Genetic diversity in two introduced biofouling amphipods (*Ampithoe valida* & *Jassa marmorata*) along the Pacific North American coast: investigation into molecular identification and cryptic diversity

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ABSTRACT

Aim We investigated patterns of genetic diversity among invasive populations of *A. valida* and *J. marmorata* from the Pacific North American coast to assess the accuracy of morphological identification and determine whether or not cryptic diversity and multiple introductions contribute to the contemporary distribution of these species in the region.

Location Native range: Atlantic North American coast; Invaded range: Pacific North American coast.

Methods We assessed indices of genetic diversity based on DNA sequence data from the mitochondrial cytochrome c oxidase subunit I (COI) gene, determined the distribution of COI haplotypes among populations in both the invasive and putative native ranges of *A. valida* and *J. marmorata*, and reconstructed phylogenetic relationships among COI haplotypes using both maximum parsimony and Bayesian approaches.

Results Phylogenetic inference indicates that inaccurate species level identifications by morphological criteria are common among *Jassa* specimens. In addition, our data reveal the presence of three well supported but previously unrecognized clades of *A. valida* among specimens in the northeastern Pacific. Different species of *Jassa* and different genetic lineages of

Ampithoe exhibit striking disparity in geographic distribution across the region as well as substantial differences in genetic diversity indices.

Main conclusions Molecular genetic methods greatly improve the accuracy and resolution of identifications for invasive benthic marine amphipods at the species level and below. Our data suggest that multiple cryptic introductions of *Ampithoe* have occurred in the northeastern Pacific and highlight uncertainty regarding the origin and invasion histories of both *Jassa* and *Ampithoe* species. Additional morphological and genetic analyses are necessary to clarify the taxonomy and native biogeography of both amphipod genera.

Keywords: benthic invertebrate, cryptic species, DNA barcode, invasive species**,** marine amphipod

INTRODUCTION

The taxonomy and biogeography of many coastal invertebrate taxa, particularly those associated with anthropogenic range expansions, remains poorly understood (Carlton, 2009). For many coastal habitats, intercoastal and transoceanic shipping predates taxonomic studies of their benthic marine invertebrates by several hundred years. Faunistic studies of the benthos in these locales have occurred long after many non-native fauna have successfully colonized and these non-native taxa are then often mistaken as natural members of the ecosystem (Carlton, 2009). Persistent questions thus remain regarding the native origins of many coastal benthic species, and many still cannot reliably be assigned native or introduced status in parts of their global range (Geller *et al.,* 2010). The rise in molecular DNA studies of benthic invasive invertebrates has further complicated matters by uncovering cryptic species in such disparate groups as hydrozoans (Folino-Rorem *et al.*, 2009), jellyfish (Holland *et al.*, 2004; Dawson *et al.*, 2005), mussels (Geller *et al.*, 1994; Rawson & Hilbish, 1995; Hilbish *et al.*, 2000), bryozoans

53 (Davidson & Haygood, 1999; Schwaninger, 2008), and tunicates (Caputi *et al.*, 2007). These
54 unanswered questions regarding taxonomy, native biogeography and cryptic diversity have
55 critical importance for understanding the relationships between biological invasions and
56 historical changes to benthic community structure.

57 The benthic marine amphipods *Ampithoe valida* Smith 1873 (family Ampithoidae) and
58 *Jassa marmorata* Holmes 1903 (family Ischyroceridae) are both tube-building biofoulers that
59 have invaded many shallow to intertidal habitats around the world (Table 1). Both are considered
60 invasive species along the Pacific coast of North America where their initial introductions are
61 presumed to have occurred in San Francisco Bay (Cohen & Carlton, 1995) followed by
62 expansion north and south along the coast. These two species can occur in very dense
63 aggregation in the benthos; populations of *Jassa* may have densities above 10,000 individuals/m^2
64 (Franz & Mohamed, 1989). Identifying individuals of these two species requires considerable
65 taxonomic expertise as species in both *Ampithoe* and *Jassa* are often distinguished
66 morphologically by differences in the gnathopods (legs modified for grasping) of adult males
67 (Chapman, 2007). These adult males, however, may be infrequently encountered (Scinto *et al.*,
68 2007), complicating the identification of collected specimens of these taxa.

69 Both taxa are members of genera that have experienced unsettled taxonomy. Prior to
70 revision (Conlan, 1990), all species in the genus *Jassa* had been lumped into a single species
71 (Reid, 1951) mainly due to the difficulties of identifying juveniles and females into separate
72 species. Conlan's revision resurrected many *Jassa* species and described fourteen new species.
73 The genus *Ampithoe* has not undergone any recent revisions, but recent work on Australian
74 species suggests that the genus is in need of further taxonomic scrutiny. Peart (2007) moved four

Australian species to other genera and described seventeen new species of *Ampithoe* bringing the total number of species for Australia from three to twenty.

Both *A. valida* and *J. marmorata* thus have the hallmarks of invasive benthic marine taxa that are likely to harbour unrecognized diversity: 1) they have a history of taxonomic uncertainty; 2) there are reasons to suspect that morphological criteria may be insufficient for accurate species level identification except in ideal cases (e.g. presence of major males or accessibility of specialized taxonomic expertise); 3) they are widespread with contemporary distributions shaped in large part by anthropogenic dispersal. Given these considerations, we sought to investigate the genetic diversity in non-native populations of these two amphipods along the Pacific North American coast with the aim of assessing the accuracy of morphological identifications and determining whether or not cryptic diversity and/or multiple introductions contribute to the current distribution of these taxa in the region. Using the standard DNA barcoding locus (COI), we examined whether phylogenetic reconstructions based on this locus could reliably distinguish *A. valida* and *J. marmorata* from native Pacific *Ampithoe* and *Jassa* species, respectively. We also examined the geographic distribution of genetic variation in each species across its introduced range and compared this to genetic diversity observed in putative native populations in the northwestern Atlantic. These approaches together provide strong evidence for the utility of molecular genetic data in clarifying species level identifications and recognizing cryptic diversity within populations of invasive marine amphipods.

METHODS

Specimens of invasive amphipods were collected as part of grab samples in the summer of 2006 from localities along the Pacific coast of North America (Table 2) as well as two sites from the Atlantic coast (the putative native range of *A. valida* and *J. marmorata*), and were

99 stored in 95% ethanol. Although collection efforts were targeted toward *A. valida* and *J.*
100 *marmorata*, at some sites additional specimens from congeneric species were also collected.
101 Specimens were identified by morphologists as *Ampithoe lacertosa* (Bate) 1858, *A. valida*, *Jassa*
102 *staudei* Conlan 1990, or *J. marmorata,* although some of these identifications were considered
103 tentative due to the difficulties associated with identifying these taxa accurately to species,
104 particularly in the case of juvenile individuals (Chapman, 2007). Identifications of *Jassa* species
105 are most readily accomplished, although not exclusively, using major males (Conlan, 1990;
106 Chapman, 2007), which are larger males that have species-specific modifications of their
107 secondary gnathopods for male-male competitions (Fig. 1), as opposed to identifying juveniles,
108 females, or minor ("sneaker") males.
109 Prior to DNA extraction, tissue samples of large individuals or the entire specimen of
110 small juveniles were vacuum-dried. All extractions were performed with the QIAgen DNeasy
111 Tissue Kit following the manufacturer's protocol. PCR amplification of the COI locus took place
112 in 20 μL volume reactions with the following conditions: 3mM $MgCl_2$, 200 pM dNTPs, 1 unit of
113 *Taq* polymerase, 1 mM for each primer, standard PCR buffer, and approximately 20 ng of
114 template DNA. Initial amplification of COI used the standard DNA barcoding Folmer primers
115 LCO 1490F and HCO 2198R (Folmer *et al.,* 1994). Some samples did not amplify well with the
116 standard primers, and *Ampithoe*- and *Jassa*-specific primers were designed for PCR and DNA
117 sequencing (Table 3). The PCR amplification program included an initial step of 94°C for 150
118 sec, followed by 35 cycles of 94°C for 30 sec, 46°C for 60 sec, and 72°C for 60 sec, with a final
119 step of 72°C for 10 min. PCR products were cleaned with the QIAquick PCR kit on a BioRobot
120 3000 from QIAgen. Sequencing reactions were done with the ABI Big Dye Terminator Cycle
121 Sequencing Ready Reaction Kit 3.1. The sequenced products were purified with DyeEx 96 Kit

from QIAgen, dried and re-eluted with formamide, and run on an ABI Prism 3730xl DNA Analyzer. All COI sequences were deposited into GenBank (see Table 2 for accession numbers).

