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MOLECULAR AND MORPHOLOGICAL MARKERS FOR DISTINGUISHING THE SYMPATRIC INTERTIDAL GHOST SHRIMP *NEOTRYPAEA CALIFORNIENSIS* AND *N. GIGAS* IN THE EASTERN PACIFIC

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ABSTRACT

The vigorous burrowing activities of many thalassinidean shrimp have such dramatic effects on their habitats of soft sediment that these animals are often considered ecosystem engineers. Because they strongly interact in these communities, basic information about their life histories and population dynamics is needed to effectively manage the habitats in which they live. These data can only be obtained if the shrimp can be accurately identified. On the west coast of the United States, two species of burrowing intertidal shrimp in the genus *Neotrypaea*, *N. californiensis* and *N. gigas*, often co-occur and are not easily differentiated morphologically except as adult males by characters of the major claw (which is often lost in collection). Here we describe and validate (using mtDNA data from the cytochrome b gene) an allozyme marker (LDH) that can be scored rapidly and inexpensively for the identification of these species. We used this marker to generate a large sample of molecularly-identified specimens that we then used to evaluate a variety of morphological characters in an effort to differentiate the two species. With the exception of characters associated with the male major claw, most of the morphological characters examined here were not useful in distinguishing members of the two species. The exceptions were two simple and robust characters associated with the eyestalks—length, and shape of the distal outer edges. These could be used to reliably differentiate between the two species regardless of sex, and over a wide range of sizes. We hope that these characters will facilitate future studies of the distribution, habitat preference, and comparative biology of these two often co-occurring species.

KEY WORDS: allozymes, mtDNA, *Neotrypaea*, Thalassinidea

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INTRODUCTION

Two species of burrowing ghost shrimp in the genus *Neotrypaea*, *N. californiensis* (Dana, 1854) and *N. gigas* (Dana, 1852), are currently recognized from soft-sediment intertidal habitats in the northeastern Pacific from southern Alaska to Baja California (Campos et al., 2009). Because they create and constantly modify deep and extensive burrow systems, and because they frequently occur at high densities, members of both species likely have major effects on the physical structure of the habitat and the population biology of other co-occurring organisms (Posey, 1986; Dumbauld and Wyllie-Echeverria, 2003). Thus studies of these two ''ecosystem engineers'' are important in understanding the ecology of the intertidal soft-sediment communities in which they occur. However, attempts at exploring species-specific differences in habitat preferences, burrow architectures, feeding, life histories, and other aspects of their biology have been difficult for a simple reason—namely, it is difficult to distinguish most individuals of these two species of ghost shrimp. Large males (greater than about 10 mm carapace length) are clearly distinguishable by the distinctive form of the major claw (Stevens, 1928), but it has long proven difficult to identify juveniles of either sex, adult females, or males that have lost their major claw (loss of this claw is a frequent consequence of collection). This is somewhat surprising, given that a diverse array of ''non-male major claw'' morphological characters have been proposed as useful in identifying members of the two species (Kozloff, 1987; Sakai, 1999; Tudge et al., 2000; Kuris and Sadeghian, 2007, Campos et al., 2009). In practice, however, it appears that the juvenile and female individuals of *Neotrypaea* spp. present in a given population (together, these usually represent well over 50% of the individuals present) cannot be identified using existing morphological characters. Because the two species routinely coexist in some regions, species-resolved studies of the natural history and ecology of these two important crustaceans have thus not been possible.

Taxonomists in Southern California have been aware of of this problem for several years, but its resolution has proven difficult (D. Cadien, personal communication). During a study of the phylogeography of *Neotrypaea californiensis* across much of the west coast of the United States, Pernet et al. (2008) sequenced mitochondrial DNA of both *N. californiensis* and *N. gigas*. [To our knowledge, original types for these species are no longer in existence, and no neotypes have been designated (D. Felder, personal communication), so Pernet et al. (2008) named specimens by comparison of the morphology of adult males with that described in the primary systematic literature.] Molecular markers for identifying the two species were thus available, and we reasoned that these could help in identifying morphological characters that might be useful in distinguishing the two species. Therefore we decided to carry out this study, with two goals. First, because Pernet et al.

Table 1. Characteristics of the *Neotrypaea* spp. samples collected for this study. ''Bait Shop'' samples were purchased alive from a shop in southern California, but were not local. According to the shop owner, they had been imported from a population on Whidbey Island, Washington State.

Site	Date collected	Sample size	# Males	# Females	Size range (carapace length, mm)
Bait Shop (animals from Washington)	10 April 2008	10	10	0	14.2-17.5
Cabrillo mudflat, San Pedro, California 33°42′N 118°17′W	21 July 2008	96	42	54	7.2-14.1
Anaheim Bay, Sunset Beach, California 33°43′N 118°04′W	20 July 2008	92	29	63	7.4-13.6
Agua Hedionda Lagoon, Carlsbad, California 33°08′N 117°19′W	4 August 2008	47	16	31	7.9-15.6
Dog Beach, San Diego, California 32°45′N 117°14′W	2 August 2008	100	37	63	7.2-13.3

(2008) had used a relatively expensive and time-consuming DNA sequencing approach, we sought to identify another molecular marker that could be used to reliably distinguish members of the two species more rapidly and less expensively. Here we report on an allozyme marker that appears to meet these criteria. Second, we used this new marker to generate a large sample of molecularly-identified ghost shrimp of both species (including specimens of both sexes and a range of sizes); we then used this sample to systematically evaluate morphological characters that had previously been suggested to be useful in distinguishing juvenile and female individuals of the two species. In addition, one of the authors (LH) studied a subset of these identified specimens to identify new characters that might distinguish the two species; these new characters were also then systematically evaluated using the larger sample of molecularly-identified specimens.

