Species Identification Using Genetic Tools: The Value of Nuclear and Mitochondrial Gene Sequences in Whale Conservation

S. R. Palumbi and F. Cipriano

DNA sequence analysis is a powerful tool for identifying the source of samples thought to be derived from threatened or endangered species. Analysis of mitochondrial DNA (mtDNA) from retail whale meat markets has shown consistently that the expected baleen whale in these markets, the minke whale, makes up only about half the products analyzed. The other products are either unregulated small toothed whales like dolphins or are protected baleen whales such as humpback, Bryde's, fin, or blue whales. Independent verification of such mtDNA identifications requires analysis of nuclear genetic loci, but this is technically more difficult than standard mtDNA sequencing. In addition, evolution of species-specific sequences (i.e., fixation of sequence differences to produce reciprocally monophyletic gene trees) is slower in nuclear than in mitochondrial genes primarily because genetic drift is slower at nuclear loci. When will use of nuclear sequences allow forensic DNA identification? Comparison of neutral theories of coalescence of mitochondrial and nuclear loci suggests a simple rule of thumb. The "three-times rule" suggests that phylogenetic sorting at nuclear loci is likely to produce species-specific sequences when mitochondrial alleles are reciprocally monophyletic and the branches leading to the mtDNA sequences of a species are three times longer than the average difference observed within species. A preliminary test of the three-times rule, which depends on many assumptions about the species and genes involved, suggests that blue and fin whales should have species-specific sequences at most neutral nuclear loci, whereas humpback and fin whales should show species-specific sequences at fewer nuclear loci. Partial sequences of actin introns from these species confirm the predictions of the three-times rule and show that blue and fin whales are reciprocally monophyletic at this locus. These intron sequences are thus good tools for the identification of these species and will afford a chance to identify putative hybrid blue/fin whales thought to have entered the retail market after 1989.

International fisheries represent a global resource that is increasingly threatened by overexploitation. There are over 3 million fishing vessels operating in the world, and 69% of fisheries stocks are either fully exploited or overfished (FAO 1995). International agreements to protect oceanic ecosystems from overfishing have become more numerous in recent years, but it is not clear if these agreements will have a dramatic effect. For example, the United Nations Convention on the Law of the Sea, implemented in 1994, requires nations to ensure that the living marine resources within their exclusive economic zones are not endangered by overexploitation. However, the impact of this treaty is limited, especially because 4 of the top 20 fishing nations (including the United States) have yet to sign it.

One of the first international fishery resources to be recognized as overexploited were the great whales. Whales have traditionally been part of the diet and culture of many nations around the world, but industrialization of whaling in the 19th and 20th centuries led to the collapse of all of the world's populations of great whales. The International Whaling Commission (IWC) was established in 1948 to regulate and maintain the whaling industry through a set of international agreements about whaling quotas and practices. Species that were particularly threatened came under international protection in the 1960s (blue whales, gray whales, humpback whales), and commercial whaling was halted in 1985 in order to allow all whale stocks to recover.

Despite these regulations, whale hunt-

From the Department of Organismic and Evolutionary Biology and Center for Conservation and Evolutionary Genetics, Harvard University, Cambridge, MA. 02138 (Palumbi and Cipriano) and Earthtrust, Kailua, Hawaii (Cipriano). We thank Scott France, Matt Hare, Francis Villablanca, and two reviewers for discussion and criticism of an earlier draft. We also thank C. S. Baker, R. Brownell, A. Dizon, A. Helden, G. Lento, L. Medrano, J. Reyes, and L. Siemann for tissue samples or unpublished reference sequences. Monitoring of whale meat markets is supported by Earthtrust. Development of nuclear markers and the theory of species identifications is supported by the National Science Foundation. Address correspondence to Dr. Palumbi at the address above or e-mail: spalumbi@oeb.harvard.edu. This paper was delivered at a symposium entitled "Conservation and Genetics of Marine Organisms" sponsored by the American Genetic Association at the University of Victoria, Victoria, BC, Canada, June 7, 1997.

