

The tip of the tail: molecular identification of seahorses for sale in apothecary shops and curio stores in California

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Abstract Millions of seahorses a year are traded internationally, with many eventually sold at retail shops as curios, as aquarium pets, and especially for use in traditional medicine. The entire genus is now protected by CITES, but conservation measures have been limited in part by difficulties with species identification and incomplete understanding of trade patterns. In this study, we use molecular techniques to make species- and population-level identifications of 56 seahorses sampled from both traditional medicine and curio shops in San Francisco and central California. Seahorses from medicinal sources included unexpectedly large numbers of the eastern Pacific *Hippocampus ingens*, a species poorly protected by current CITES export recommendations. Curio shops were dominated by *H. barbouri*, a spiny species that has been reportedly confused in trading records as *H. histrix*. Specific populations of origin could be inferred for several species through comparison with publicly available phylogeographic data. Our results underscore the strengths and weaknesses of current recommended export regulations, and we suggest that molecular forensics can help in verifying trade documentation and developing more effective conservation measures.

Keywords Molecular forensics · Conservation · Seahorses · *Hippocampus ingens* · Phylogeography

Introduction

Seahorses (genus *Hippocampus*) are threatened by a growing international trade (Vincent 1996). While there is potential in at least some species for relatively fast recovery due to early age at maturity and short generation times, other elements of their biology, such as high mate fidelity and low dispersal ability, make them vulnerable to sustained exploitation (Foster and Vincent 2004; Curtis and Vincent 2006). Seahorses are distributed worldwide, with much of the species diversity centered in the Indo-Pacific (Lourie et al. 2004). An estimated 20 million individual seahorses enter the international market every year, many dried and sold for traditional Chinese medicine (TCM), but with significant numbers also dried as curios or sold live in the aquarium trade (Vincent 1996). Most seahorses are caught incidentally as bycatch (e.g. Baum et al. 2003; Baum and Vincent 2005), but some of the poorest fishers in developing countries also target seahorses as a critical source of daily income (Vincent 1996). These factors are contributing to a global decline in seahorse populations (Vietnam: Giles et al. 2006; Latin America: Baum and Vincent 2005; Philippines: Perante et al. 2002; Meeuwig et al. 2003).

Most of the 34 listed seahorse species are taken as part of this international trade (Vincent 1996), and all but the eight species deemed Vulnerable or Endangered are considered “data deficient” by the IUCN (IUCN 2006). In recognition of the threats to many individual species of seahorses, CITES formally listed the entire *Hippocampus* genus for protection effective 2004 under the Appendix II

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designation (CITES 2002a), requiring that all international trade be restricted to sustainable levels (CITES 1973).

In order to help parties certify their exports as sustainable, CITES adopted a suggested single 10 cm minimum height limit (measured from the top of the head to the tip of the tail) for the genus (CITES 2004). A single genus-wide limit for seahorses is in contrast to typical fisheries regulations that are based on size at first reproduction of single species, but offers several advantages. First, species identification is particularly difficult among seahorses (Lourie et al. 2004), so a single size limit simplifies enforcement. Second, the 10 cm limit affords protection to most targeted species, because most seahorses mature at a smaller size (Foster and Vincent 2005). As a result, the single size limit is one management option favored by affected parties (Martin-Smith et al. 2004). However, the largest seahorse species mature above 10 cm, and are thus not well protected by the regulation (Foster and Vincent 2005).

Unfortunately, the limited data on current seahorse take and export make evaluation of management options such as the 10 cm limit difficult. Because CITES depends on self-reporting at the national export level and cannot address illegal trade, available data probably underestimate total seahorse take and often lack population-level information. The latter issue could be especially important for conservation efforts in geographically extensive nations like Indonesia and the Philippines, both of which contain genetically distinct populations of individual species (e.g. Lourie and Vincent 2004b; Lourie et al. 2005).

Molecular forensic analysis has proved to be a useful technique for vetting official trade data and resolving species or population-level differences for which morphological identification is impossible or impractical (e.g. whale flesh in Japanese meat markets, Baker and Palumbi 1994; Baker et al. 1996). In this study, we apply these tools to the seahorse trade by using mtDNA sequencing to characterize the composition of seahorse species in the traditional Chinese medicine market in San Francisco and nearby coastal curio shops. Our goals are to generate a genetic snapshot of local market composition and to make inferences about locations of origin utilizing publicly available genetic data.