Materials and Methods

Primary Collections

In July and August of 2008, we collected ghost shrimp using yabby pumps at four intertidal sites in Southern California (Table 1). We retained all shrimp that were greater than 7 mm in carapace length (in order to facilitate morphological analyses) and were not obviously missing appendages or otherwise damaged, as we were concerned with obtaining a sample suitable for thorough analysis of morphology. The smallest individuals in our samples were likely juveniles (see below, Evaluation of Morphological Characters, for more detail). We also purchased a few living *N. californiensis* from a bait shop in Southern California; these shrimp had been imported from Washington State, and were used for comparison with local populations. Characteristics of the primary sample are detailed in Table 1.

Collected animals were each measured (carapace length) and sexed (females have pleopods on all abdominal segments, while males lack pleopods on the second pleomere. While animals were alive, we removed a tissue sample from each animal for subsequent allozyme surveys. A small piece of muscle (about one segment's worth) was removed from one side of the abdomen, homogenized in 200 μL of cold extraction buffer (0.2 M Tris-HCl, pH 9.0), centrifuged, then stored at −80°C until analysis. Each animal was then preserved in 95% ethanol for later DNA and morphological analyses.

Allozyme Surveys

Instead of using starch gels for allozyme electrophoresis, we used cellulose acetate gels, following the protocols of Hebert and Beaton (1993). Cellulose acetate gels can be run much more rapidly (in our case, in 1-2 h) than starch gels, facilitating the rapid scoring of large numbers of samples.

In initial surveys to identify reliably stained and scored enzyme loci, we surveyed eight loci: alcohol dehydrogenase, aldehyde oxidase, carbonate dehydrogenase, glucose-6-phosphate isomerase, lactate dehydrogenase, malate dehydrogenase, superoxide dismutase, and xanthine dehydrogenase. Of these, only two stained reliably, glucose-6-phosphate isomerase (GPI) and lactate dehydrogenase (LDH). Preliminary surveys revealed that only two differentiable alleles were present at the LDH locus (a ''fast'' and a ''slow'' allele), in contrast to GPI, where at least five alleles were present. Because it was much easier to score the LDH alleles, we focused on that locus for further surveys. All 345 individuals collected (Table 1) were genotyped at the LDH locus.

DNA Sequencing and Phylogenetic Reconstruction

To test the hypothesis that allozyme scores served as a good indicator of species identity, a short fragment of mitochondrial DNA was amplified from a subset of specimens (42, selected so as to include about 50% putative *N. californiensis* and 50% putative *N. gigas* based on allozyme genotypes), and allozyme genotypes were mapped onto a phylogeny generated from mtDNA sequences. Genomic DNA was extracted from pleonal muscle of ethanol-preserved specimens using DNeasy™ kits (Qiagen). A fragment of the cytochrome b gene (cyt b) was amplified using the primers UCYTB151F and UCYTB270R (Merritt et al., 1998), with each 50 μL reaction consisting of 1× PCR Buffer B (Fisher Scientific #BP-6113), 2 mM $MgCl_2$, 0.12 mM of each dNTP, 0.2 μM of each primer, 1.5 units TAQ polymerase (New England Biolabs), and 1 μL of template DNA. Amplifications consisted of 94°C for one min, followed by 40 cycles of 94° for 30 s, 48°C for one min, and 72°C for one min, with a final extension at 72°C for three min. Negative controls (no template added) were included in each PCR run. Amplification products were cleaned using QIAquick PCR Purification kits (Qiagen), then sequenced in one direction by Macrogen (Seoul, South Korea). For use as out-groups, two cyt b sequences from *Neotrypaea biffari* (Holthuis, 1991) were obtained from GenBank (EU341515 and EU341516). Sequences were aligned using ClustalW (the ''emma'' module of Emboss Explorer: http://embossgui.sourceforge.net/), and verified by eye. A neighbor-joining phylogeny was constructed using MEGA 4.1 (Kumar et al., 2008) from a matrix of pairwise genetic distances (Kimura 2-parameter), with robustness of tree topology assessed with 1000 bootstrap replicates. Pairwise sequence divergence calculations were done in MEGA 4.1, with 500 bootstrap replicates used to estimate standard errors.

Evaluation of Morphological Characters

We surveyed the systematic literature and regional guides for qualitative and quantitative characters previously used to distinguish *N. californiensis* and *N. gigas* (Table 2). Though we were particularly interested in assessing the utility of characters that have been suggested to work for separating juvenile and female individuals of the two species, we also included characters that are typically used to distinguish large males. In addition, one of us (LH) studied the molecularly-characterized specimens described above in order to identify novel characters that might differ between the two species consistently.

We then evaluated all of these characters in a subset of 122 of the molecularly-characterized specimens in the primary sample described in Table 1. The subset was chosen so as to include about 60 individuals of each species, with males and females of each species approximately equally represented. The subset was also chosen to include as wide a size range of specimens as possible. The sample evaluated for morphological characters included 30 female (ranging in carapace length from 7.8-15.6 mm) and 32 male (7.2-17.5 mm) *N. californiensis*, and 30 female (7.2-14.5 mm) and 30 male (7.7-13.6 mm) *N. gigas*. Because relatively little is known about the timing of sexual maturity in these two species, we do not know exactly how many of the individuals that we examined were juvenile vs. mature. However, some estimates can be made from previously published data. In a study of a Washington State population of *N. californiensis*, Dumbauld et al. (1996) found that females smaller than

Table 2. Morphological characters (both qualitative and quantitative) evaluated using molecularly-identified specimens of *Neotrypaea californiensis* and *N. gigas*. Characters used specifically to separate males of the two species are italicized; other characters have been used more generally to separate members of the two species (presumably including juveniles and females). Where sources are marked with an asterisk (*), it indicates that the character was derived from comparing illustrations in that source. Though the relative length of the antennal peduncles is really a quantitative character, it was difficult to measure and was thus treated qualitatively.