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ing continues and whale products are legally traded. Currently, whaling is sanctioned by the IWC for aboriginal use or scientific research. Whales taken for research purposes may be sold in domestic commercial markets. In addition, some member nations continue commercial whaling by resigning from the IWC or by lodging an objection to IWC regulations. International sale and transport of whale products taken legally under IWC regulations is regulated by the Convention for International Trade of Endangered Species (CITES), and all international transactions involving whales must be conducted under CITES permits.

Thus hunting of whales is under strict international regulation, as is trade in whale products. These two modes of regulation are a seemingly effective and balanced approach to preserving whale species, as well as preserving the whaling industry, and might serve as models for the regulation of other high seas fisheries. However, there is a critical gap in the net of regulatory scrutiny that surrounds modern whaling. This gap—pirate whaling and smuggling of whale meat products—historically has been difficult to document and, as a result, has received little scrutiny from regulatory agencies. In this case, genetic approaches have proven invaluable in directly monitoring the retail market itself, and can be used to determine if international regulations on hunting and trade are reflected in the whale products sold to consumers.

Genetic Monitoring of Whaling

Once whale products reach retail markets, they have been processed to such an extent that morphological identification is often impossible. Canned meat, dried whale jerky, bacon, or thin strips of blubber with skin show few of the morphological features that distinguish the great whale species. Yet persistent reports that massive hunts of whales have gone unreported to the IWC (Yablokov 1994), and that whale meat is smuggled internationally (Anonymous 1993, 1994a–d) suggest that species-level identification is required to verify the composition of commercial whale products.

Using genetics to identify whale products is a straightforward application of molecular tools developed to understand the relationships between species (Hillis et al. 1996). DNA sequence data from test samples can be compared to sequences collected from animals of known species identity. We have concentrated on collecting sequence data from the mitochondrial control region because it is highly variable among cetacean species and has also been shown to vary among conspecific populations (Baker et al. 1993; Baker and Palumbi 1994). Thus mtDNA sequences can be used in "forensic" identification of whale products from local markets. A drawback of mtDNA sequencing is that the mitochondrial genome is inherited maternally in most animals, and so hybrids possess only the genetic signature of their maternal parent. In cases where natural hybridization occurs (Arnason et al. 1991), mitochondrial data must be supplemented with molecular tools based on biparentally inherited nuclear genes.

Following International Regulations

The choice of genes to analyze is only one of the issues that needs to be surmounted in order to establish a molecular monitoring program for whales. CITES regulations cover the trade of endangered species or their derivatives, and this includes their DNA. For researchers to claim immunity from international regulations designed to protect species is an unacceptable position. As a result, genetic monitoring of whale markets required us to develop protocols that allow the identification of DNA without moving it across international boundaries. To accomplish this we took advantage of the ability of the polymerase chain reaction (PCR) to rapidly copy template DNA. PCR products of whale meat are not derivatives of whales, but are synthetic, partial copies of particular genes. We and others have argued successfully (Bowen and Avise 1994) that pure PCR products should not be regulated under CITES and should be transportable without permits (Jones 1994).

However, the PCR reaction cocktail containing synthetic products is not free of the derivatives of whales. Instead, it includes a small amount of starting DNA template, and this template falls under CITES regulations. To remove this template from the PCR mixture, we took advantage of a second property of the PCR process: the incorporation into each PCR product of one of the oligonucleotide primers used as starting material for the PCR synthesis. These primers can be synthesized so that they include the molecule biotin, which is never found in native DNA. As a result, the resulting copies of whale DNA also contain biotin. Biotinylated DNA copies can be separated from nonbiotinylated whale template DNA by mixing the PCR product with a slurry of tiny magnetic beads coated with the protein strepavidin. Biotin binds tightly to the strepavidin and thus the biotinylated, copied DNA becomes attached to the beads. The copied DNA can then be separated from the native whale DNA by pulling the beads out of the slurry with a magnet (Bowman and Palumbi 1993). After repeated washing, the beads retain abundant copies of the particular gene synthetically copied by PCR, but no native whale DNA.