We used Sequencher 4.6-4.8 (Gene Codes, Ann Arbor, MI) to combine forward and reverse reads into contiguous sequences and then to align the different haplotypes. Genetic distances and neighbour-joining cluster analysis (both Kimura 2-parameter—standard for DNA barcode data sets) of the data set were generated using MEGA 4.0 (Kumar *et al.,* 2004). The data set also was analyzed with Arlequin 3.11 (Excoffier *et al.,* 2005) to produce measures of haplotype and nucleotide diversity. Effective haplotype number (k) was calculated as:

$$k = 1 / \sum p_i^2 ,$$

where p_i is the frequency of the ith haplotype. As outgroups were necessary for some analyses, an *Ischyrocerus* COI sequence (accession number DQ889106) was chosen as an outgroup for the *Jassa* samples (both *Ischyrocerus* and *Jassa* are members of Ischyroceridae), and *Ampithoe longimana* (Smith 1873) and *A. lacertosa* COI sequences were used as outgroups for the *A. valida* samples. Maximum parsimony analysis was run using Winclada 0.9 (Nixon, 1999) and Nona 0.99 (Goloboff, 1999) with the following commands: `rs 0; hold 10000; hold/100; mult*30; max;`. Bootstrap support (BSS) based on 1000 pseudoreplicates was generated for the data set using Nona. Models of evolution for each data set (*Ampithoe*: GTR + G; *Jassa*: GTR + I) were determined with jModeltest 0.1 (Guindon and Gascuel, 2003, Posada, in press). Bayesian analyses of each data set were performed with MrBayes 3.1 (Ronquist & Huelsenbeck, 2003) using the models from jModeltest with two runs of four chains each for three million generations (runs converged with average standard deviation of the split frequencies below 0.01) sampled every 100 generations and burn-in periods of 50,000 generations.

RESULTS

Jassa *spp. populations*

For *Jassa* species, we generated COI sequences for 183 individuals from five sites on the Pacific coast of North America along with 35 individuals from two sites on the Atlantic coast. The *Jassa* sequences were 606-658 bp in length for most specimens, dependent upon which PCR primer pair was used. Including samples from both coasts, there were 24 different COI haplotypes (Table 4), which grouped into four distinct, well-supported clades (BSS = 100%, posterior probability = 1.00) in neighbour-joining, maximum parsimony, and Bayesian analyses (Fig. 2). The largest group (N = 143) of Pacific coast samples included most of the specimens identified by morphology as *J. marmorata*, although 12 individuals from Coos Bay that were part of this clade had been tentatively identified as *J. staudei*. The next largest group (N = 25) comprised all individuals from Puget Sound that had been identified as *J. staudei*. The smallest group (N = 15) included individuals from Tomales Bay and Moss Landing that had been initially identified as *J. marmorata*. These specimens were grouped in their own clade distinct from *J. marmorata*, and subsequent re-identification of adult major males (Fig. 1) from these sites found these individuals to be *Jassa slatteryi* Conlan 1990. For the two Atlantic coast sites of putative native *J. marmorata* samples, the specimens (N = 17) from Millstone Point, CT did group within the *J. marmorata* clade, but the Charleston Harbor, SC specimens (N = 18) formed their own clade distinct from the other three *Jassa* species.

Although 24 COI haplotypes were found among all *Jassa* specimens, only six different haplotypes were found in the 143 individuals confirmed by phylogenetic inference as *J. marmorata*. One of these haplotypes dominated *J. marmorata* populations at all five Pacific coast localities (Fig. 3), occurring in 81.8% of all individuals and ranging in frequency from 69.2-100% in these populations. A single haplotype was also found shared among all 17

specimens of *J. marmorata* from the Atlantic coast. Genetic distances between *J. marmorata* COI haplotypes were small and ranged from 0.2-0.6% (Table 5). The low number of haplotypes in the Pacific coast populations of *J. marmorata* was reflected in low mitochondrial diversity indices for these populations (see Table 4). No single Pacific coast population of *J. marmorata* contained more than three COI haplotypes, and effective haplotype number ranged only from 1.00 to 1.89 (mean = 1.08) (Table 4).

Though the two other Pacific coast *Jassa* species were collected in much lower numbers than *J. marmorata*, they possessed as many or more different COI haplotypes as the *J. marmorata* samples. The lone population of *J. staudei* (Puget Sound) exhibited eight COI haplotypes, corresponding to an effective haplotype number of 4.56 (Table 4). We found six different COI haplotypes in the two *J. slatteryi* populations. These populations shared a dominant haplotype (Tomales Bay frequency = 45.5%; Moss Landing frequency = 75%) (Fig. 3), and the remaining five haplotypes were unique to their collection sites. Diversity measures for both *J. staudei* and *J. slatteryi* were higher than those observed for *J. marmorata* (Table 4). Intraspecific genetic distances ranged from 0.2 to 1.6% in *J. staudei* and 0.2 to 1.1% in *J. slatteryi* (Table 5). When compared across known *Jassa* species, genetic distances were much larger than intraspecific variation, ranging from 14.6% to 16.0% (Table 5). The Charleston Harbor, SC *Jassa* differed from *J. marmorata* (including both invasive and putative native specimens) by at least 14.0%, suggesting that although these specimens were originally identified as *J. marmorata*, they represent a distinct species.

A. valida *populations*

For *A. valida* species, we generated COI sequences for 314 individuals from eight sites on the Pacific coast of North America along with 25 individuals from two sites on the Atlantic

coast. These COI sequences were 610-658 bp in length for most specimens, dependent upon which PCR primer pair was used. We found 25 COI haplotypes among the *A. valida* populations from the Pacific North American coast, and these haplotypes formed three separate, well-supported clades in neighbour-joining, maximum parsimony, and Bayesian analyses (Fig. 4). The first group (hereafter Clade A) consisted of 37 specimens, most of which (N = 32) were collected in Humboldt Bay. The second group (Clade B) consisted of 63 specimens that were all collected only in San Francisco Bay. The last group (Clade C) contained 213 individuals collected from all Pacific coast sites except for San Francisco Bay (Fig. 5). The two "native" (Atlantic coast) sites of *A. valida* (N = 25) contained haplotypes that grouped with Clade A.

Haplotype numbers, frequencies, and diversities varied between the three clades of *A. valida*. Clade A had one dominant shared haplotype that occurred in all but one individual in Humboldt Bay (frequency 96.9%) and was the sole Clade A haplotype present outside of that Bay (4 individuals in San Francisco Bay and one individual in Willapa Bay). This same dominant haplotype also occurred in all the "native" *A. valida* in Millstone Point, CT and six of seven individuals in Charleston Harbor, SC. Clade B, found only in San Francisco Bay, had one dominant haplotype (frequency of 90.5%) with the other six haplotypes occurring in only one specimen each. Clade C was the most widespread, including a dominant haplotype found at all seven Pacific coast sites with an overall frequency of 93.4% that ranged from 85.7 to 100% in these populations. Clade C also had 14 other COI haplotypes, but these occurred in only 1-2 specimens and were not shared among different sites. Diversity values ranged widely across sites, with the highest values of H_e and k occurring at sites harbouring Clade C (Table 4).

