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Molecular identification of two species of the carnivorous sea slug *Philine*, invaders of the US west coast

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Abstract Open-coast and deep-water ecosystems have been less disrupted by invasion than estuaries, but a notable exception is the establishment of multiple species of the sea slug genus *Philine* in the northeastern Pacific. These large slugs spread from San Francisco Bay to the whole of the US west coast in 5 years during the 1990s, and are abundant from intertidal mudflats to soft-sediment bottoms >300 m deep along the open coast. Voracious predators that secrete acid, *Philine* spp. have few natural enemies and substantial impacts on native bivalve communities. Despite their ecological significance, the identity and number of invasive *Philine* spp. in the US has remained controversial. Here, we adopt a molecular approach to identify the species commonly found along the US west coast. We compared mitochondrial 16S gene sequences of reference specimens from the native range of seven possible invaders against sequences of 66 specimens collected from southern California to Oregon from intertidal and subtidal habitats. All slugs from southern California and Oregon were confirmed as *P. auriformis*, and most shared haplotypes with samples from New Zealand. Larger slugs from San Francisco, Tomales Bay and Bodega Harbor, believed to represent 3–4 species, were all identified as *P. orientalis*. Molecular data support a recent morphological analysis that erected two proposed species, *P. paucipapillata* from Hong Kong and *P. quadripartita* from Europe, which are genetically distinct from anatomically similar species. For taxonomically challenging groups like sea slugs, genetic data can illuminate cryptic invasions and provide a backdrop for further studies of the ecological impacts of introduced marine species.

Keywords DNA bar-coding · Heterobranch · Marine invasion · Opisthobranch · Philine · Sea slug

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Introduction

Accurate identification of introduced species is critical to determine sources, vectors of introduction, and the ecological and genetic characteristics that facilitate invasion success (Holland 2000; Martel et al. 2004; Roman 2006; Lindholm et al. 2005; Stepien and Tumeo 2006). Taxonomic uncertainty in groups such as marine invertebrates impedes our ability to

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recognize invasions and identify non-native species or highly invasive strains (Carlton 1996a, b; Bax et al. 2001). Failure to recognize the establishment of alien species has obscured the historical record and impaired our understanding of biological invasion in the marine realm (McDonald and Koehn 1988; Geller 1999). The ecological disruption and economic costs of marine invasions are evident in ports such as San Francisco Bay, which contains representatives of >85 % of the invasive marine invertebrate and algal species known from western North America (Cohen and Carlton 1995, 1998; Pimentel et al. 2000; Ruiz et al. 2000).

Molecular methods offer powerful tools for identifying species and strains, and may compensate for a lack of taxonomic expertise and resolve disagreements among specialists. Genetic studies have successfully uncovered cryptic invasions and identified sources for diverse marine organisms (Geller et al. 1997; Hanfling et al. 2002; Holland et al. 2004; Voisin et al. 2005; Bastrop and Blank 2006; Andreakis et al. 2007; See and Feist 2010). Molecular approaches also offer great promise in recognizing or detecting microscopic larvae in ballast water, due to the species-specificity and sensitivity achievable with polymerase chain-reaction (PCR) methods (Deagle et al. 2003). In particular, DNA bar-coding approaches have been introduced to screen preemptively for alien pests, and may allow rapid identification in groups where taxonomy is unclear and experts cannot unambiguously identify specimens of unknown origin; however, effective use of bar-coding requires reference sequences from candidate invaders against which invasives can be compared (Armstrong and Ball 2005; Darling and Blum 2007; Saunders 2007).