DNA bound to magnetic beads can be sequenced directly or it can be reamplified and sequenced (Bowman and Palumbi 1993). These sequences are compared to a database of sequences from individuals from known populations and species, and the relationships between sequences from unknown and known samples are evaluated by parsimony or maximum likelihood criteria (Swofford et al. 1996). Reliability of sequence relationships is established using bootstrap procedures, in which random subsets of the original data (chosen with replacement) are reanalyzed and compared. Relationships that are overwhelmingly supported in the randomly chosen, reconstructed datasets are statistically reliable (Hillis and Bull 1993).

Monitoring of Asian Markets: 1993–1996

Whale products (n = 237 samples total) were purchased throughout central Japan in 1993, 1995, and 1996 using randomized collection protocols in which samples are chosen independent of label or appearance. As in previous collaborative surveys, a variety of commercial outlets were visited by independent conservation agents. Sample collection was funded through and coordinated by the international conservation organization Earthtrust. Two or three packages of whale meat products were purchased from each vendor. In 1995 and 1996, collectors made an effort to obtain samples throughout the cities of central Japan from Fukuoku to Miyagi Prefecture, and many of the same stores were revisited in both years. Although not completely random, these protocols provide a cross-section of available products. Descriptions of products from Japan and Korea included sashimi, salted and partially cooked meat, whale bacon, and sliced blubber strips with attached skin.

An initial survey of whale products available for sale in Japan showed that the

Table 1. Whale products identified in surveys, 1993–1996

Species	Korea		Japan			Total
	1994	1995	1993	1995	1996	
N. minke whale	13	13	3	4	9	42
S. minke whale	0	2	22	37	46	107
Pygmy Bryde's whale	2	0	0	0	0	2
Bryde's whale	0	2	1	1	0	4
Humpback whale	0	0	1	0	0	1
Fin whale	0	0	7	3	2	12
Blue whale	0	0	1	1	0	2
Baird's beaked whale	0	0	0	10	5	15
Cuvier's beaked whale	1	0	1	0	1	3
Dolphins	1	13	5	5	18	42
Porpoises	0	0	0	1	6	7
Total	17	30	41	62	87	237