Morphological characters	*N. californiensis*	*N. gigas*	Literature sources
qualitative			
rostrum: shape	bluntly rounded	sharp	Kozloff, 1987; Kuris and Sadeghian, 2007
eyestalk: length relative to 2nd article of antenna 1	extends to base of 2nd article	extends ½-¾ L of 2nd article	this study
eyestalk: shape of outer distal edge	concave (sometimes straight)	convex	this study
antenna 1: ventrally directed setae	dense brush	no dense brush	Tudge et al., 2000
antennae: peduncle lengths	ant. 1 ped. L > ant. 2 ped. L	ant. 1 ped. L < ant. 2 ped. L	Tudge et al., 2000
maxilliped 3: crista dentata	more prominent	less prominent	Tudge et al., 2000
female major claw: ventral merus tooth shape	small, triangular	small, conical	Campos et al., 2009
male major claw: carpus dorsal ridge	strongly incurved	not incurved; straight	Hart, 1982; Sakai, 1999
male major claw: conspicuous gape	present	absent	Kozloff, 1987
minor claw: ischium ventral denticulation	absent	present	Tudge et al., 2000
3rd pereopod: propodus proximal lower corner shape	''heel''	more oval	Tudge et al., 2000
female pleopod 1: segment number	2-segmented	3-segmented	Tudge et al., 2000
male pleopod 1: present/absent	present	absent	Hart, 1982; Tudge et al., 2000
pleopods 3-5: appendices internae	embedded	stubby, projecting	Tudge et al., 2000
abdominal somites 3-5: lateral setae	dense tufts present	dense tufts not present	Tudge et al., 2000
final abdominal somite: setae	one pair	two pairs	Hart, 1982*
telson: marginal setae	posterior and lateral	posterior only	Hart, 1982*
quantitative			
male major claw: carpus & propodus length	chela L > carpus L	chela L = carpus L	Sakai, 1999
male major claw: carpus height & length	carpus H = carpus L	carpus H < carpus L	Hart, 1982*
minor claw: propodus & carpus length	propodus L < carpus L	propodus L > carpus L	Hart, 1982
minor claw: carpus height & merus height	carpus H > merus H	carpus H = merus H	Hart, 1982; Kozloff, 1987; Sakai, 1999
2nd pereopod: pollex height & dactyl height	pollex H = dactyl H	pollex H > dactyl H	Stevens, 1928; Hart, 1982; Kuris and Sadeghian, 2007
abdominal somites 2 & 6: length	abd 2 L > abd 6 L	abd 2 L = abd 6 L	Tudge et al., 2000
telson: length & width	telson L = telson W	telson L < telson W	Tudge et al., 2000

9 mm in carapace length were not reproductive. Of the 62 *N. californiensis* that we used in our analysis of morphological characters, 16 were 9 mm or less in carapace length; thus, assuming that relationships similar to those documented by Dumbauld et al. (1996) hold in our populations, 26% of the individuals of that species that we examined were likely juveniles. To our knowledge there are no published data on the relationship between body size and reproductive maturity in *N. gigas* so that we cannot make inferences about the proportion of juvenile shrimp in our samples, but 13 of the 60 (22%) *N. gigas* used in our analysis of morphological characters were 9 mm or less in carapace length.

The first author scored qualitative characters as suggested by the original source. We evaluated most quantitative characters by removing the relevant structure, viewing it in a consistent orientation with a dissecting microscope, and tracing its outline using a camera lucida. An image of a stage micrometer was also traced to serve as an absolute indicator of size. Tracings were digitized using a flatbed scanner, and the relevant dimensions between easily identifiable landmarks (see results for illustrations) measured using the program ImageJ. The first author made all tracings and measurements so as to ensure consistency. Because the pleon was often flexed and always brittle in preserved specimens, the pleomeres and the telson were difficult to orient consistently for drawing using a camera lucida; thus, these were measured using digital calipers to the nearest 0.05 mm. Finally, though the relative length of the peduncles of antennae 1 and 2 is in essence a quantitative character, accurate measurement of these structures, which again were typically flexed and brittle in preserved specimens, was difficult, so we treated it qualitatively, e.g., the peduncle of antenna 1 was longer than that of antenna 2, vice versa, or the two were equal in length. We did the same for the character describing the length of the eyestalks relative to the second article of antennae 1.

Validation of Eyestalk Characters

In order to further validate morphological characters judged as potentially useful from the analyses described above, we made a second ''test'' collection of ghost shrimp in May 2009. We collected 40 animals as described above, 20 from Cabrillo mudflat (San Pedro), and 20 from Dog Beach (San Diego). Tissue samples were prepared for allozyme electrophoresis as above and stored frozen until analysis by the second author (AD). The first author (BP), who was blind to knowledge of either collection site or the allozyme genotype of the specimens until after the analysis, did the morphological analyses of the test specimens. Predictions of species identity based on the morphological characters were then compared to identifications based on allozyme genotype.

Results

Allozymes

All individuals were easily scored at the lactate dehydrogenase locus except for one shrimp from Cabrillo mudflat whose extract showed no enzyme activity at all; thus, total sample size for the allozyme survey was 344 individuals. Only two alleles were resolvable, Fast (F) and Slow (S). All individuals scored were homozygotes for one or the other of these alleles. All ten individuals from Washington State had the SS genotype, but all Southern California populations included both FF and SS individuals, though in differing proportions (Table 3).

Table 3. LDH genotypes of *Neotrypaea* spp. collected in this study. We failed to obtain the allozyme genotype from one collected individual from Cabrillo mudflat, as the extract displayed no enzyme activity.