Genetic distances observed within *A. valida* clades were much smaller (0.2-0.4%) than the inter-clade distances (at least 4.1%), although the latter were still smaller than those found

when comparing *A. valida* clades to the outgroups *A. lacertosa* (at least 18.0%) and *A. longimana* (at least 21.5%) (Table 6).

DISCUSSION

Species level Distinctions and Morphological Mis-identification in *Jassa*

Our results show that the COI DNA barcode locus is useful for identification of *Ampithoe* and *Jassa* specimens of the Pacific North American coast to species level or below. For *Jassa,* the COI sequence data provide the first molecular corroboration of Conlan's (1990) revision of the genus. Prior to this revision, the taxonomy of the genus had been in flux, with one author actually synonymizing all *Jassa* of the world into a single species (Reid, 1951). The large genetic distances (>10%; Table 5) between *J. marmorata*, *J. slatteryi*, and *J. staudei* strongly support these taxa as separate species, and suggest morphological characters such as the shape of the second gnathopod in adult *Jassa* (Conlan, 1990) indeed are useful for delineating and identifying individuals to species. Rough divergence estimates based on a widely adopted molecular clock for crustacean COI sequences (1.4% per million years (Knowlton & Weigt, 1998) with a generation of time of ~1 year for *Jassa*) place the split between *J. marmorata* and *J. slatteryi* at least ~10 MYA and the split between *J. marmorata* + *J. slatteryi* and *J. staudei* at least ~11 MYA.

The mis-identification of some *Jassa* samples in this study, however, illustrates the difficulties associated with species level identification in the genus. The most prominent characters for species identification are found in adult major males due to enhancement of the second gnathopod for male-male competition (Borowsky, 1985; Conlan, 1990). *Jassa* species, however, lack a 1:1 sex ratio because of the male castes (Nair & Anger, 1980), and major males are rarely collected, making identification of *Jassa* populations problematic. For instance, in one

237 year-long study, less than 2% of collected individuals of *J. falcata* were major males, with nearly
238 80% of the samples being juveniles (Scinto *et al.,* 2007). Male *Jassa* do not exhibit major status
239 until after their final moult (Kurdziel & Knowles, 2002), and although juveniles, minor males,
240 and females of *Jassa* can be identified to species (Conlan, 1990), such identifications require
241 considerable morphological and taxonomic skill relative to the ease of designating major males
242 to species (see Fig. 1). The molecular results of this study, however, show the COI locus to be
243 very useful for identifying *Jassa* specimens of any age or sex to species.

244 These challenges explain the high frequency of misidentification exhibited in the current
245 study. Our molecular genetic approach was able to correct three different errors in morphological
246 identification. First, 12 out of 143 *J. marmorata* specimens (8.4%) were incorrectly identified as
247 the presumed native *J. staudei*, although these identifications were listed as "tentative." Second,
248 15 individuals identified morphologically as *J. marmorata* were definitively identified by genetic
249 criteria as *J. slatteryi*. This case is particularly interesting, because subsequent re-evaluation of
250 diagnostic morphological criteria revealed that these specimens did indeed possess
251 morphological features consistent with *J. slatteryi*. Finally, all 18 individuals collected from
252 Charleston Harbor, South Carolina, were incorrectly identified as *J. marmorata*. Genetic criteria
253 clearly indicate that these specimens are members of a distinct *Jassa* lineage. Furthermore, given
254 that divergence at the COI locus between this lineage and other *Jassa* lineages is comparable to
255 divergence between lineages recognized as independent species, we believe that it may represent
256 an additional, as yet unidentified, species of *Jassa*. Consistent with the hypothesis of independent
257 species status for this lineage, we refer to it here as *Jassa* sp.

258 These findings offer important insights regarding the shortcomings of invasive species
259 detection based on traditional morphological identifications. At least three of the *Jassa* species

260 involved in the current study (*J. marmorata*, *J. staudei*, and *J. slatteryi*) are well defined by
261 diagnostic morphological criteria, and thus technically not "cryptic" species (Conlan, 1990).
262 However, difficulties associated with identification of life stages other than major males may
263 render these species "effectively cryptic" for the generalist taxonomists typically tasked with
264 field identification of non-native taxa. This phenomenon has been observed previously for other
265 invasive marine taxa. For instance, an introduced population of the convex slippershell limpet
266 *Crepidula convexa* in Humboldt Bay was recently mistaken for its (also introduced) sibling
267 species *C. fornicata* despite existing diagnostic morphological criteria; molecular genetic
268 analysis subsequently corrected the mis-identification (McGlashan *et al.*, 2008). Similarly, the
269 presence of the Mediterranean green crab *Carcinus aestuarii* in South Africa was mistaken for
270 the already recognized invasive European green crab *C. maenas* (Geller *et al.,* 1997), although
271 morphological characteristics are known to clearly differentiate the two species (Yamada &
272 Houk 2000).
273 These examples and the current study all suggest that targeted collections aimed at any
274 particular invasive taxon may bias identifications toward that target when diagnostic
275 morphological criteria are subtle or absent. In the case of *Jassa*, most errors in identification
276 were errors of commission and the only errors of omission were accurately tagged as "tentative"
277 identifications. In fact, several errors were committed despite the presence of morphological
278 characteristics enabling correct identification on further inspection. Similarly, the presence of
279 both *Crepidula convexa* and *Carcinus aestuarii* may have been overlooked because the sibling
280 species *Crepidula fornicata* and *Carcinus maenas* were already known to be present and thus
281 expected to appear in faunal surveys. DNA-based clarification of mistaken identifications, both
282 here and in other studies, indicates that molecular genetic data can aid substantially in

overcoming these potential biases. Further, the problems associated with distinguishing between closely related taxa in practice may derive in part from the necessity in rapid faunal surveys of relying on single (or few) diagnostic characters rather than systematic statistical analysis of morphological variation. Although the latter is standard for species definition and description, it may be rendered difficult or impossible in situations of species identification, where resources often must be spread broadly over numerous taxa and numerous sampling sites. Fortunately, by helping to recognize difficulties associated with species identification, molecular data can provide the impetus for future refinement of protocols adopted for morphological identification. In at least one recent case species identifications based in part on molecular genetic data have led researchers to question the validity of previously accepted diagnostic morphological characters (Grigorovich *et al.*, 2008).

Cryptic Diversity and Multiple Introductions in *Ampithoe*

Reports of previously undescribed diversity within invasive populations, particularly in marine systems, is becoming a common feature of the invasion biology literature (reviewed in Geller *et al.*, 2010). The observed genetic variation among invasive *A. valida* populations clearly indicates the presence of three distinct well-supported evolutionary lineages that have been diverging for some time. Applying the same molecular clock as above, the three clades diverged ~3 MYA. Given the substantial genetic distances between clades and the fact that these distances are greater than ten times those observed within clades, the divergence between *A. valida* lineages in fact may be sufficient to warrant their consideration as separate species, especially if applying either the phylogenetic species concept (Eldredge & Cracraft, 1980) or the genetic species concept (Baker and Bradley, 2006). This hypothesis is further supported by the observation that *A. valida* inter-clade distances fall into the range (greater than 4.4%) applied to

306 cryptic species within the amphipod genus *Hyallela* (Witt *et al.,* 2006). Unfortunately, the
307 discovery of cryptic diversity during the study of invasive populations frequently leaves
308 unresolved issues regarding the taxonomic status of newly described evolutionary lineages
309 (Meusnier *et al.,* 2002; Zardus & Hadfield, 2005, Folino-Rorem *et al.,* 2009). While the
310 designation of new species is clearly beyond the scope of this work, we feel that the three *A.*
311 *valida* lineages described here bear further investigation by integrated genetic and morphological
312 taxonomic approaches such as the addition of data from nuclear DNA markers coupled with
313 morphometric analyses to determine if they are indeed separate species and which, if any, of the
314 three clades ought to be considered *A. valida*.