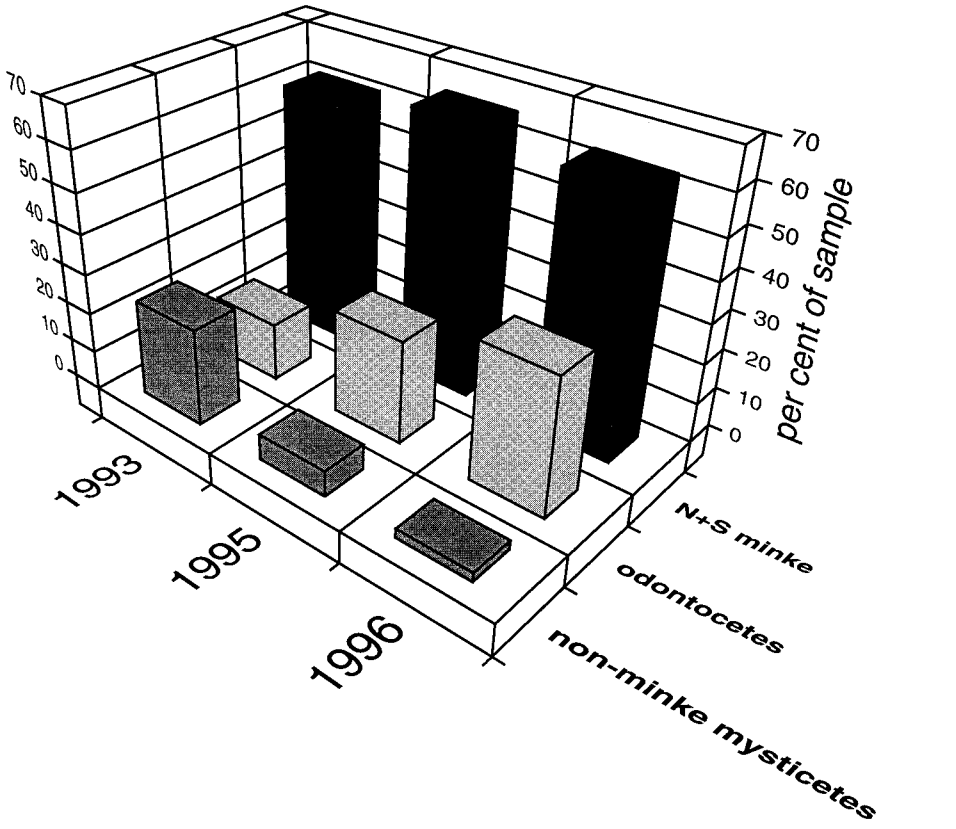

Figure 1. Apparent decline in number of protected species found in Japanese markets from 1993 to 1996, with concomitant increase in number of small odontocetes. Data are summarized from Table 1.

expected legal population (minke whales taken under scientific permit in the southern hemisphere) made up slightly more than half of the commercial market (Baker and Palumbi 1994). Repeated sampling of the Japanese market confirmed these results. The 1993 and 1995 surveys in Japan showed that about 60– 70% of the samples were derived from minke whales, 15–25% were small odontocetes, and 10–25% were from baleen whale species that are currently under IWC protection (Table 1).

Other markets have very different patterns. In Korea, the bulk of the samples were minke whales from the northern Pacific, with few animals from the Japanese hunt in the southern oceans (Baker et al. 1996). Korea presently has no legal whaling industry, so the presence of any type of whale meat for sale is a surprise (Baker et al. 1996).

Of the other baleen whale species, we have found sequences from humpback, blue, Bryde's, and fin whales on the Japanese market. Fin whales, probably from the North Atlantic, are the most common species, appearing in 12 out of 170 baleen whale samples. This population has not been hunted legally since 1989, and has not been traded legally since 1991, yet fin whales have appeared in every Japanese sample we have analyzed. Other baleen whale species have not occurred as frequently as fin whales. The initial 1993 survey of 19 samples identified tissue from a northern Pacific humpback whale in a mixture of dried, ground meat (Baker and Palumbi 1994). We have yet to find another humpback in subsequent samples (see however Lento et al. 1997). However, surveys in both 1993 and 1995 discovered a single sample each that had the mtDNA signature of a blue whale.

Trends in Species Found in the Japanese Whale Meat Market

Overall results of our market survey analysis from 1993 to 1996 suggest a decline in the number of non-minke mysticetes (i.e., baleen whales other than minke whales) available on the Japanese market. The frequency of samples from protected baleen whales has dropped from 24% in 1993 to 2.5% in 1996 (Table 1, Figure 1). The drop in the availability of products from protected whales may be due to increased efforts to prevent illegal hunting and importation of whale products, and to decreases in stockpiles of products of protected species. Continued surveys with larger sample sizes are needed to determine whether this decline reflects a significant decrease or is due to random fluctuations in the market that cannot be distinguished with small samples. The number of minke whales taken by Japan has recently expanded, with an increased hunt in the Antarctic and a new hunt in the North Pacific that was established in 1994. However, the apparent decline in non-minke mysticetes is not accompanied by an increase in the proportion of minke whale samples identified, but instead is at the expense of small odontocetes. In the latest sample, 28% was dolphin and porpoise meat being sold as whale meat (Table 1). Together with beaked whales, currently unprotected odontocetes accounted for 35% of the Japanese market in our 1996 survey (Table 1, Figure 1).