Materials and methods

Seahorse specimens were collected at various end-markets in California's San Francisco Bay area in February 2005. Dried seahorses intended for use in traditional Chinese medicine were collected in San Francisco's "Chinatown" district. Lacquered specimens were collected at curio shops catering to tourists in Monterey Bay and Santa Cruz, California. Height of all specimens was measured according

to CITES (2004) specifications. We sequenced 46 specimens intended for use in Chinese medicine and 10 specimens from curio shops. In addition, 32 dried specimens of *H. ingens* from Mexico (9), Guatemala (10), Ecuador (7), and Peru (6) were obtained through the Redpath Museum at McGill University (courtesy Sara Lourie) and sequenced. Two types of pills listing seahorses as an active ingredient were also collected, but failed to yield amplifiable genomic DNA.

A small amount of tissue from the tail of each seahorse was removed and macerated for extraction. DNA was extracted using NucleoSpin® columns (Machery-Nagel) following the published protocol for isolation of genomic DNA from cells and tissue (BD Biosciences Clontech) with the following modifications: half of the recommended volumes of all reagents were used; during cell lysis with Proteinase K, the tissue was incubated at 65°C for 2 h without agitation for dried specimens and overnight for lacquered specimens; and all centrifugations were performed at 2000 × *g*, except the last, which was performed at 500 × *g*. Genomic DNA was electrophoresed on a 1% agarose gel to determine the amount and quality of extracted DNA.

A portion of the cytochrome *b* gene was amplified using seahorse specific primers for all specimens collected from traditional medicine shops and curio shops. We also amplified cytochrome *b* from 13 of the museum specimens. The primers used were forward primer shf 5'-CTACCTGCACCATCAAATATTTTC-3' or shf2 5'-TTGCAACCGCATTTTCTTCAG-3' and reverse primer shr2 5'-CGGAAGGTGAGTCCTCGTTG-3' (Lourie and Vincent 2004a). In addition, a portion of the mitochondrial control region's right domain was amplified for 19 museum specimens. The primers used were forward primer HCAL2 (5'-CACACTTTCATCGACGCTT-3') and reverse primer HCAH2 (5'-TCTTCAGTGTATGCTTTA-3') (Teske et al. 2003). These primers were used to generate sequences comparable to those in previous seahorse phylogenetic studies, and because this portion of the control region has been reported to be more readily amplified in seahorse specimens with degraded DNA (Teske et al. 2003).

PCR amplifications were done in a 25 µl reaction with 1 µl of genomic DNA extract and the following reaction concentrations: 1x AmpliTaq polymerase buffer (Applied Biosystems), 0.2 mM dNTPs, 0.4 mM each forward and reverse primers, and 0.5U AmpliTaq polymerase (Applied Biosystems). PCR was done using a Biometra T Gradient thermal cycler under the following conditions: 2 min initial denaturation followed by 39 cycles with the following thermal profile: 94°C 30 s, 55°C 30 s, 72°C 1 min. To ensure against contamination, a negative control was run in each PCR. Successful amplification products were then sequenced using an Applied Biosystems 3100 Genetic Analyzer.

Using Sequencher 4.1 (Gene Codes Corporation), we aligned and visually cleaned all sequences. Final edited sequences for all specimens in this study have been submitted to GenBank (accession numbers DQ912699–DQ912786). Sequences from the medicinal and curio specimens were then compared to 22 reference sequences from Casey et al. (2004). Sample sequences were compared to reference sequences by using PAUP*4.0b10 to generate maximum parsimony trees. The pipefish *Corythoichthys haematopterus* (AY166830) was included as an outgroup for the species-level identification tree. A heuristic bootstrap analysis (1,000 replicates) was run on the parsimony tree.

Sequences for species with available phylogeographic data, including *H. barbouri* (AY495716–AY495738), *H. spinosissimus* (AY495739–AY495825), and *H. trimaculatus* (AF192699–AF192703, AY322434–AY322476 excluding AY322436, AY322451, and AY322460) were obtained from GenBank as additional reference sequences for population-level analysis. Separate maximum parsimony trees were created for specimens identified as *H. barbouri*, *H. spinosissimus*, and *H. trimaculatus* to establish likely regions of origin for those individuals based on Lourie et al. (2005). These trees were rooted with closely related distinct seahorse species according to the phylogeny in Casey et al. (2004), using *H. whitei*, *H. camelopardalis*, and *H. kelloggi*, respectively.