Sea slugs (or opisthobranchs) are marine heterobranch molluscs with reduced or absent shells, and consequently lack many characters used in gastropod taxonomy. As a result, the systematics and taxonomy of many sea slug groups remains unstable, and morphological criteria alone are often insufficient to confirm species identity (Hirano and Hirano 1991; Morrow et al. 1992; Sisson 2002; Krug et al. 2007, 2008). The inability to identify sea slugs is also an applied problem, as species from several groups are successful marine invaders. Aeolid nudibranchs that feed on fouling community taxa are transported on boat hulls, and at least five species have been introduced to ports on the US west coast (Carlton 1979; Behrens 1991; Gosliner 1995). One herbivorous species in clade Sacoglossa, an unidentified *Costasiella*, has recently colonized bays near Miami, Florida (A. DuPont and P. Krug, unpubl. data). A member of the shelled clade Cephalaspidea was described as *Haminoea calledigenita* (Gibson and Chia 1989) from Washington State, USA, but was later identified as an invasive population of *H. japonica*, an invader now found in San Francisco Bay and Europe (Gosliner and Behrens 2006). Molecular approaches are therefore warranted to study the invasion success of this taxonomically challenging group.

Estuaries are especially prone to invasion, due largely to their fluctuating conditions and human use as ports (Lavoie et al. 1999; Grosholz 2002). In contrast, invasions of open coast ecosystems are comparatively rare. An exception is the explosive invasion of the US west coast in the mid-90s by sea slugs in the genus *Philine*, which occur from intertidal mudflats to depths below 300 m (Cadien and Ranasinghe 2003). *Philine* spp. are large carnivorous sea slugs that secrete an acidic discharge making them unpalatable to predators (Chow 2001). Non-native *Philine* spp. were first detected in San Francisco Bay in 1992 (Gosliner 1995). Invasive *Philine* were absent from trawls and benthic grabs in coastal waters and ports of the Southern California Bight (SCB) from 1972–1993, first appearing in Ventura, CA in 1994, in Los Angeles and San Diego, CA in summer 1995, and in Coos Bay, Oregon in 1998 (Goddard 1998; Cadien and Ranasinghe 2003). *Philine* specimens in the SCB reached densities of up to 140 slugs/m^2 at depths of 25–305 m, and were the 7th most-sampled invertebrate in the port of Los Angeles in 1998. Abundance of *Philine* fluctuated in the SCB after establishment but were near historic highs in 2001, the last year for which sampling data were published (Cadien and Ranasinghe 2003). Densities of small infaunal bivalves, the preferred prey of *Philine*, declined by two orders of magnitude in parts of the SCB following the establishment of *Philine* spp., highlighting the ecological impact of this ongoing invasion (Cadien and Ranasinghe 2003). Gut content analyses revealed that non-indigenous *Philine* are flexible consumers that eat foraminifera, gastropods and ophiuroids when bivalves are scarce, meaning this invasion could have community-wide impacts that are not yet appreciated.

Based on morphology of the gizzard plates, radular teeth, internal shell, and anatomical features, Gosliner

(1995) identified invasive slugs in San Francisco Bay as *P. auriformis* (Suter 1909), described from New Zealand. However, this identification was challenged by Rudman (1998), who argued the radula and gizzard plates were half as large as corresponding features from comparably-sized New Zealand slugs. It remains a point of contention among experts whether the first reported invasives are truly *P. auriformis*. Gosliner (1998) subsequently reported 2–3 additional *Philine* spp. had invaded northern California, tentatively identified as *P. orientalis* (Bodega Bay), *P. japonica* (Tomales Bay), and *P. aperta* (San Francisco Bay). However, taxonomic uncertainty plagued these identifications, as all three species have similar gizzard plates and the extent of intra-specific variation in key traits is unknown. A recent revision of *Philine* based on morphology synonymized *P. orientalis* and *P. japonica*, described anatomically similar specimens from Hong Kong as *P. paucipapillata*, and resurrected *P. quadripartita* as the correct name for *P. aperta*-like animals from Europe (Price et al. 2011). However, it remains unclear which of these species are actually invasive in the US.