Many more non-minke mysticetes have been identified in the recent surveys of Japan and Korea by Gina Lento and Scott Baker, who in 1996–1997 focused on questionable sources and products that were openly labeled or advertised as protected species (Lento et al. 1997). Our randomized surveys give a general description of potential trends in the market but are unlikely to detect such extraordinary species of baleen whales from samples sizes of less than a hundred individuals. Expanded surveys, with sample sizes increased up to 500 or so individual products analyzed per year, would give more precise estimates of changes in market composition over time and the prevalence of protected species being sold on the Japanese market. A whale meat monitoring program, such as the one proposed in a resolution passed

at the 47th IWC meeting (Dublin, Ireland, 1995), which utilizes random sampling could be used to document trends in whale markets.

Sample sizes of past surveys have been limited by the cost and difficulty of sequencing large numbers of samples. Improvements in our analysis methods (improved extraction techniques, species-specific primers), technology (automatic sequencing, dot-blot techniques), and software (improved sequence analysis and database software) will increase the speed and decrease the cost of sequence identification analysis by about one order of magnitude when fully implemented. Although the cost of conducting market surveys is still high, it is now possible to increase sample sizes to the level that both market trends and the incidence of protected species may be estimated with better precision in a single survey of about 500 products.

The Need for Nuclear Loci

Using nuclear primers to investigate species identities in forensic studies has the advantage that conclusions can be verified by examining a number of independent loci. This ability is particularly important in cases where PCR contamination is a possibility, or when hybridization between species is known to occur (e.g., Wayne 1996).

For example, blue and fin whales are known to have hybridized to form viable offspring (reviewed in Bérubé and Aguilar 1998). Four of these offspring have been examined in detail, three of which had blue whale mothers (Bérubé and Aguilar 1998). Because mtDNA is inherited maternally, these animals have blue whale mtDNA sequences, and if present in the Japanese market, they would appear to be blue whales despite their hybrid origin. In fact, of the 237 tissue samples we have analyzed for mtDNA, two showed sequences that clustered with those of blue whales (Baker at al. 1996). Partial control region sequences from these two samples are indistinguishable from one another (Baker et al. 1996) and are indistinguishable from the sequence obtained from a hybrid blue/fin whale studied by Arnason and colleagues in 1991 (Arnason et al. 1991; Spilliaert et al. 1991). Did we find the same hybrid whale in our Japanese market samples in 1993 and 1995? Or did we find a pure blue whale that happened to have the same sequence as the hybrid? Sequencing of variable nuclear DNA regions might allow us to distinguish the two blue whale samples from one another and to distinguish them from the animal described by Spilliaert et al. (1991).

Table 2. Determinants of genetic drift in mitochondrial and nuclear loci under the standard neutral model

Parameter	mtDNA	Nuclear DNA	Reference
Effective gene number	N_f	$2N_e$	Birky et al. 1989
Mean time to fixation (generations)	$2N_f$	$4N_e$	Birky 1991
Time to reciprocal monophyly of ~95% of loci (generations)	$4N_f$	$8N_e$	Neigel and Avise 1986; Nei 1987
Average divergence between alleles	$2N_f\mu$	$4N_e\mu$	Kuhner et al. 1995

Times are in generations; μ is the per generation mutation rate.

Using nuclear sequences to distinguish different species of whales is more complicated than using mtDNA sequences. Nuclear sequences typically evolve more slowly than mtDNA in most animals (Vawter and Brown 1986; Wilson et al. 1985). In addition, nuclear loci have a fourfold higher effective population size than mitochondrial loci and as a result, genetic drift occurs more slowly among alleles at a nuclear locus. These differences have two important consequences for forensic studies. First, different populations diverge in mitochondrial gene frequencies more rapidly than they do for nuclear alleles (Birky et al. 1989). For example, humpback whales show strong population structure within and between ocean basins for mtDNA variants (Baker et al. 1993), but for intron variation at an actin locus, only populations from different ocean basins differ strongly (Palumbi and Baker 1994). Second, closely related species are more likely to have fixed differences for mitochondrial haplotypes than they are for nuclear gene variation (Neigel and Avise 1986). This is because lower effective population size at mitochondrial loci leads to much shorter coalescence times within species than at nuclear loci.