Site	# FF individuals	# SS individuals	Total
Bait Shop (animals from Washington)	0	10	10
Cabrillo mudflat, San Pedro, California	89	6	95
Anaheim Bay, Sunset Beach, California	89	3	92
Agua Hedionda Lagoon, Carlsbad, California	7	40	47
Dog Beach, San Diego, California	1	99	100

Correspondence of Allozyme and DNA Data

To determine whether or not LDH genotype was a useful indicator of species identity, we generated a phylogeny based on mtDNA sequences of a subset of the specimens and mapped allozyme genotype onto the phylogeny. Sequencing yielded a 273 bp fragment of the cyt b gene from 42 individual shrimp. Neighbor-joining phylogenetic analysis yielded a tree comprised of two clades of *Neotrypaea* spp. that were very strongly supported in bootstrap analyses (Fig. 1). Allozyme genotypes showed perfect concordance with the boundaries of these clades—individuals in one clade were all homozygous for the F allele at the LDH locus, and individuals in the other clade were all homozygous for the S allele.

Large males with the FF allozyme genotype had major claws that were typical of those described for *Neotrypaea gigas*, while large males with the SS allozyme genotype had major claws typical of those described for *N. californiensis*. In the rest of this paper we apply those names to members of each clade/allozyme genotype. Mean pairwise sequence divergences (Kimura-2-parameter) between members of the examined taxa were 0.085 (standard error 0.018) between *N. californiensis* and *N. gigas*; 0.116 (0.021) between *N. californiensis* and *N. biffari*; and 0.118 (0.021) between *N. gigas* and *N. biffari*.

Morphological Analyses

We used 122 of the molecularly-characterized specimens in the primary sample to evaluate 22 morphological characters that had previously been described as useful in distinguishing between *N. californiensis* and *N. gigas*, as well as two new morphological characters described in this study. Because the sample included specimens of a range of size (~ 7-15 mm CL), we could also evaluate whether or not the utility of these characters varied as a function of body size. We were able to successfully score almost all individuals for each trait examined, but occasionally a specific trait could not be scored or measured on several individuals because the relevant structure was broken or missing.

Of the 17 qualitative characters evaluated (Table 2), only four proved reliably useful in distinguishing between members of the two species. Two of these were characters of the male major claw, and thus useful only for mature specimens of this sex. In *N. californiensis*, the dorsal edge (or ridge) of the carpus of the major claw is strongly incurved in males larger than 10 mm CL; in *N. gigas*, this edge is not incurved. Also, in *N. californiensis*, there is usually a conspicuous gape between the dactyl and propus

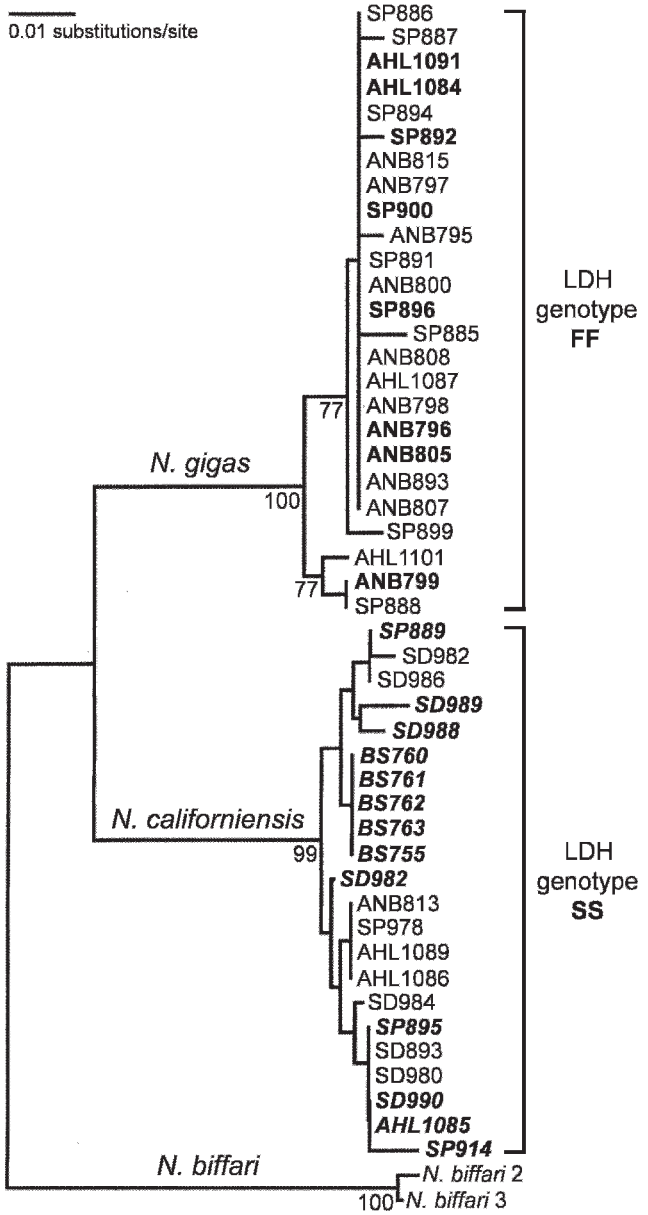

Fig. 1. Mitochondrial DNA phylogeny and genotype at LDH allozyme locus of *Neotrypaea* spp. collected from soft-sediment habitats. Neighbor-joining phylogeny based on 273 bp of cyt b mtDNA, and rooted with sequences from two individuals of *N. biffari* collected from rocky intertidal zone near Santa Barbara, CA. Numbers below branches = percentage of 1000 bootstrap replicates that support the relevant node, with values less than 70% not shown. Individuals identified with site code (AHL = Agua Hedionda Lagoon, ANB = Anaheim Bay, BS = bait shop, SD = San Diego, and SP = San Pedro) followed by individual-specific number. **Bold** identifiers indicate males > 12 mm carapace length with major claw morphology of *N. gigas*; ***Bold italic*** identifiers indicate males > 12 mm carapace length with major claw morphology of *N. californiensis*. All individuals in clade including large males of *N. gigas* with FF genotype at the LDH locus; all individuals in clade including large males of *N. californiensis* with SS genotype.

of the major claw when closed, while this gape is absent or very small in *N. gigas*. Again, this difference is only reliably present in males larger than 10 mm CL.