Given the ecological impact of introduced *Philine* spp. and the taxonomical controversies surrounding these invasives, we adopted a DNA bar-coding approach to identify which species are present in the USA. We compared a portion of the mitochondrial 16S gene from specimens collected in California and Oregon to sequences of candidate species sampled from their native range: *P. auriformis* and *P. angasi* from New Zealand and Australia, *P. aperta* from South Africa, *P. quadripartita* Europe, *P. orientalis* from Japan, the recently named *P. paucipapillata* from Hong Kong, and an undescribed *Philine* sp. from Australia. Our results confirmed the identity of two invasive species, and provide a baseline of sequence data against which future specimens can be compared to determine the taxonomic breadth of this ongoing invasion.

Materials and methods

Collection of organisms

Specimens were collected alive by the authors or colleagues and stored in 95–100 % ethanol, or obtained from the Natural History Museum (London), prior to extraction (Table 1). Unknown invasives (Fig. 1) were collected in southern California from intertidal mudflats (Anaheim Bay and the mudflat adjacent to Long Beach Harbor), and during shallow-water or deep-water trawls of the Los Angeles Harbor in 2005–2006. Samples from Tomales Bay, Bodega Harbor, San Francisco Bay (California), and Coos Bay (Oregon), were collected intertidally in 2008, except for specimens from south San Francisco Bay that were trawled in 1999 (Chow 2001).

Native specimens of *P. auriformis* (n = 3) and *P. angasi* (n = 6) were sampled intertidally from two sites in New Zealand in 2005, and three specimens of *P. aperta* were collected from South Africa in 2008. Material similar in appearance to *P. orientalis* (n = 5; hereafter termed "*Philine* sp.") was collected from the Northern Territories, Australia in 2006 by R. Willan. Current evidence supports the existence of only one large-bodied, shallow-water species of *Philine* in Japan. From the British Museum of Natural History (BMNH), we obtained tissue from a specimen that had been identified as *P. japonica* from Nagasaki, Japan, which is herein termed *P. orientalis* due to their recent synonymy; the same material that we sequenced was used in the morphological analysis of Price et al. (2011). A specimen of *P. paucipapillata* from Hong Kong and three unidentified slugs from western Australia were also obtained from the BMNH and used in molecular analyses. Sequenced individuals that we collected, and additional specimens collected but not sequenced, were deposited in the invertebrate zoology collection of the California Academy of Sciences; voucher identification numbers are given in Supplemental Table 1.

DNA sequencing

From specimens preserved in 95 % ethanol, genomic DNA was extracted with a QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA) and stored in extraction buffer at −20 °C. Polymerase chain reactions (PCR) were used to amplify a portion of the mitochondrial large ribosomal subunit (16S) rRNA gene using primers 16Sar-5′ and 16Sbr-3′ (Palumbi 1996). We used 16S because universal primers did not reliably amplify the cytochrome *c* oxidase I gene, the more commonly used barcoding locus, from *Philine* spp. The 16S locus is variable enough to distinguish among

Table 1 Collection localities and information for specimens in the genus *Philine* sampled in the present study

Species	Location	Collector	Date	*n*
P. angasi	Parengarenga Harbor, NZ	M. Morley	9/20/2005	2
	Wattle Bay, NZ	M. Morley	10/17/2005	4
(*P. angasi*)	Rottnest Island, Perth, Western Australia, Australia	J. D. Taylor E. A. Glower	1/1996	3
P. aperta	Long Beach, Simonstown, False Bay, South Africa	T. Gosliner	1/6/2008	2
P. auriformis	Farm Cove, Auckland, NZ	M. Morley	8/31/2005	3
(***P. auriformis***)	Coos Bay, Oregon, USA	J. Goddard	9/23/1998	3
	Santa Barbara Point, CA	J. Goddard	11/1/2000	1
	Los Angeles Harbor, CA:	J. Asif		
	Shallow-water trawl (~5 m)		5/6/2006	6
	Deep-water trawl (~20 m)		5/6/2006	6
	Long Beach harbor, CA	B. Pernet	4/29/2006	6
	Anaheim Bay mudflat, CA	B. Pernet	6/2005	6
P. orientalis	Nagasaki, Japan	Leningrad Acad. of Sci.	3/29/1897	1
(***P. orientalis***)	Tomales Bay, CA:	T. Gosliner		
	Shell Beach		10/2007	3
	Inverness		10/2007	3
	San Francisco Bay, CA:	T. Gosliner		
	San Mateo (south Bay)		5/5/2007	2
	China Camp (north Bay)		4/7/2007	1
Philine sp.	Darwin Harbor, Northern Territory, Australia	R. Willan	8/12/2006	5
P. paucipapillata	Mirs Bay, Hong Kong	N. J. Morris	1983	1
P. quadripartita	Murcia, Spain	C. Grande unknown	1	