315 Observations of cryptic invasive lineages not only draw attention to shortcomings in our
316 understanding of the taxonomy and biogeography of numerous marine taxa, they also serve to
317 clarify the invasion histories of recipient ecosystems. Our results indicate that genetic methods
318 are crucial for assessing the full extent to which Pacific North American coastal waters have
319 been invaded by introduced *Ampithoe* lineages. Unlike *Jassa* species, there exist no known
320 morphological criteria by which the observed lineages of *A. valida* clades can be accurately
321 identified. Unless integrated taxonomic approaches can determine diagnostic morphological
322 criteria corresponding to the observed genetic divergence between lineages, managers tasked
323 with monitoring the spread of these invaders will require genetic methods to accurately
324 determine changes in their future distribution. The observed evolutionary divergences also
325 recommend additional studies to assess whether or not relevant ecological differences exist
326 which might help explain the widely different distributions of these lineages on the Pacific coast
327 of North America. Previous descriptions of cryptic diversity within invasive populations have
328 been accompanied by observations of apparent evolutionary divergence in ecological traits

329 (Meusnier *et al.*, 2002; Kelly *et al.*, 2006; Folino-Rorem *et al.*, 2009). Such differences could
330 have dramatic implications for management strategies targeting different invasive lineages.
331 The presence of three distinct evolutionary lineages of *A. valida* in the northeast Pacific
332 and the non-uniform distribution of these lineages among sampling sites (Figure 5) also suggests
333 the likelihood of multiple introductions to the region. Our genetic results are clearly not
334 consistent with the hypothesis of a single introduction to San Francisco Bay followed by
335 expansion along the Pacific coast. *A. valida* was first recorded in the northeast Pacific from the
336 San Francisco Bay region in the early 1940's, long before populations were observed in Oregon
337 and Washington, suggesting a scenario of initial introduction to San Francisco Bay followed by
338 subsequent secondary spread to northern sites. However, the clear dominance of Clade C
339 throughout northern sites, along with its virtual absence from southern sites at Humboldt Bay
340 and San Francisco Bay (dominated instead by Clades A and B respectively), presents two
341 plausible alternative hypotheses for the invasion history of *A. valida* in the region. It is possible
342 that Clade C was introduced initially to the San Francisco area, whence it spread throughout the
343 region eventually reaching as far north as the Strait of Georgia. More recently, cryptic Clades A
344 and B have been introduced to California and have displaced Clade C in San Francisco and
345 Humboldt Bays. Alternatively, Clade B represents the initial introduction to San Francisco Bay,
346 but has failed to spread to northern sites. The apparent expansion of *A. valida* to the north has
347 occurred via cryptic secondary introduction of Clades B and C, the latter of which has spread
348 dramatically among northern sites.
349 Given limits to our sampling, the absence of a particular clade from a sampling site
350 cannot be taken as strong evidence for the general absence of that clade from a particular locale.
351 There is the possibility, for instance, that Clade B exists in San Francisco Bay but that the three

352 *A. valida* clades have largely non-overlapping spatial distributions in the Bay. More thorough
353 sampling would test the robustness of the observed disjunct distribution patterns. Nonetheless,
354 the likelihood of multiple introductions to the region is supported not only by these patterns, but
355 by the presence of well differentiated monophyletic clades among invasive populations. *A. valida*
356 thus joins the ranks of the numerous coastal marine taxa for which genetic evidence implicates
357 multiple introductions in the establishment of invasive populations (Geller *et al.,* 2010),
358 providing further illustration of the importance of genetic data in fully accounting for the
359 frequency with which recipient environments suffer incursions of non-native taxa. Recognition
360 of multiple introductions can have serious implications for the management of invasive
361 populations, as a growing body of evidence suggests that admixture of previously allopatric
362 lineages can lead to increases in heritable quantitative genetic variation and the emergence of
363 novel genotypes (Dlugosch & Parker, 2008), in some cases facilitating rapid adaptive evolution
364 of introduced populations (Kolbe *et al.*, 2007; Facon *et al.*, 2008; Lavergne & Molofsky, 2007)
365 **Historical Biogeography**
366 Given the unsettled state of taxonomy for the genus *Jassa* prior to Conlan's revision and
367 the difficulty associated with morphological identification to species level, the fact that questions
368 remain regarding the historical biogeography of many *Jassa* species is perhaps not surprising.
369 Both *J. marmorata* and *J. slatteryi* are known invasive species with broad disjunct distributions
370 and established populations throughout the world, mainly at sites harbouring major commercial
371 ports (Table 1). However, the native ranges of these two species are not well characterized
372 (Chapman, 2007). Populations of *J. slatteryi* on the Pacific North American coast have been
373 considered either introduced or cryptogenic (Boyd *et al.*, 2002), whereas *J. staudei* is considered
374 native to the region and *J. marmorata* invasive (Chapman, 2007). Our analysis indicates that the

genetic diversity present in Pacific *J. slatteryi* populations is similar to that observed in *J. staudei*, and both species exhibit diversity measures dramatically higher than *J. marmorata* (Table 4). Despite intensive sampling of *J. marmorata*, the observed number of haplotypes (N_h=6, N=143) remained equal to or lower than that observed in small samples of *J. staudei* (N_h=8, N=25) and *J. slatteryi* (N_h=6, N=15). Because introduction events transfer only a small subset of native genetic diversity, invasive populations frequently exhibit lower diversity than native sources (Dlugosch & Parker, 2008). The low genetic diversity found in Pacific *J. marmorata* is thus consistent with this expected founder effect. The number of haplotypes, haplotype diversity, and intraspecific genetic distances for the *J. slatteryi* populations, however, are similar to those found for the presumed native *J. staudei* population, suggesting the possibility that the Pacific coast of North America could represent the native range of *J. slatteryi*. However, the relationship between genetic diversity and native or introduced status is suggestive at best (Roman & Darling 2007; Geller *et al*., 2010), and the limited sampling for both *J. staudei* and *J. slatteryi* in the current study preclude high confidence in this hypothesis pending further study.

Atlantic coast samples of *J. marmorata* were included in the current study as putative potential sources because the Pacific coast populations have been classified as native transfers from the Atlantic (Cohen, 2004). Interestingly, we observed no genetic diversity at all in the single Atlantic *J. marmorata* population from Millstone Point, CT, and the presumed native *J. marmorata* population from Charleston Harbor, SC was clearly shown by DNA sequence analysis to derive from a separate *Jassa* lineage, possibly representing a currently unidentified independent species. Unfortunately, the limited sample size of these Atlantic *Jassa* is insufficient to draw detailed conclusions regarding biogeographic patterns in the putative native range of *J.*

marmorata, although clearly multiple *Jassa* lineages currently inhabit this range. Whether the putative unidentified species from Charleston Harbor represents a second native or a recent introduction to the northwest Atlantic will clearly require more extensive sampling.