The mean time to fixation of a new mitochondrial mutant is about $2N_f$, where N_f is the genetically effective number of females in the population (Birky 1991). For species with an 1:1 sex ratio and the same variance in reproductive success between males and females, $2N_f \approx N_e$. This is fourfold lower than the fixation time for nuclear loci because mtDNA is haploid and inherited largely through the female parent in most animals [Table 2, but see Birky (1991, pp. 121–122) for important exceptions.] Thus, even if mtDNA sequences can be used to discriminate among species, nuclear loci from the same species may fail to be distinct.

We recently proposed a simple rule of thumb for using mtDNA data to predict when nuclear loci should be monophyletic (Palumbi SR et al., manuscript in preparation). Species with mtDNA branches at least three times longer than the mtDNA diversity within that species are likely to show nuclear alleles that are also monophyletic. By contrast, species with mtDNA branches less than three times as long as the intraspecific diversity are less likely to show a majority of nuclear loci that are monophyletic (Figure 2). This "three-times rule" makes many assumptions that may not be met in practice, including complete neutrality, constant population size, equal sex ratios, equal variance in reproductive success, no recombination, and no hybridization. In addition, because lineage sorting occurs randomly, there is wide variation in fixation times among neutral loci (Nei 1987), and thus not all nuclear loci will coalesce at the same time. Recombination of nuclear genes will also affect coalescence times. Nevertheless, the rule provides a yardstick with which we can use mtDNA data to make predictions about the genealogies of nuclear loci.

Can we use nuclear genes to tell blue whales from fin whales and thereby test whether samples from putative blue whales from the Japanese market are hybrids instead? Are blue and fin whales likely to show the reciprocally monophyletic nuclear loci required for this test? Sequences from the mtDNA control region of blue, fin, and humpback whales are monophyletic (Figure 3). Moreover, these blue and fin whales satisfy the three-times rule. A maximum likelihood estimate of distances between species shows that the sum of the distance between blue and fin whales (7.3%) is more than three times the sum of their intraspecific diversities (2.1%). This suggests that most nuclear loci will also be monophyletic in comparison of these species. However, a fraction of nuclear loci are not predicted to be monophyletic. This is because there is a wide