Samples in parentheses were identified to species in the present study; *bold* samples are from invaded sites

NZ New Zealand, *CA* California, USA, *BMNH* British Natural History Museum, *N* number of slugs sequenced

species and is phylogenetically informative in diverse marine heterobranch clades, and was thus an appropriate alternative marker for species delimitation in this study (Turner and Wilson 2007; Anthes et al. 2008; Krug et al. 2012).

Reaction conditions for mtDNA amplifications followed Ellingson and Krug (2006), using a solution of 1× Promega PCR buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 0.1 % Triton X-100), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer, and 1 U Promega Taq DNA Polymerase (Promega Corporation, Madison, WI). Total reaction volume was 50 μL, of which 2 μL was template DNA (1 % of total DNA extracted). PCR was run on a thermocycler heated to 95 °C prior to loading samples; the profile started with an initial denaturation step of 95 °C for 5 min, followed by 40 cycles of a 20 s denaturation step at 95 °C, a 30 s annealing step at 55 °C, and a 70 °C extension step for 60 s, with a final extension at 70 °C for 10 min. A negative control (no template) was included in each reaction.

Products of PCR were visualized by electrophoresis on a 1 % agarose gel and purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). Purified products were directly cycle-sequenced in both directions using PCR primers and Big Dye Terminator 3.1 Cycle Sequencing chemistry, and electrophoresed on an ABI 3100 Avant Capillary Sequencer (Applied Biosystems, Foster City, CA). Total volume of the sequencing reaction was 10 μL, including 3 μL of water, 2 μL of template DNA, 2 μL of ABI sequencing buffer (Applied Biosystems, Foster City, CA), 1 μL of 5 μM primer, and 2 μL Big Dye Terminator v3.1. After an initial denaturation step of 2 min at 96 °C, each sample was cycle-sequenced using 20 cycles of the following thermal cycle profile: 30 s at 95 °C, followed by 40 s at 55 °C. One final step of 5 min at 55 °C was added to complete extension.