Ampithoe valida is similarly thought to be a native of Atlantic North America (Chapman, 2007). Specimens of *A. valida* from both Connecticut and South Carolina belong to Clade A and share the dominant Clade A haplotype observed on the Pacific coast, suggesting that this clade may indeed have a western Atlantic origin. As is the case of *J. marmorata*, Atlantic samples of *A. valida* also show surprisingly low diversity for presumably native taxa, although sampling was again limited in this range. The presence of the same dominant haplotype in both Connecticut and South Carolina is also interesting. While this may reflect the absence of strong native biogeographic structure in this species, anthropogenic mixing of different populations along the Atlantic coast also could have homogenized genetic variation across the range, both eliminating signatures of past structure and further complicating any work aimed at determining which localities in the native range gave rise to the invasive populations along the Pacific coast. The apparent widespread distribution of Clade A in the western Atlantic along with the absence of Clades B and C from samples in that range leaves the native origins of those clades unresolved. More extensive sampling in the western Atlantic may uncover native sources for these clades; alternatively, observed divergence between clades is sufficient to support the hypothesis that Clades A, B, and C represent multiple *Ampithoe* species with distinct and currently unknown biogeographic distributions.

CONCLUSION

The results of this study illustrate the value of molecular genetic approaches for 1) clarifying species level identifications in introduced taxa unresolved by available morphological

criteria; 2) recognizing multiple introductions of evolutionarily divergent but morphologically cryptic invasive lineages; and 3) highlighting uncertainties regarding the historical biogeography of benthic marine invertebrates with histories of anthropogenic range expansions. Unfortunately, the amphipod taxa studied herein are representative of many widespread invasive marine invertebrates poorly understood in terms of their taxonomy, native biogeography, and invasion histories (Carlton, 2009). However, our analyses demonstrate that the DNA barcode region of COI is useful for differentiating amphipod lineages, and numerous other studies suggest that it may be similarly useful for many other marine invertebrates (Geller *et al.*, 2010). Morphological study alone is unlikely to untangle how human activity has radically altered the distributions of invasive benthic species such as *A. valida, J. marmorata* and others like them. Molecular genetic data can thus play a critically important role in overcoming existing impediments to understanding the taxonomy and native biogeography of these taxa.

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Biosketches

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Author contributions: Both authors conceived the study; E.P. collected the data; both authors analyzed the data; both authors wrote the paper.

590 **Table 1.** Current* putative distributions of invasive *Ampithoe* and *Jassa*.

	Ampithoe valida	*Jassa marmorata*	*Jassa slatteryi*
"Native" Range	Atlantic North America	Atlantic North America and Gulf of Mexico	Cryptogenic, possibly from Pacific North America
Introduced Range	Argentina Japan Pacific North America (British Columbia to California)	Pacific North America (Alaska to California) Mexico (Sea of Cortez) Ireland England France (Mediterranean & Atlantic) Germany Sweden Denmark Spain (Mediterranean & Atlantic) Italy Yugoslavia China (Zhangiao Bay) Japan Russia (Sea of Japan) Chile Brazil Argentina Uruguay Gambia South Africa Senegal Australia (New South Wales & Tasmania) New Zealand	Mexico (Sea of Cortez) Ireland France (Mediterranean & Atlantic) Croatia Japan South Korea Galapagos Islands Chile Brazil South Africa Australia (New South Wales & Tasmania) New Zealand

591 *These distributions are based on past publications and therefore likely underestimate the number of introduced populations.

592 **Table 2.** Sample collection data.

Species	Locality	Latitude/Longitude	N	Accession Numbers
Ampithoe lacertosa Bate	Willapa Bay, OR, USA	46.54°N 123.99°W	3	GU048166-GU048168
	Puget Sound, WA, USA	47.94°N 122.53°W	2	GU048164-GU048165
	Coos Bay, OR, USA	43.41°N 124.21°W	1	GU048163
A. longimana Smith	Millstone Point, CT	41.30°N 72.17°W	1	GU048169
A. valida Smith	San Francisco Bay, CA, USA	37.72°N 122.28°W	67	GU048411-GU048477
	Tomales Bay, CA, USA	38.17°N 122.91°W	31	GU048478-GU048508
	Humboldt Bay, CA, USA	40.72°N 124.24°W	33	GU048378-GU048410
	Coos Bay, OR, USA	43.41°N 124.21°W	15	GU048179-GU048193
	Yaquina Bay, OR, USA	44.62°N 124.02°W	9	GU048170-GU048178
	Willapa Bay, WA, USA	46.54°N 123.99°W	96	GU048257-GU048352
	Grays Harbor, WA, USA	46.95°N 124.04°W	7	GU048250-GU048256
	Puget Sound, WA, USA	47.94°N 122.53°W	56	GU048194-GU048249
	Charleston Harbor, SC, USA	32.75°N 79.90°W	7	GU048371-GU048377
	Millstone Point, CT, USA	41.30°N 72.17°W	18	GU048353-GU048370
Jassa marmorata Holmes	Moss Landing, CA, USA	36.81°N 121.79°W	66	EU243692-EU243731 GU048119-GU048144
	Tomales Bay, CA, USA	38.17°N 122.91°W	39	EU243732-EU243765 GU048114-GU048118
	Humboldt Bay, CA, USA	40.72°N 124.24°W	6	EU243686-EU243691
	Coos Bay, OR, USA	43.41°N 124.21°W	31	EU243666-EU243670 EU243671-EU243685 EU243766-EU243777
	Puget Sound, WA, USA	47.94°N 122.53°W	1	EU243671
	Millstone Point, CT, USA	41.30°N 72.17°W	17	GU048097-GU048113
J. slatteryi Conlan	Moss Landing, CA, USA	36.81°N 121.79°W	4	EU243814-EU243815 GU048095-GU048096
	Humboldt Bay, CA, USA	40.72°N 124.24°W	11	EU243803-EU243812
J. staudei Conlan	Puget Sound, WA, USA	47.94°N 122.53°W	25	EU243778-EU243802
Jassa spp.	Charleston Harbor, SC, USA	32.75°N 79.90°W	18	GU048145-GU048162

593
594

595 **Table 3.** Oligoncleotide primers developed for PCR and DNA sequencing in this study.

Name	Sequence
Ampval COIF	5'-GAC TTT ATA TTT TAT TTT AGG TGG-3'
Ampval COIR	5'-AAA TAA RTG TTG RTA TAA AAT AGG-3'
Jasmar COIF	5'-CTT TAT ATT TTA TTT TAG GTA TTT GG-3'
Jasmar COIR	5'-AAA TAA ATG TTG GTA TAA GAT AGG-3'

Table 4. Summary population genetic statistics by species and site. Number of individuals (N), number of haplotypes (N_h), effective number of haplotypes (k).