For museum *H. ingens* specimens, population structure was investigated using maximum parsimony trees for both sequenced loci and analyzed with Arlequin 2.0 (Schneider et al. 2000). Analyses were carried out on 13 museum specimens for cytochrome *b* and 19 museum specimens for the control region.

Results

Cytochrome *b* sequences of 46 samples collected from San Francisco's traditional Chinese medicine market and 10 samples collected from curio markets in Santa Cruz and Monterey ranged in length from 492 to 741 bp. Several samples failed to amplify; all from specimens that appeared to have been bleached. Pills said to contain seahorse material yielded no genomic DNA, and could not be sequenced.

Most obtained sequences clustered with a known seahorse cytochrome *b* sequence with high bootstrap reliability (Fig. 1). The parsimony consensus tree produced by a heuristic search reveals 29 (63%) of the traditional Chinese medicine specimens cluster with the reference sequence of *H. ingens* with a bootstrap value of 97%. Also represented in our sample pool were six *H. spinosissimus* (13%), four *H. comes* (9%), three *H. trimaculatus* (6%),

and a single *H. hippocampus* (2%). The remaining three specimens clustered with both *H. reidi* and *H. algiricus* but could not be distinguished between these closely related species. All of the bootstrap values were high, the lowest being 73% for the *H. comes* cluster. For the ten curio specimens, nine clustered with the reference sequence of *H. barbouri* and one with *H. trimaculatus*, all with bootstrap values of 100%.

The traditional Chinese medicine specimens ranged in height from 8 to 19 cm; all but one of these exceeded 10 cm, with a mean height of 14.1 cm. The mean height for specimens identified as *H. ingens* was 15.1 cm, with a mean of 12.4 cm for the remaining species.

Phylogeography within species

All individuals of the three species in our sample for which existing phylogeographic data are available could be resolved to a single biogeographically significant clade, with most specimens of *H. barbouri* and *H. spinosissimus* sharing a single, previously documented haplotype.

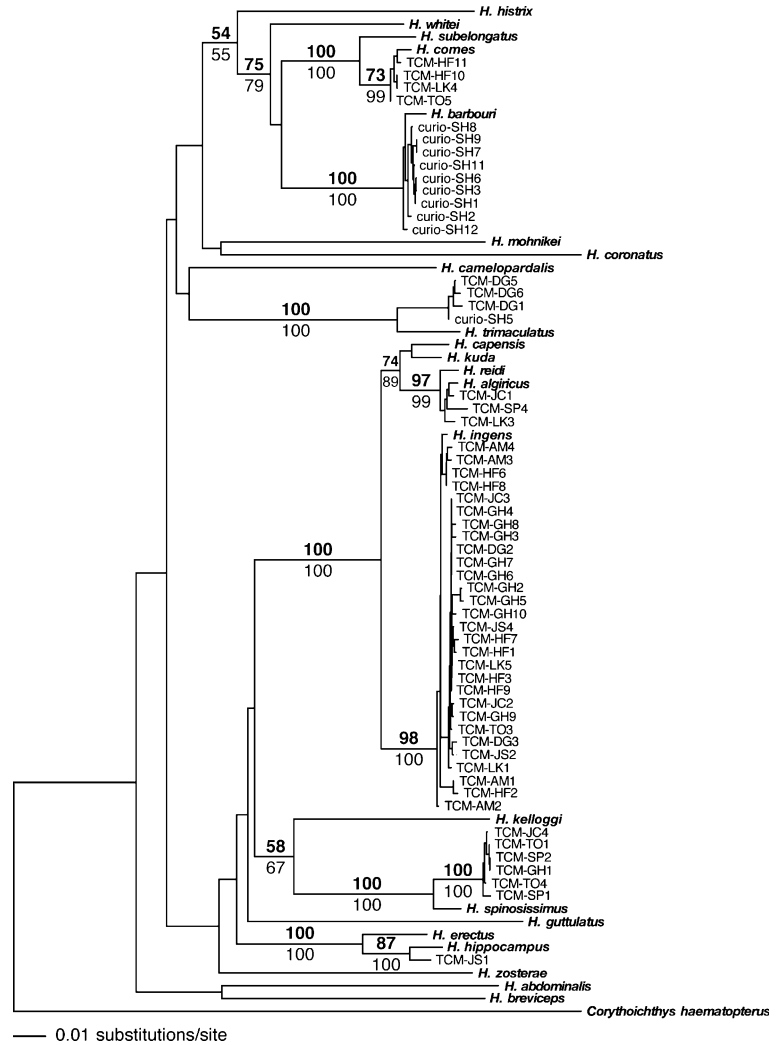
In the case of *H. barbouri*, all nine curio specimens belonged to the B lineage, a group extending south from the Sulu Sea in the Philippines (Lourie et al. 2005). Eight of our specimens shared the B16 (2 specimens) or B17 (6 specimens) haplotype found by Lourie et al. (2005) in the Sulu Archipelago of the southern Philippines. The remaining individual has a novel haplotype one change removed from B16.