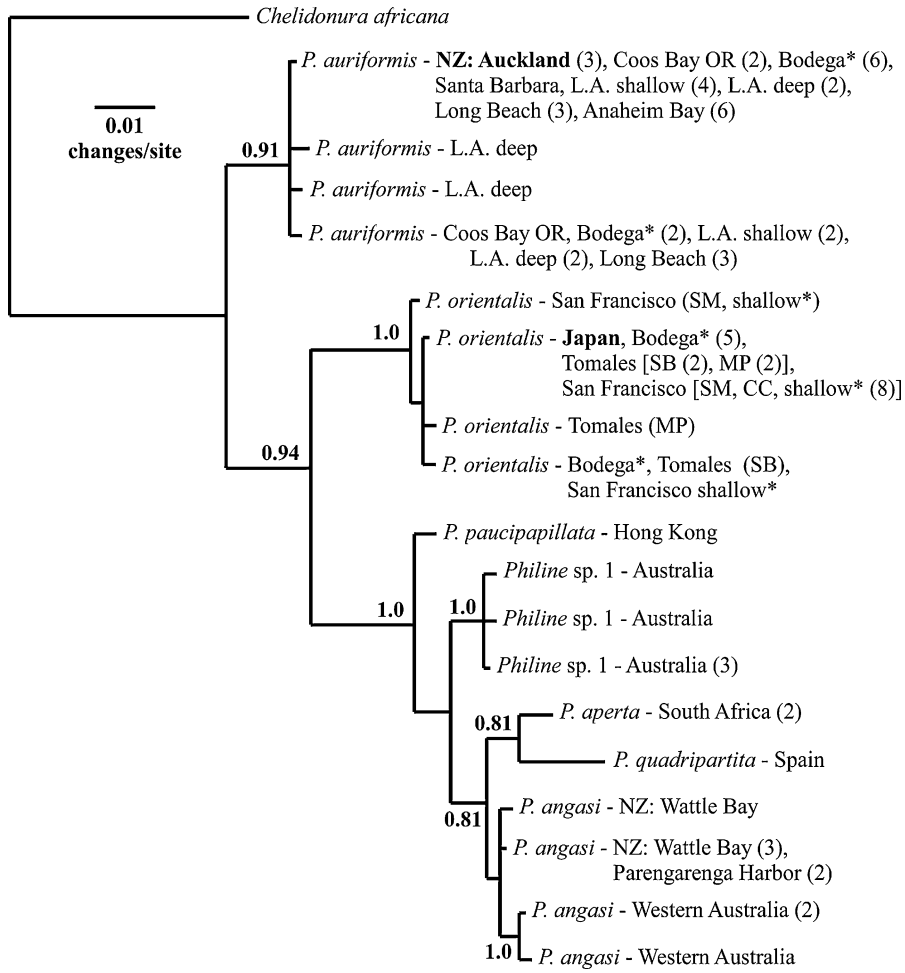

Fig. 1 Species identifications and evolutionary relationships among invasive and native *Philine* spp. Relationships among haplotypes of the mitochondrial large ribosomal subunit (16S) gene were inferred using Bayesian Inference, and are depicted as a 50 % majority-rule consensus phylogram with mean branch lengths (pooled from four independent MCMC analyses). Posterior probabilities above 0.8 are given to the left of supported nodes. Locations from which a given haplotype was sampled are given, with sampling frequency in parentheses if multiple specimens shared a haplotype. Native-range sampling sites of invasive haplotypes are *bolded*. *Asterisks* denote sites where a shorter version of the indicated haplotype was sampled by Chow (2001). Sites within Tomales Bay are Shell Beach (SB) and Inverness (IN); sites in San Francisco Bay are China Camp state beach (CC), San Mateo (SM), or a south-bay trawl at 4 m depth ("shallow"). Los Angeles Harbor trawls are given as shallow or deep

Phylogenetic analyses

Alignments were initially done in ClustalX (Thompson et al. 1997) using default parameters, and adjusted by eye using a model of 16S secondary structure based on published secondary structures for gastropods, to maintain base-pairing interactions in stem regions (Lydeard et al. 2000; Medina and Walsh 2000; Valdés 2003); alignments are available from PJK upon request. Aligned sequences of 413 bp were obtained for all but one taxon; NCBI accession numbers are given in Supplemental Table 1. The ends of a South African haplotype that was slightly shorter than other haplotypes were coded as missing data. Unique haplotypes were identified with Collapse 1.2 (Posada 2004). Among ingroup sequences, four positions in the alignment had indels of 1–2 bp which were coded as gaps.

Unpublished shorter 16S sequences (307 bp) were obtained for putative *P. auriformis* from San Francisco Bay (n = 8), *P. orientalis* from Bodega Harbor (n = 9), and *P. aperta* from a trawl of south San

Francisco Bay (n = 10) (Chow 2001). All shorter sequences were identical to longer haplotypes recovered from our samples, and the 3′ region missing from these shorter sequences was invariant across all species in our 413 bp alignment. We therefore collapsed the shorter haplotypes with our longer sequences to estimate the frequency of each haplotype across populations and to identify the samples in Chow (2001).