	Site	Clade/Species	N	N_h	Haplotype Diversity	Shannon Diversity	k	Nucleotide Diversity
***Jassa* spp.**	Moss Landing	*J. marmorata*	66	2	0.3021	0.4741	1.42	0.0009
		J. slatteryi	4	2	0.5000	0.5623	1.60	0.0053
	Tomales Bay	*J. marmorata*	39	3	0.4845	0.8262	1.89	0.0018
		J. slatteryi	11	5	0.7636	1.3667	3.27	0.0238
	Humboldt Bay	*J. marmorata*	6	1	0.0000	0.0000	1.00	0.0000
	Coos Bay	*J. marmorata*	31	3	0.1269	0.2839	1.14	0.0002
	Puget Sound	*J. marmorata*	1	1	0.0000	0.0000	1.00	0.0000
		J. staudei	25	8	0.8133	1.7551	4.56	0.0105
	Charleston Harbor, SC	*Jassa* sp.	18	3	0.3922	0.6837	1.59	0.0022
	Millstone Point, CT	*J. marmorata*	17	1	0.0000	0.0000	1.00	0.0000
***A. valida* groups**	San Francisco Bay	Clade A	4	1	0.0000	0.0000	1.00	0.0000
		Clade B	63	7	0.1828	0.4851	1.22	0.0003
		total	67	8	0.2754	0.6823	1.37	0.0053
	Tomales Bay	Clade C	31	4	0.1871	0.4243	1.22	0.0050
	Humboldt Bay	Clade A	32	2	0.0625	0.1391	1.06	0.0085
		Clade C	1	1	0.0000	0.0000	1.00	0.0000
		total	33	3	0.1193	0.2706	1.13	0.0110
	Coos Bay	Clade C	15	2	0.1333	0.2449	1.14	0.0099
	Yaquina Bay	Clade C	9	1	0.0000	0.0000	1.00	0.0000
	Willapa Bay	Clade A	1	1	0.0000	0.0000	1.00	0.0000
		Clade C	95	6	0.1227	0.3341	1.14	0.0052
		total	96	7	0.1410	0.3886	1.16	0.0061
	Grays Harbor	Clade C	7	2	0.2857	0.4101	1.32	0.0004
	Puget Sound	Clade C	56	4	0.1052	0.1814	1.15	0.0002
	Charleston Harbor, SC	Clade C	7	2	0.2857	0.4101	1.32	0.0004
	Millstone Point, CT	Clade C	18	1	0.0000	0.0000	1.00	0.0000

Table 5. COI genetic distances for *Jassa*. Ranges for intraspecific distance are shown on the diagonal; interspecific distances are shown above the diagonal.

	Jassa sp.	*J. marmorata*	*J. slatteryi*	*J. staudei*
Jassa sp.	0.2-1.1%	14.0-15.2%	15.8-17.4%	17.6-18.8%
J. marmorata		0.2-0.6%	14.6-16.1%	15.9-17.2%
J. slatteryi			0.2-1.1%	16.0-17.8%
J. staudei				0.2-1.6%

Table 6. COI genetic distances for *Ampithoe*. Ranges for intra-clade distance are shown on the diagonal; inter-clade distances are shown above the diagonal. Only a single haplotype was considered for *A. longimana*.

		A. valida			
	Clade A	Clade B	Clade C	*A. lacertosa*	*A. longimana*
Clade A	0.2-0.4%	4.6-4.9%	4.4-5.5%	19.6-20.4%	23.1-23.4%
Clade B		0.2-0.3%	4.1-4.8%	18.0-18.7%	22.6-22.9%
Clade C			0.2-0.3%	18.4-19.7%	21.5-21.9%
A. lacertosa				0.2-0.8%	21.6-22.1%
A. longimana					n/a

Figure Captions.

Figure 1. Qualitative comparison of secondary gnathopods of major males and juveniles of *Jassa*, illustrating differences in these diagnostic morphological characteristics between life stages. These gnathopods are used in male-male competitions for mating dominance and have species-specific morphologies based on the size, shape, and angle of the "thumb" (marked with arrows in A) major male *J. marmorata*, and B) major male *J. slatteryi*). In juveniles, the morphological distinction between species (C) juvenile *J. marmorata*; D) juvenile *J. slatteryi*; E) juvenile *J. staudei*) is less apparent as the "thumb" does not appear until the final moult .

Figure 2. Bayesian phylogram based on *Jassa* species COI haplotypes. Numbers before each node represent 1000 bootstrap support pseudoreplicates for neighbour-joining and maximum parsimony analyses, and posterior probabilities from Bayesian analysis, respectively. The node marked with a small asterisk was collapsed in the most parsimonious strict consensus tree. The haplotypes marked with large asterisks were found only in *Jassa* specimens collected along the Atlantic coast of North America.

Figure 3. Haplotype distribution in *Jassa* species along the Pacific Coast of North America (A) and the Atlantic Coast (B). Blue, *J. marmorata*; green, *J. slatteryi*; red; *J. staudei*; orange, unidentified *Jassa sp*. The dark blue portions of the *J. marmorata* populations represent the frequencies of the dominant shared haplotype. The light blue segments represent less frequent haplotypes that were part of the *J. marmorata* clade, and the medium blue marks the haplotype found only on the Atlantic Coast. The dark green segments of the *J. slatteryi* populations represent the frequencies of the dominant shared haplotype. The lighter green portions are private haplotypes at those localities.

Figure 4. Bayesian phylogram based on *Ampithoe* species COI haplotypes. Numbers before each node represent 1000 bootstrap support pseudoreplicates for neighbour-joining and maximum parsimony analyses, and posterior probabilities from Bayesian analysis, respectively. The haplotype marked with the asterisk is the dominant Clade A haplotype that was found in samples from both the Atlantic and Pacific coasts of North America. The haplotype marked with the cross was only found on the Pacific coast while the haplotype marked with the double cross was found only on the Atlantic coast.

Figure 5. Haplotype distribution in *Ampithoe* lineages along the Pacific Coast of North America (A) and the Atlantic Coast (B). Orange, Clade A; green, Clade B; blue, Clade C. The darker orange portions represent the frequencies of the dominant shared haplotype for Clade A. The lighter orange segments are private haplotypes to those localities. The green portions represent the haplotypes of Clade B. The dark blue portions represent the frequencies of the dominant shared haplotype for Clade C. The lighter blue portions are private haplotypes at those localities.

Figure 1.

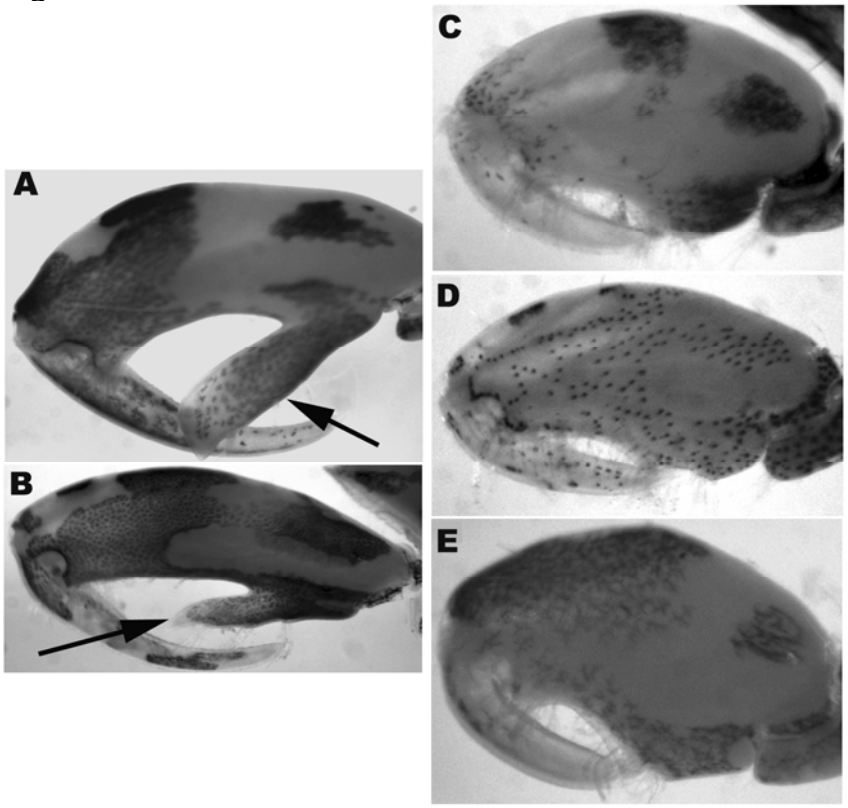


Figure 2.