Hippocampus spinosissimus has a generally less geographically structured population than *H. barbouri*, but all six of our traditional Chinese medicine sequences clustered within the A lineage, a minor group concentrated in the central Philippines (Lourie et al. 2005). Five of our samples were of the A03 haplotype, with the sixth representing a novel haplotype two changes removed from A03.

H. trimaculatus in the wild is divided into two primary lineages, one on either side of Wallace's Line in Indonesia (Lourie and Vincent 2004a). The three traditional Chinese medicine and one curio *H. trimaculatus* specimens all grouped with the A lineage, indicating an origin on the Asiatic side of Wallace's Line. Two traditional Chinese medicine specimens were of documented haplotypes (A18 and A31).

In order to ascertain the geographic origins of the *H. ingens* samples from medicinal shops, we compared sequences with Redpath Museum *H. ingens* specimens collected throughout Latin America. The cytochrome *b* locus was amplified for 13 of these specimens, providing sequences of 587 bp. Comparing cytochrome *b* sequences of museum and traditional Chinese medicine specimens shows a low level of variation. Among 587 bp, there are only 14 variable positions, with 0–7 base differences

Fig. 1 Kimura 2-parameter neighbor-joining tree of seahorses from San Francisco traditional Chinese medicinal shops (denoted TCM-***) and curio shops near Monterey Bay, California. Cytochrome *b* sequences from shop specimens are compared to GenBank sequences from reference samples. Bootstrap percentages in bold above branches derive from 1,000 heuristic parsimony bootstrap searches using 10 random-order data entry replicates per bootstrap; those below branches derive from 1,000 K2P-corrected distance bootstrap replicates



between sequences, and a maximum percent sequence divergence of 0.8% (Tamura-Nei distance, $\gamma = 0.5$). Phylogenetic analysis shows most sequences (nine museum samples and 19 market samples) fall into a single major cluster with four minor sequence variants (Fig. 2). Greater sequence divergence is seen among the remaining four museum and eight market specimens. However, there is no strong geographic structure obvious in the phylogenetic tree and analysis of sequence variation shows low F_{ST} among museum localities ($F_{ST} = 0.0$).

We then explored whether a higher level of sequence variation in the mitochondrial control region would provide better geographic resolution. Out of 340 bases compared for 19 individuals, there were six variable positions and a maximum of 1.2% divergence (Tamura-Nei distance). As in the cytochrome *b* dataset, a major haplotype occurred in 8 of 19 individuals—other haplotypes differed from this one by 1–2 substitutions. No geographic subdivision among museum collection localities was present ($F_{ST} = 0.0$).

Discussion

Market composition

We found that *H. ingens* dominated the traditional Chinese medicinal market in San Francisco’s Chinatown, accounting for almost two-thirds of 46 identified seahorses. This was a surprising result. Based on previous market research (Vincent 1996) and patterns of seahorse diversity (Lourie et al. 2004), we expected that most of our specimens would originate in the Indo-Pacific. In contrast, *H. ingens* is an Eastern Pacific species, ranging from southern California to Peru. Only 29% of our sample (*H. spinosissimus*, *H. comes*, and *H. trimaculatus*) originated in Asia.