Evolutionary relationships of 16S haplotypes were reconstructed to determine the identity of invasives and the genetic distance within and among species, critical for effective bar-coding identifications (DeSalle et al. 2005; Meyer and Paulay 2005). No closely related genera from family Philinidae were available, so the cephalaspidean *Chelidonura africana* (family Aglajidae) was used as an outgroup; in preliminary phylogenetic analysis, *Chelidonura* grouped more closely to *Philine* than representatives of the aglajid genera *Philinopsis* and *Melanochlamys*. The 16S sequence of *C. africana* was downloaded from GenBank (AY098930). Relationships among haplotypes were inferred using Bayesian Markov Chain Monte Carlo (MCMC) methods, with a mixture model implemented in BayesPhylogenies software (Pagel and Meade 2004; www.evolution.rdg.ac.uk/BayesPhy.html). Mixture models allow rate heterogeneity among sites without partitioning data a priori, using a likelihood approach to assign one of a user-specific number of general time-reversible models with among-site rate heterogeneity to each position. Model parameters and base frequencies were estimated from the data during MCMC runs.

Preliminary analyses indicated that one mixture model with four gamma-distributed rates was sufficient to capture the pattern of sequence evolution in our data; adding a second model did not improve log-likelihood scores. Following Pagel and Meade (2004), we performed four replicate runs of one Markov chain apiece, each lasting 5×10^6 generations. The posterior distribution was sampled by saving a tree every 10^3 generations for the final 10^6 generations, from which the harmonic mean of log-likelihood scores was calculated and nodal support estimated by constructing a 50 % majority-rule consensus tree in the program BayesTrees (http://www.evolution.reading.ac.uk). All four chains converged independently on the same region of tree space based on the posterior distribution of trees, and all likelihoods were within one log-unit; the last 10^3 trees from all four runs were therefore combined, and a consensus tree with mean branch lengths generated. Posterior probability values ≥90 % were taken as statistical support for a clade (Douady et al. 2003; Huelsenbeck and Rannala 2004; Simmons et al. 2004).

To determine whether there was any overlap in genetic distance within versus between putative species groups, mean pairwise maximum likelihood genetic distances were calculated in Mega 5.0 (Tamura et al. 2011) for collapsed, full-length haplotypes, excluding shorter sequences from Chow (2001). Mean ML distance between haplotypes was determined for each species pair based on phylogenetic analysis. Mean ML distance among haplotypes within each species was determined for the four taxa represented by multiple 16S haplotypes, treating New Zealand and Australian *P. angasi* as conspecific (see "Results").

An unrooted statistical parsimony network also was generated for 16S haplotypes from invasive populations of (A) *P. auriformis*, and (B) *P. orientalis* (see "Results"), using a 95 % parsimony criterion in TCS v1.21 (Clement et al. 2000). Unlike phylogenetic reconstructions, which posit extinct ancestral nodes from which terminal sequences are descended, parsimony networks infer mutational connections among extant haplotypes. Invasions that spread following an initial bottleneck are expected to produce haplotype networks with a characteristic star-shape reflecting an abrupt increase in coalescence rate, with a common haplotype in the central (ancestral) position connected to rarer descendant haplotypes that differ by only 1–2 mutational steps (Galtier et al. 2000). Distributions of pairwise sequence differences (mismatches) are similarly expected to be more unimodal in a rapidly expanding population than in a population at equilibrium (Rogers and Harpending 1992); departures from equilibrium expectations were modeled in Arlequin v3.5 (Excoffier and Lischer 2010) and tested using a parametric bootstrap procedure with 1000 pseudoreplicates.