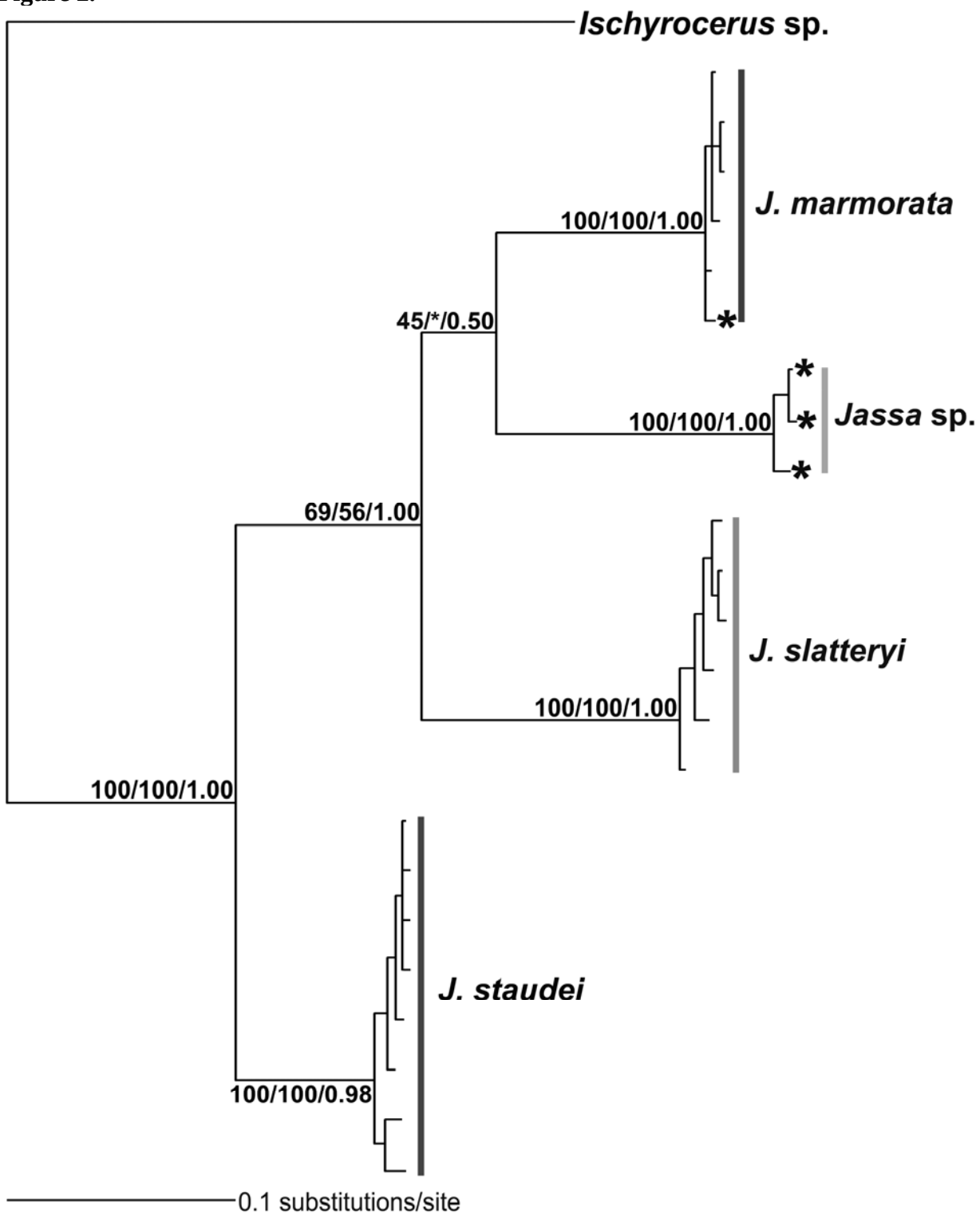

Ischyrocerus sp.
J. marmorata
100/100/1.00
45/*/0.50
Jassa sp.
100/100/1.00
69/56/1.00
J. slatteryi
100/100/1.00
100/100/1.00
J. staudei
100/100/0.98
0.1 substitutions/site

Figure 3.

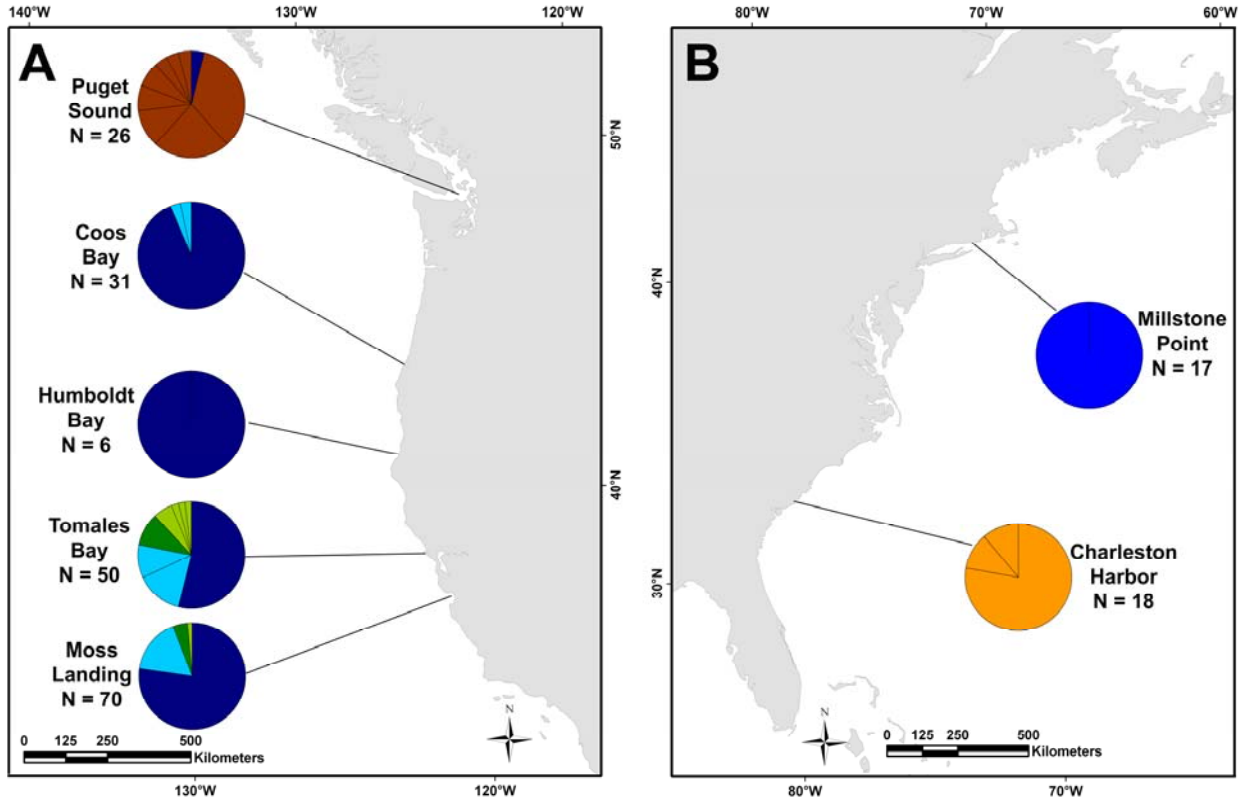

A
Puget Sound N = 26
Coos Bay N = 31
Humboldt Bay N = 6
Tomales Bay N = 50
Moss Landing N = 70
140°W
130°W
120°W
50°N
40°N
0 125 250 500
Kilometers
B
Millstone Point N = 17
Charleston Harbor N = 18
80°W
70°W
60°W
40°N
30°N
0 125 250 500
Kilometers

Figure 4.