A decade ago, Latin American countries were seen as only peripherally involved in international seahorse trade (Vincent 1996). In 2002, the CITES listing of the genus *Hippocampus* indicated Mexico as the only Latin American major exporter (> 1 tonne dried) of seahorses (CITES 2002b). More recent trade data, however, suggest that this

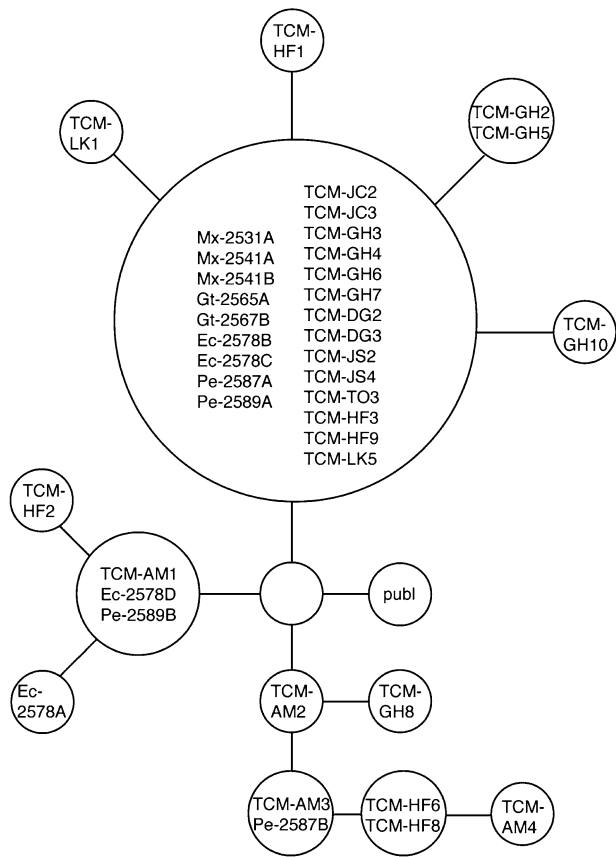


Fig. 2 Minimum spanning network of cytochrome *b* sequence variants among *H. ingens* from market samples and museum specimens. Origin of museum specimens is indicated by a two-letter country code (Mexico = Mx, Guatemala = Gt, Ecuador = Ec, Peru = Pe) preceding the museum accession number; market specimens are denoted TCM-***. “Publ” indicates the *H. ingens* reference sequence from fig. 1. Each circle represents a haplotype: diameters increase with increasing sample size. All lines between haplotypes represent one base pair substitution. The single empty circle represents an inferred haplotype not present in the data set

is changing: for 2004, the first year for which CITES data are available for seahorses, Peru reported exports of over 500,000 individuals (UNEP-WCMC 2006). At a rate of approximately 350 dried individuals per kilogram (Vincent 1996), this represents a total export of at least 1.5 tonnes.

Our results, though only a snapshot of one market at one moment in time, are consistent with an increase in the number of exports from Pacific Latin America. Broader market surveys and comparisons with official trade data will be necessary to make rigorous statements about global trade dynamics, but the observation that essentially all of Peru’s 2004 documented exports of seahorses were to China and Hong Kong (UNEP-WCMC 2006) suggests that any increase in representation of *H. ingens* is probably not confined to American markets.

Seahorses from curio shops in the Monterey Bay region showed markedly different results, with almost all

specimens belonging to *H. barbouri* (90%) and one specimen to *H. trimaculatus* (10%). The small sample size makes generalization to the market level impossible, but the origins of both species in the Indo–Pacific is consistent with past analyses of U.S. curio trade documentation (Grey et al. 2005). The dominance of *H. barbouri*, however, runs counter to trade records suggesting that *H. histrix* is more common (Grey et al. 2005). Both *H. histrix* and *H. barbouri* are spiny seahorses that are more favored in aquarium and curio trades: smoother seahorses are favored in medicinal trade (Vincent 1996). However, Lourie et al. (1999) suggest that several seahorse types may be identified in trade as *H. histrix* no matter their true identity. Our results suggest that *H. histrix* is not always the dominant spiny seahorse in trade, and provide some evidence that the species misidentification noted by Lourie et al. (1999) may be common.