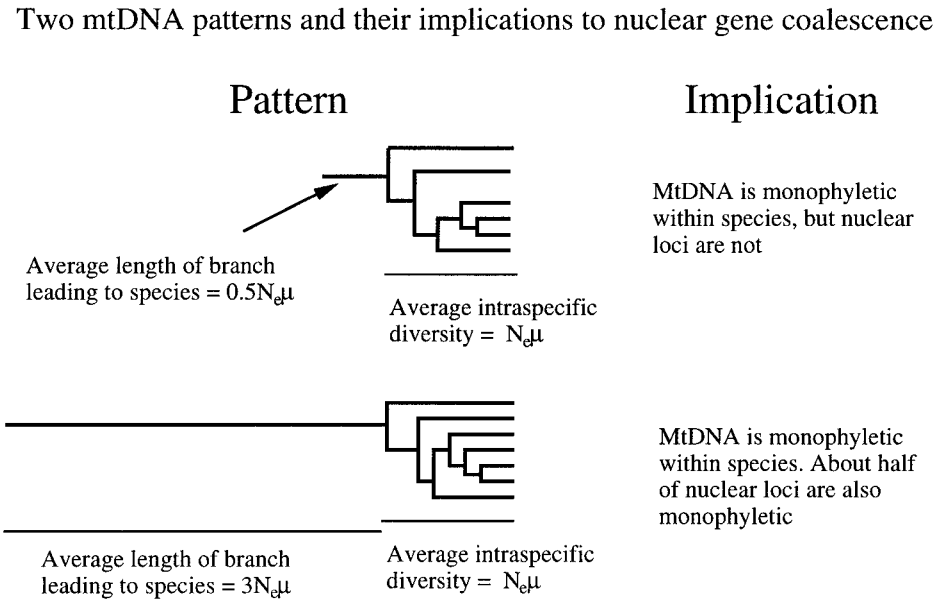

Figure 2. Two possible mtDNA phylogenetic patterns and their implications to coalescence at neutral nuclear loci. In both cases, average mtDNA diversity within the species depicted is equal to $N_e\mu$. In the upper figure the branch leading to the intraspecific mtDNA cluster is short compared to the diversity within the species (in this example, we have depicted this branch length as 0.5 $N_e\mu$). In this case we expect few nuclear loci to be monophyletic within this species, because not enough time has elapsed for nuclear gene "fixation." In the lower figure, the branch leading to the intraspecific cluster is three-times longer than the intraspecific diversity and we expect the average nuclear locus to be monophyletic. Note that patterns of monophyly or coalescence can only be examined with respect to one or more outgroup taxa, which are not shown in this simplified schematic.

variance around average coalescence times (Nei 1987) and not all nuclear loci will behave the same. As a result, we need to carefully choose which locus to use.

Actin Introns in Blue, Fin, and Humpback Whales

To empirically test the above predictions of the three-times rule and to develop tools to potentially identify blue/fin whale hybrids in retail markets, we amplified a region of an actin intron previously described (Palumbi and Baker 1994) in a number of fin and blue whales and compared these sequences to those previously reported. We amplified, cloned and sequenced a 309 bp portion of the first actin intron for blue, fin, and humpback whales (see Palumbi and Baker 1994 for details). Fin and blue whale sequences were monophyletic with respect to one another, with seven fixed differences between them (Figure 4). By contrast, humpback sequences were not strongly monophyletic with respect to the other whales (Figure 4). This result was due to a sequence reversal in one humpback allele that showed a reversion to nucleotide state shared with blue whales at position 517 (numbered as in Palumbi and Baker 1994). All humpback sequences share a unique derived substitution at position 365, and so they may be considered monophyletic by this criterion. Nevertheless, the reversion at 517 caused maximum parsimony procedures to occasionally group the revertant alleles outside of the main humpback cluster, and so the humpback sequences can not be considered strongly monophyletic.

These results, strong monophyly of blue versus fin whales with a weak monophyletic or paraphyletic pattern among humpback and fin whales are consistent with the predictions of the three-times rule. More importantly, they suggest that analysis of actin alleles can distinguish fin and blue whale sequences with reasonable certainty and that this technique is a powerful one for determining the hybrid status of meat samples with blue or fin whale mitochondrial sequences from retail markets. More tests of the three-times rule are needed before it can be used in any predictive sense, and more data on nuclear genetic variation among closely related species (Hey and Kliman 1993) need to be gathered to test empirically how closely the coalescence behavior of these loci corresponds to neutral expectations. Nevertheless, nuclear loci such as actin should be useful in distinguishing fin and blue whales and in identifying particular hybrid whales on the international whale meat market.

Future Needs

Conservation research often focuses on the basic biology of threatened species in order to more precisely design strategies to manage and stabilize small populations. In many cases, the goal is to document current problems so that regulations necessary to protect the species can be devised or habitat critical to it's survival can be defined. It is important to recognize, however, that establishment of national or international guidelines for the protection of a species is not the final stage in the protection of that species. Instead, conservation biology should continue to play a strong role in monitoring the effectiveness of these guidelines by (1) studying the biological impact of the guidelines on the target populations, and (2) providing information about how well the guidelines are implemented.