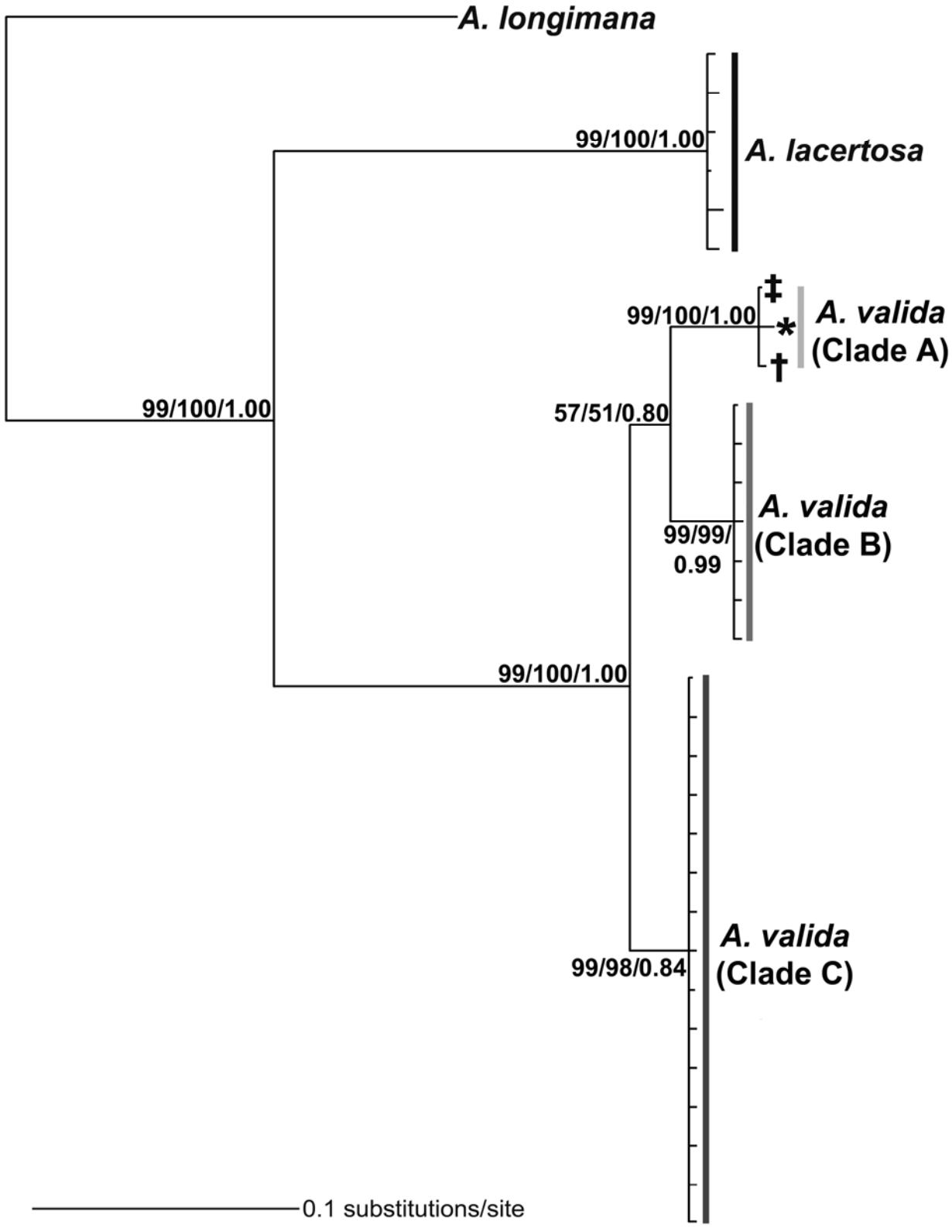
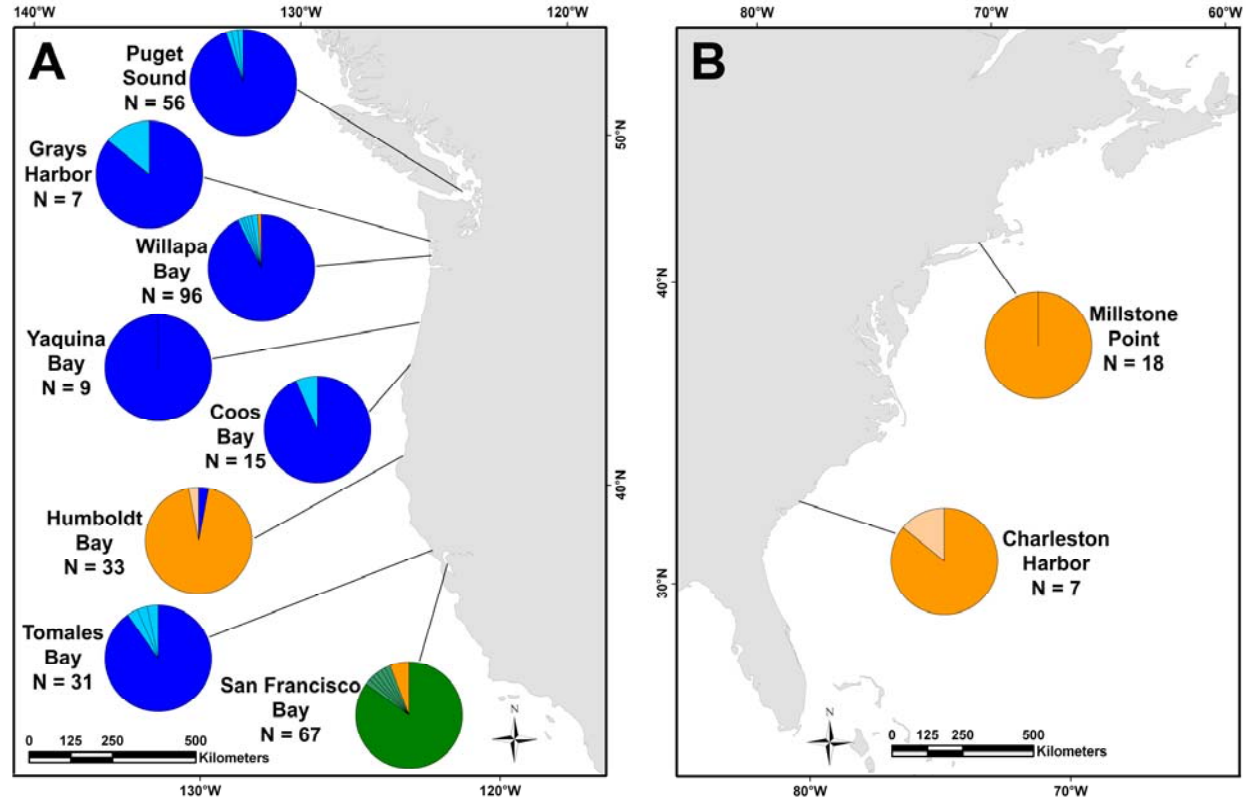

A. longimana
99/100/1.00
A. lacertosa
99/100/1.00
‡
*
†
A. valida
(Clade A)
99/100/1.00
57/51/0.80
A. valida
(Clade B)
99/99/
0.99
99/100/1.00
A. valida
(Clade C)
99/98/0.84
0.1 substitutions/site

Figure 5.


A
Puget Sound N = 56
Grays Harbor N = 7
Willapa Bay N = 96
Yaquina Bay N = 9
Coos Bay N = 15
Humboldt Bay N = 33
Tomales Bay N = 31
San Francisco Bay N = 67
140°W
130°W
120°W
50°N
40°N
0 125 250 500
Kilometers
B
Millstone Point N = 18
Charleston Harbor N = 7
80°W
70°W
60°W
40°N
30°N
0 125 250 500
Kilometers