Two qualitative characters were useful in distinguishing between members of the two species regardless of sex or

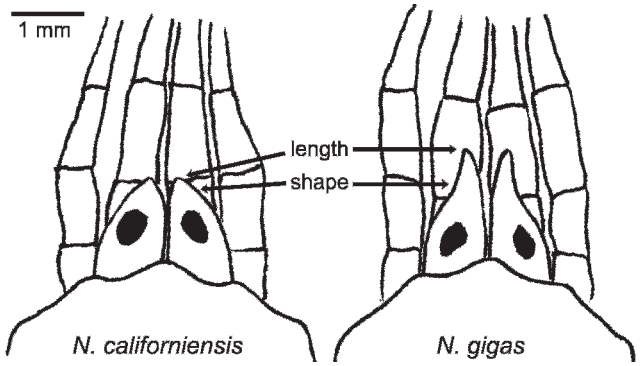

Fig. 2. Dorsal view of anterior ends of *N. californiensis* and *N. gigas*, showing two characters of eyestalks that appear to reliably differentiate members of the two species. In *N. californiensis*, eyestalks extend only to the base of article 2 of the first antennae, but in *N. gigas* they extend 1/3-2/3 to the base of article 2. Also, in *N. californiensis*, the distal outer edge of each eyestalk is slightly convex in outline, while in *N. gigas* it is slightly concave. Drawings made using camera lucida of female specimens, each 10 mm in carapace length.

size (within the size range examined). These two characters had the added benefit of being associated with the eyestalks, which are rarely lost during collection (in contrast to the major chela or other appendages). The first was the length of the eyestalks relative to the second article of the first antennae. In *N. californiensis*, the tip of the eyestalks typically protruded only to the base (rarely as far as ¼ the length) of the second article of the first antennae; in *N. gigas*, the tip of the eyestalk routinely protruded 1/3-¾ of the length of the second article of the first antennae (Fig. 2). This character appeared to be quite robust. In our sample of 122 individuals, classification by this character alone was sufficient to correctly identify all 62 *N. californiensis*, and 57 of 60 (95%) *N. gigas*. The three mis-classified individuals of *N. gigas* (all females, ranging in CL from 7.2-9.3 mm) were initially identified as *N. californiensis* on the basis of this character because their eyestalks protruded beyond the base of the second article of the first antenna only ¼ of the length of second article; they were thus slightly shorter than is typical for *N. gigas*.

The second eyestalk character was also robust, but was slightly more difficult to score. In *N. californiensis*, of the distal outer edge of each eyestalk was slightly convex (or sometimes straight) in outline, while in *N. gigas* the distal outer edge of each eyestalk was slightly concave (Fig. 2). Like the length of the eyestalk, this character proved very reliable in differentiating members of the two species. Classification by this character alone was sufficient to correctly identify all 122 specimens in our sample. Both of these eyestalk characters were subsequently validated using a separate ''test'' collection (see below).

The other 13 qualitative characters assessed were not as useful in differentiating between either females or males of the two species, at any body size. Most of these characters were based on the shapes of structures, or the prominence of particular features, and for these we generally found no consistent qualitative differences between individuals of the two species. In some cases the character was essentially invariant either within or between species, e.g., the prominence of the crista dentata on maxilliped three. In other cases intraspecific variation was so great as to swamp previously-described interspecific variation. We describe briefly below specific patterns of variability in each of these characters.

Rostrum: Shape.— Rostrum shape in specimens of both species was typically ''bluntly rounded,'' with very few individuals of either species having a rostrum that could be characterized as sharply pointed.

Antenna 1: Ventrally Directed Setae.—All individuals of both species possessed ''reasonably dense'' setal brushes on antenna 1 (Tudge et al., 2000).

Antennae 1 and 2: Relative Length of the Peduncles.— Though this is really a quantitative character, we found it difficult to accurately measure the lengths of the antennal peduncles and so treated it as a qualitative character, classifying the peduncles of the first antennae as longer than or equal in length to those of the second antennae. In almost all individuals of both species, the peduncles of the first antennae were clearly longer than those of the second antennae (a trait that Tudge et al. [2000] noted was characteristic of *N. californiensis*), though the magnitude of the difference varied. However, the magnitude of the difference did not vary consistently between the two species. Some individuals of *N. californiensis* had antennal peduncles that were nearly equal in length, and many individuals of *N. gigas* had peduncles of the first antennae that were substantially longer than those of the second antennae. The substantial intraspecific variation in this trait means that it is not useful for distinguishing individuals of the two species.

Maxilliped 3: Crista Dentata.—The crista dentata of all individuals of both species were similar in prominence.

Female Major Claw: Shape of Ventral Tooth on Merus.— Campos et al. (2009) state that this small tooth is ''triangular'' in female *N. californiensis*, but ''conical'' in *N. gigas*. In their illustration (their fig. 2b, e), however, the teeth look quite similar. We saw little variation in the form of this tooth, and classified all specimens of both species as possessing triangular teeth. We did, however, notice some females (11 of our sample of 30) of *N. gigas* that had unusually large teeth on the merus of major claw. In a few cases, these teeth approached the sizes of those typically found on the major claws of males.