Results

Identifying invasive *Philine* spp.–*P. auriformis*

All invasive *Philine* specimens from southern California and Oregon were identified genetically as *P. auriformis*, native to New Zealand and Australia (Fig. 1). One 16S haplotype was shared by three

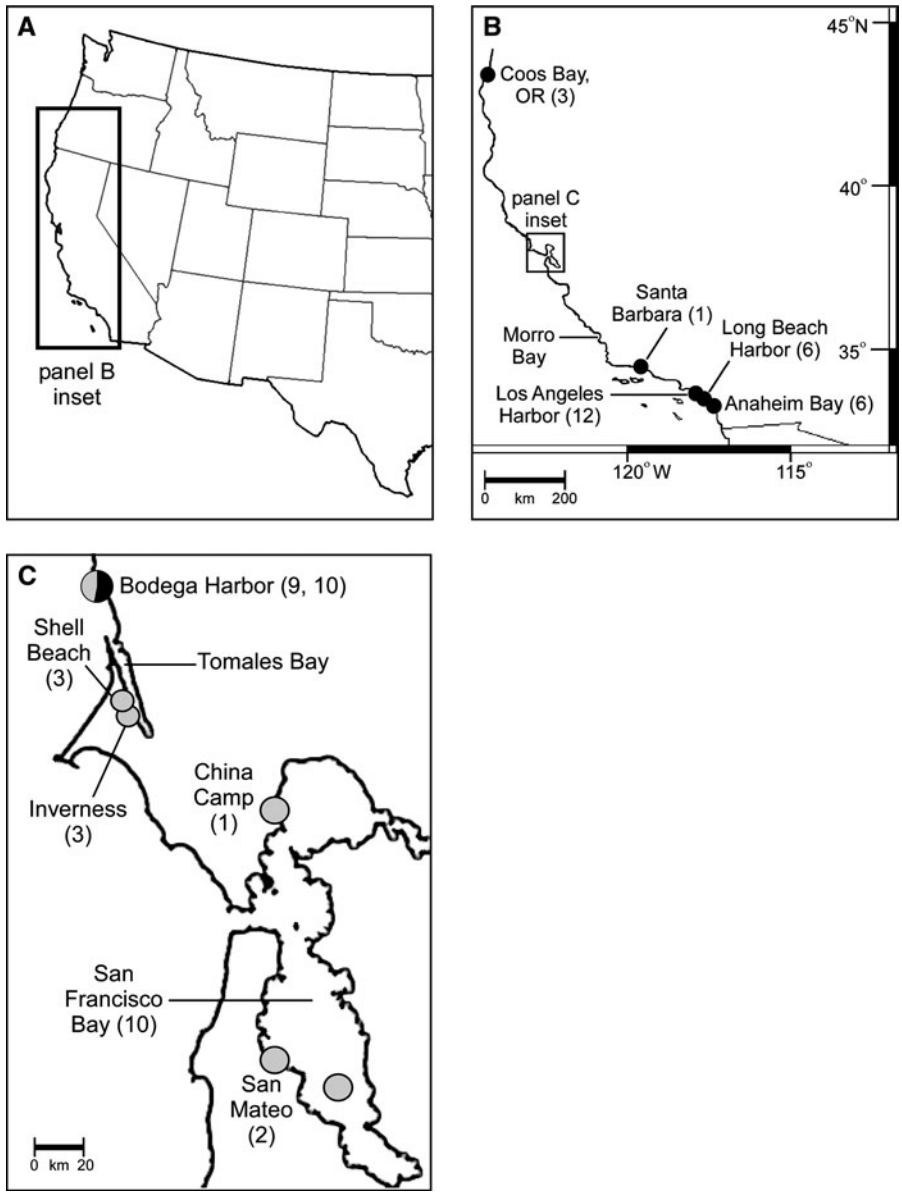

Fig. 2 Sites invaded by *Philine* spp. along the US west coast. Pie graphs indicate the proportion of sampled slugs that were identified as *P. auriformis* (*black*) or *P. orientalis* (*grey*), with the corresponding sample size. **a** Pacific coast of the United States where invasive *Philine* spp. were sampled, with inset showing region expanded in (**b**). **b** Sampling frequency of *P. auriformis* along the US west coast, outside of the inset area. Specimens of *P. auriformis* were previously reported to occur in Morro Bay, indicated without a *circle*. **c** Close-up of the inset region in (**b**), showing the proportion of each *Philine* spp. sampled from northern California. The *circle* in south San Francisco Bay represents samples caught by a shallow-water trawl (Chow 2001)