Applying molecular techniques to the seahorse trade

Our results show that mtDNA analysis is a robust tool for market characterization and verification in the seahorse trade. Easy DNA extraction and amplification from all but bleached individuals and pills indicates that mtDNA is left relatively intact through the supply chain. Cytochrome *b* divergence among seahorse species is relatively high (Kimura 2-paramater distances range from 1.3 to 23.2% [Casey et al. 2004]), and high bootstrap support for sequences clustering with all but the most closely related species indicates that the cytochrome *b* locus is adequate for most species-level forensic analysis. Distinguishing between genetically similar species like *H. reidi* and *H. algericus* (Teske et al. 2004) will require additional sequence data.

The capability of population-level identification afforded by molecular analysis may ultimately be more useful than simple species identification. Large island nations like Indonesia and the Philippines harbor genetically distinct regional populations of certain seahorse species (Lourie et al. 2005). Conservation regulations targeted at the population level may help these nations balance the economic benefits of seahorse export with the challenges of ecological sustainability, but would be difficult to enforce. The results of this study, along with a growing body of seahorse phylogeographic data (Teske et al. 2003; Lourie and Vincent 2004a; Lourie et al. 2005), offer one way to make such enforcement tenable. For example, we were able to identify the four *H. trimaculatus* specimens in our sample as more closely related to the A clade of the species than to the B1 or B2 clades (see Fig. 1 in Lourie and Vincent 2004a). The A clade is limited to the west of Wallace’s Line, suggesting that our specimens originated in SE Asia or western Indonesia. Populations from

species with deep structuring, like *H. trimaculatus*, may be distinguishable through RFLP analysis, allowing for a coarse but inexpensive population-level assay.

Unfortunately, at least one seahorse species that might benefit from population-level management may not be so easily characterized. Our initial study of intraspecific variation in cytochrome *b* and control region sequences for *H. ingens* shows that low genetic variation may make phylogeographic identification of origin difficult for this species. Cytochrome *b* and control region data were dominated by single haplotypes, and the genetic variation that was uncovered in our surveys did not group geographically. Specimens that were identical across 587 bp of cytochrome *b* or 340 bp of control region were found from Mexico to Peru, covering the entire range of our sample. Such large geographic spread of identical haplotypes is in marked contrast to other surveys of intraspecific phylogeography of Indo-West Pacific seahorses (Lourie and Vincent 2004a; Lourie et al. 2005), which revealed large differences in mtDNA sequence across the region for *H. barbouri*, *H. kuda*, *H. spinosissimus*, and *H. trimaculatus*. It is possible that seahorses have a low rate of control region evolution, as has been observed for other fish lineages. However, McMillan and Palumbi (1997) pointed out that low control region evolution in fish correlates with low transition/transversion mutation rates. For *H. ingens*, the ratio of transition/transversion changes in control region sequences is high (6:0), suggesting that low evolutionary rate may not limit seahorse control region variability. If the low structuring of *H. ingens* population at this locus is due to a recent geographic expansion rather than extensive and ongoing gene flow, sequencing of more variable loci or identification of microsatellite markers may yield more information.

Conservation implications: the case of *H. ingens*

Hippocampus ingens, the sole seahorse native to the Eastern Pacific, is one species that should particularly benefit from additional conservation measures. One of the six species listed as “under the greatest threat from unsustainable levels of harvest and international trade” by CITES (2002b), *H. ingens* is likely under growing pressure from recent expansion of the Latin American seahorse fishery (Baum and Vincent 2005). The high proportion of *H. ingens* in our traditional Chinese medicine market sample is consistent with such an expansion.

As one of the largest seahorses (Foster and Vincent 2005), *H. ingens* is also one of the species most poorly served by a 10 cm minimum size limit. In order to establish realistic criteria for sustainable harvest of this important species, more research must be done on its life history. Since minimum size limits are a favored conservation

mechanism, establishing accurate numbers for *H. ingens* size at sexual maturity and first reproduction, as well as size/fecundity relationships, is especially important. Because the limited sequence variation found in this study is insufficient for population-level analysis, and because of the additional levels of conservation possible with an understanding of a species’s phylogeography, additional work should be done to generate such data for *H. ingens*.

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