Minor Claw: Ischium Ventral Denticulation.—In some specimens, the ischium had no discernible denticles, but in others it bore one to four very tiny denticles on its ventral edge. These were only discernible with a dissecting microscope. Most individuals of both sexes of *N. californiensis* bore no denticles on this article (21/35 males, and 22/29 females), and those that did bear denticles rarely bore more than two. A lower percentage of individuals of *N. gigas* bore no denticles on this article (9/26 males, and 11/26 females), and those that did bear denticles often bore two or three. The overlapping variability in this trait suggests that this character does not conclusively differentiate members of the two species.

Third Pereiopod: Shape of the Lower Proximal Corner.— Tudge et al. (2000) classified the shape of the lower proximal corner of the third pereiopod of *N. californiensis* as ''heel''-like, but that of *N. gigas* as being more ''oval.'' We interpreted this to mean that in *N. californiensis* there was a more or less sharp corner, and in *N. gigas* it was more continuously curved. This difference was subtle, and we found it difficult to decide how to score many individuals. However, we certainly found many heel-like corners in individuals of *N. gigas*, and many oval corners in individuals of *N. californiensis*.

Female Pleopod 1: Two or Three-Segmented.— In our sample, all females of both species had three-segmented first pleopods.

Male Pleopod 1: Present or Absent.—Tudge et al. (2000) noted that male pleopod 1 was present in *N. californiensis* and ''unusually absent'' in *N. gigas*. In our samples, almost all male specimens of all sizes of both species bore a pair of pleopods on the first pleomere. These pleopods were quite variable in size from individual to individual, and were often difficult to see. A few males of each species lacked obvious pleopods on the first pleomere.

Pleopods 3-5: Appendices Internae.—Tudge et al. (2000) stated that pleopods 3-5 of *N. californiensis* had embedded appendices internae, while those of *N. gigas* bore ''stubby projecting'' appendices internae (illustrated in their fig. 2H, I). For consistency, we examined pleopod 3 on each of our 122 specimens, and found that this character was quite variable within each species. Some specimens of both species had clearly embedded appendices internae, while others had very slightly projecting appendices internae. Variation between these two states was continuous. [It should be noted that the genus *Neotrypaea* is defined in part by the presence of embedded appendices internae (Manning and Felder, 1991)].

Pleomeres 3-5: Lateral Setae.— All individuals of both species had dense tufts of lateral setae on these pleomeres. These tufts of setae seemed to be fairly delicate, often detaching from ethanol-preserved specimens when handled.

Final Pleomere: Setae.— Illustrations in Hart (1982: her figs. 14g, 15g) suggested that *N. californiensis* bore only one pair of tufts of setae on the ventral posterior edge of the last pleomere, but that *N. gigas* bore two pairs. In fact, all specimens of both species bore two pairs of tufts of setae on this segment.

Telson: Marginal Setae.— Again, illustrations in Hart (1982: her figs. 14g, 15g) suggested that the posterior marginal telson setae in *N. gigas* extended anteriorly to the lateral edges of the telson, but that they were restricted only to the posterior edge of the telson in *N. californiensis*. In all of our specimens of both species, these marginal setae were restricted to the posterior edge of the telson in all of our specimens.

Patterns of variation.— We examined seven quantitative characters (Table 2) as a function of carapace length, sex, and species, and these are shown in Figs. 3 and 4. Inspection of the scatter-plots suggested that patterns of variation in each quantitative character were generally similar in both species, suggesting that univariate analyses of these characters would not provide much information about species identity. Two quantitative characters showed substantial differentiation between subpopulations of the two species. First, the ratio of the height and length of the carpus of the male major claw appeared to differentiate most males of the two species that were larger than about 10 mm in carapace length, with males of *N. californiensis* having higher carpus height/length ratios than those of males of *N. gigas* (Fig. 3). Second, the ratio of pollex height and dactyl height of the second pereiopod provided some resolution in differentiating females of the two species (Fig. 4). Several authors had noted that this ratio was approximately one in *N. californiensis*, and greater than one in *N. gigas*. We found that the ratio was always much greater than one, but that most females of *N. gigas* had higher pollex height/dactyl height ratios than did most females of *N. californiensis*. However, there was substantial overlap in this character; this overlap was even more pronounced in males (Fig. 4).

Validation of Eyestalk Characters

The two characters of the eyestalk described above were further validated by examination of a separate test collection of 40 specimens from the San Pedro and San Diego sites. Allozyme surveys revealed that this set of specimens consisted of 22 *N. californiensis* (11 male, 11 female; carapace lengths 7.6-14.5 mm) and 18 *N. gigas* (5 male, 13 female; carapace lengths 7.2-13.2 mm). The first author, blind to each specimen's site of collection or genotype until after his analysis, used the length and shape of the eyestalks to predict species identity for all 40 of these specimens. Morphological identifications matched allozyme identifications for all 40 specimens.

Discussion

Since Stevens' (1928) revision of Callianassidae from the west coast of North America, *N. californiensis* and *N. gigas* have been the only two species of ghost shrimp recognized from soft-sediment intertidal habitats in the region. Stevens (1928) and some subsequent authors (Hart, 1982) have noted that it is very difficult to differentiate many specimens—in particular, juveniles and females—of the two species. Because the two species may coexist on small spatial scales, and juvenile and female shrimp typically make up 50% or more of any sample, many ghost shrimp collected from the intertidal zone in the northeast Pacific are not easily identifiable using morphological characters. This has some important consequences. Most obviously, an inability to identify many collected specimens hampers our ability to study fundamental aspects of their biology, including their distributions, burrowing and feeding activities, reproductive biology, and interactions with each other and other species in the community. Further, any previous identifications of such specimens that have been made despite the low resolution of available morphological