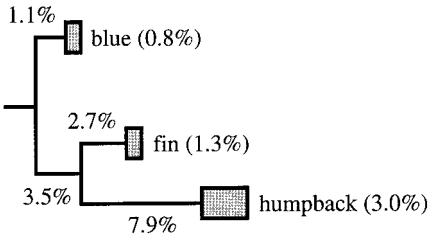

Figure 3. Maximum likelihood tree of genetic distances for control region sequences between blue, fin, and humpback whales using sequences from bowhead whales (genus *Balaena*) as an outgroup. Topological relationships and genetic distances were calculated using fastDNAml. Shown are interspecific distances from fastDNAml and within-species diversities (in parentheses) that were calculated as the average pairwise percent nucleotide variation among sequences. Data are from type sequences reported in Baker et al. (1996).

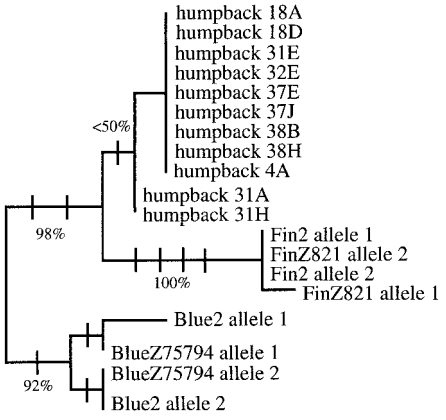

Figure 4. Maximum parsimony phylogenetic tree of partial actin intron sequences from blue, fin, and humpback whales using sequences from bowhead whales as an outgroup. Humpback sequences are from Palumbi and Baker 1994. Blue and fin whale sequences are from two individuals from each species obtained by amplification and cloning as described in Palumbi and Baker (1994). This analysis ignores noninformative substitutions to reduce the impact of *Taq* incorporation errors on the analysis (Villablanca et al. 1998). Tic marks on branches represent the number of unique substitutions placed by parsimony analysis along those branches. Percentages represent bootstrap support.

Genetic results to date suggest that both the IWC and CITES regulations are leaky. IWC regulations are violated when protected species are killed. The humpback and Bryde's whales appearing in our samples have been protected since 1966 and 1986, respectively. Fin and northern hemisphere minke whales are also protected, but are subject to aboriginal hunting in the Atlantic and scientific whaling in the Pacific, respectively. CITES regulations are violated when animals killed within the guidelines established by the IWC are transported without permits. It is possible that some of the fin whales in our analysis entered Japan this way. Small numbers of fin whales have been taken in the North Atlantic most years since the moratorium on commercial whaling. No imports of fin whales have been recorded by Japan since 1991, and so international transport of these animals, even if they were taken legally by aboriginal hunters in Greenland (see Infractions section from the Report of the International Whaling Commission in any year), would be in violation of CITES regulations. Likewise, minke whales in the north Atlantic are currently the target of commercial hunting by Norway, under an objection filed with the IWC. However, Norwegian policy currently prevents these animals from entering the international market: they are for local consumption only.

Thus both aspects of international efforts to regulate whaling and protect whale stocks face severe challenges. Legal whale meat helps cover up the existence of illegal whale meat, and the scale of the oceans makes it difficult to enforce existing regulations. One way to meet these challenges is to use molecular genetic methods to help increase compliance with international agreements. The ability to distinguish species of whales, and in some cases the populations from which they came, removes the cover provided to illegal products from legal ones. With such cover, it has in the past been easier for illegally killed or transported animals to enter the market.

Although molecular genetics provides the technology to accomplish this management goal, current methods are too expensive and time consuming to be used to identify the thousands of samples that would need to be analyzed in a comprehensive random market survey. Cheaper and faster methods, possibly using species-specific oligonucleotide probes or species-specific PCR, are being developed to allow broad-scale testing of markets.

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