2002. National Audubon Society guide to marine mammals of the world, p.8–13. Chanticleer Press, New York, NY.
32a. **Ruiz, C., S. Ven-Watson, E. Jacobson, L. Green, R. S. Wells, M. T. Walsh, B. Chittick, J. F. McBain, and H. H. Nollens.** Dev. Comp. Immunol., in press.
33. **Sasaki, T., M. Nikaido, H. Hamilton, M. Goto, H. Kato, N. Kanda, L. A. Pastene, Y. Cao, R. E. Fordyce, M. Hasegawa, and N. Okada.** 2005. Mitochondrial phylogenetics and evolution of Mysticete whales. Syst. Biol. **54:**77–90.
34. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
35. **Tryland, M., L. Kleivane, A. Alfredsson, M. Kjeld, A. Arnason, S. Stuen, and J. Godfroid.** 1999. Evidence of *Brucella* infection in marine mammals in the North Atlantic Ocean. Vet. Rec. **144:**588–592.
36. **Turvey, S. T., R. L. Pitman, B. L. Taylor, J. Barlow, T. Akamatsu, L. A. Barrett, X. Zhao, R. R. Reeves, B. S. Stewart, K. Wang, Z. Wei, X. Zhang, L. T. Pusser, M. Richlen, J. R. Brandon, and D. Wang.** 2007. First human-caused extinction of a cetacean species? Biol. Lett. **3:**537–540.
37. **Ursing, B. M., and U. Arnason.** 1998. Analyses of mitochondrial genomes strongly support a hippopotamus clade. Proc. R. Soc. Lond. B **265:**2251–2255.
38. **Van Bressem, M. F., K. Van Waerebeek, M. Fleming, and T. Barrett.** 1998. Serological evidence of morbillivirus infection in small cetaceans from the Southeast Pacific. Vet. Microbiol. **59:**89–98.
39. **Van Bressem, M. F., K. Van Waerebeek, and J. A. Raga.** 1999. A review of virus infections of cetaceans and the potential impact of morbilliviruses, poxviruses, and papillomaviruses on host population dynamics. Dis. Aquat. Organ. **38:**53–65.
40. **Veerassamy, S., A. Smith, and E. R. Tillier.** 2003. A transition probability model for amino acid substitutions from blocks. J. Comput. Biol. **10:**997–1010.