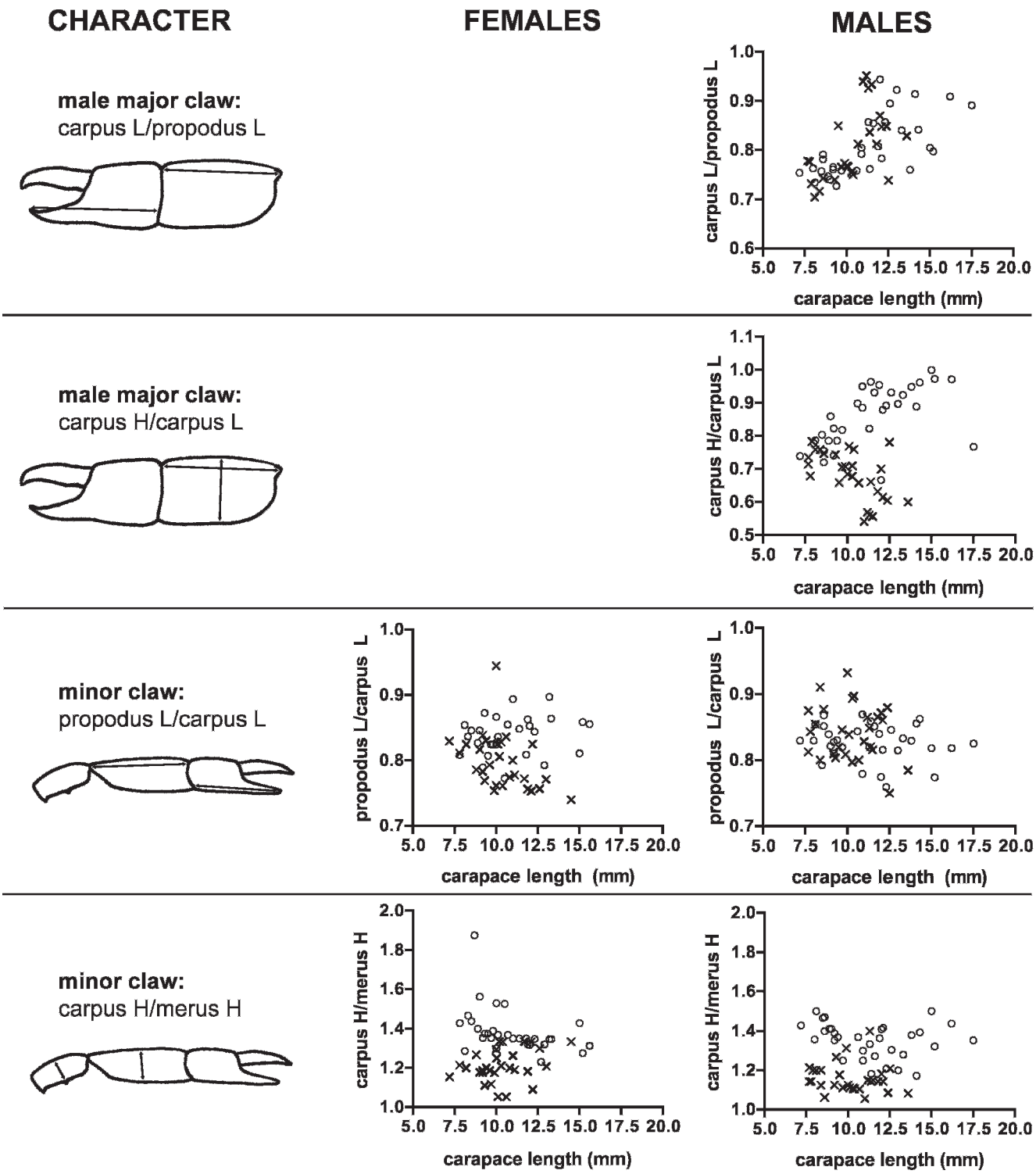

Fig. 3. Patterns of variation in four quantitative morphological characters as function of shrimp sex and size. Dimensions measured to estimate each character shown in first column, and variation in each character as a function of size and species shown for females and males in second and third columns. The symbol O = *N. californiensis*, and X = *N. gigas*.

characters should perhaps be viewed with caution, because some of them are likely to be incorrect.

The molecular and morphological markers we report on here alleviate this problem significantly. In particular, allozyme genotype at the LDH locus, which we found was perfectly correlated with mtDNA clade (Fig. 1), can be used to unambiguously identify large numbers of specimens rapidly and inexpensively. In our initial surveys using this marker, we have demonstrated that both species coexist on small spatial scales at multiple intertidal sites in

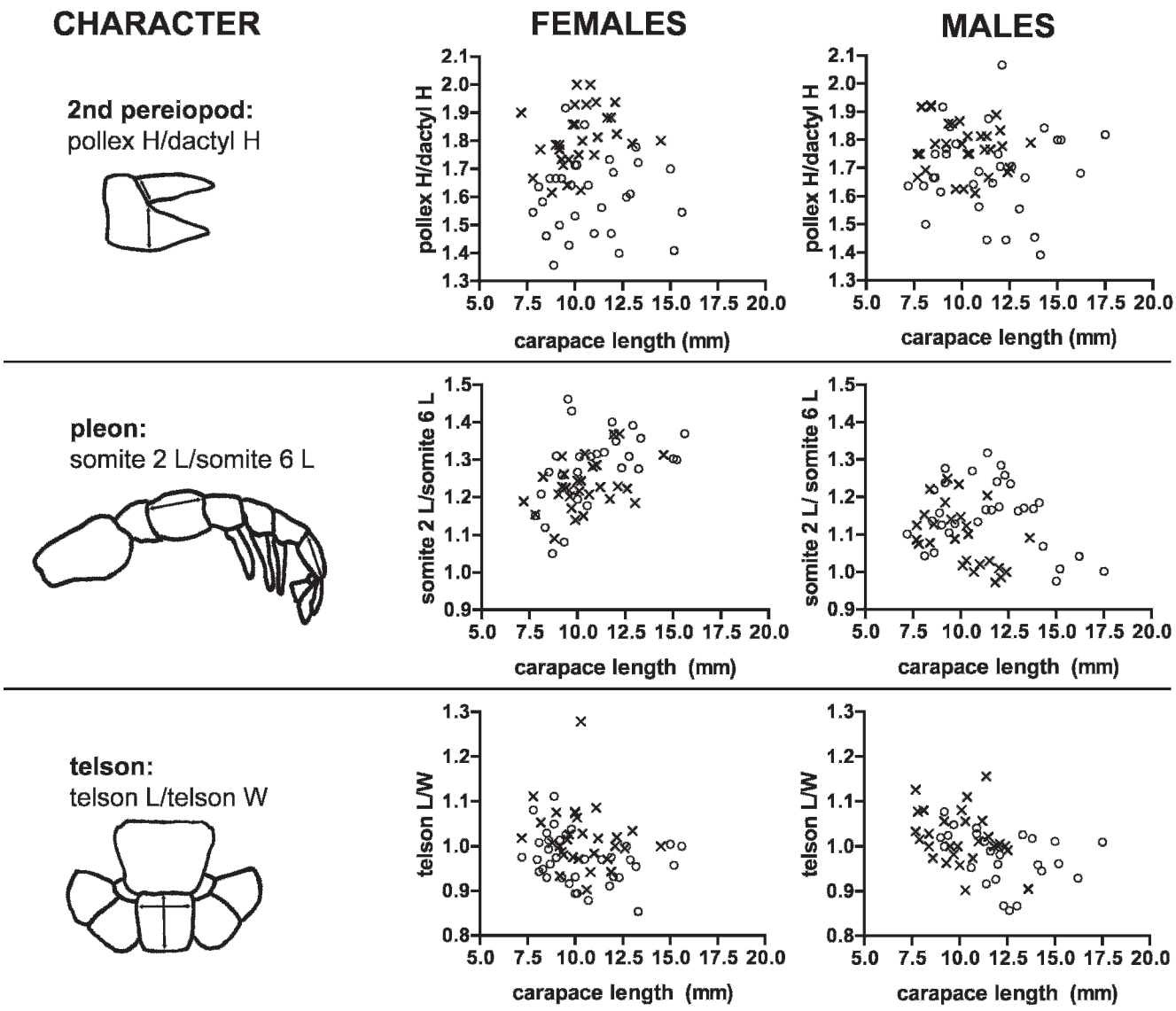

Fig. 4. Patterns of variation in three quantitative morphological characters as function of shrimp sex and size. Dimensions measured to estimate each character shown in first column, and variation in each character as a function of size and species shown for females and males in second and third columns. O = *N. californiensis*, and X = *N. gigas*.

Southern California (Table 3). Despite the utility of this marker, scoring it does require specific sample preservation protocols (freezing versus chemical fixation) and significant time in the laboratory. It would still thus be very useful to have easily-scored morphological characters so that specimens might be identified in the field alive, or in the laboratory after convenient chemical fixation.

Many morphological characters have been proposed for just this purpose (Table 2). The molecular markers we describe here permitted us to evaluate these previously-identified characters—as well as additional characters described here—systematically against specimens of both sexes and a range of sizes, and of known identity. We found that most qualitative and quantitative characters did not perform well in distinguishing between members of the two species. Most failed because rampant intraspecific variation swamped interspecific variation (Figs. 3, 4). The most effective of the previously identified characters were those based on the male major claw, e.g., degree of incurving of the dorsal carpal ridge, or shape of the carpus. As characters of mature males, though, these do not help in the identification of female or immature individuals.

The characters that proved to be most generally useful in this study were two novel characters of the eyestalks—length relative to the second article of antenna 1, and shape of the distal outer edge (Fig. 2). Both performed extremely well in separating members of the two species, including both males and females, and individuals of all sizes examined in this study (carapace lengths of 7.2-17.5 mm). To our knowledge, these characters have not previously been used to distinguish these two species, though Campos et al. (2009) illustrate both characters clearly (their fig. 3b, c). These two characters are especially useful for two reasons. First, the eyestalks and first antennae are robust

and rarely lost in collection. Second, eyestalks are sufficiently large that these characters may be useful even for identifying living shrimp in the field (perhaps with the assistance of a hand lens). We thus recommend these characters for routine identification of these two common intertidal soft-sediment ghost shrimps in the eastern Pacific.

One limitation of our study is that most of our samples originated from Southern California (San Pedro in the north, to San Diego in the south) (though ten *N. californiensis* from Washington State were also included). Thus we were unable to identify any variation in either allozyme patterns or eyestalk characters that might occur over a larger geographic scale. However, *N. californiensis* from Washington State (near the northern edge of the range) were morphologically and molecularly (in terms of both allozymes and mtDNA) indistinguishable from southern conspecifics (this study). Further, a broader phylogeographic analysis of *N. californiensis* based on mitochondrial DNA sequences suggested that there was extensive gene flow throughout most of the range of this species (Pernet et al., 2008); this gene flow is undoubtedly mediated by larval dispersal. As *N. gigas* have eggs similar in size to those of *N. californiensis*, it seems likely that their larvae also have lengthy planktonic periods and disperse widely along the west coast of North America. We thus believe that it is unlikely that there will be substantial regional differentiation in morphology in either of these two species, and that the morphological characters identified here will prove useful across their entire ranges.

At the very least, however, these characters appear to be useful in Southern California, where both species often co-occur on very small spatial scales. We hope that the molecular and morphological characters identified here will facilitate future studies of their distribution, habitat preference, and comparative biology.

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