specimens of *P. auriformis* from Auckland, New Zealand, two slugs from Coos Bay, Oregon, and a majority of specimens from southern California (Fig. 1). A second common haplotype was sampled once in Coos Bay and multiple times from Los Angeles and Long Beach harbors. Two related haplotypes were sampled once each from a deep-water trawl of Los Angeles Harbor. All four *P. auriformis* haplotypes formed a clade with significant support (PP = 0.91); mean divergence among conspecific haplotypes was 0.3 % (ML distance). Shorter versions of both common haplotypes were sampled multiple times in Bodega Harbor by Chow (2001). The haplotype shared between native and all invasive populations unambiguously confirms the identity of this invader, now widespread along the US west coast (Fig. 2a,b).

The most common 16S haplotype among invasive *P. auriformis* occupied the ancestral position in a statistical parsimony network, which exhibited the

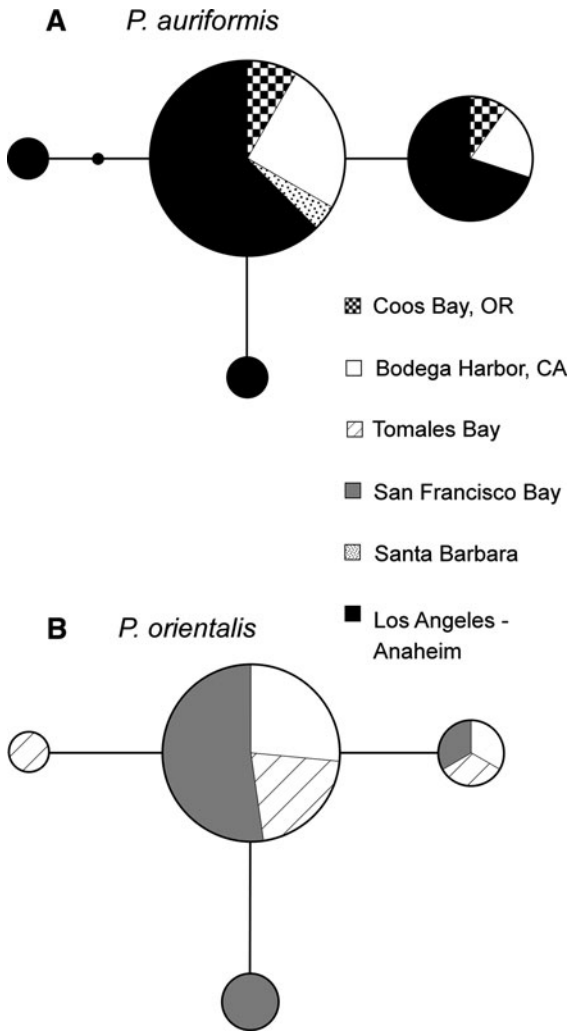

Fig. 3 Statistical parsimony network of 16S haplotypes from invasive populations of **a** *Philine auriformis*, and **b** *P. orientalis*. *Circle* area is proportional to the frequency of occurrence for each haplotype along the US west coast, with the small *black circle* representing a putative un-sampled haplotype. Line segments represent one mutational change. Sampling sites in southern California (Los Angeles Harbor, Long Beach Harbor, Anaheim Bay) were pooled into one regional population coded in *black*

star-shaped pattern characteristic of a recent bottleneck (Fig. 3a). Both common haplotypes were sampled at similar frequencies across the introduced range of *P. auriformis*. Mismatch distributions were unimodal and left-skewed compared to expected results, consistent with a rapid population expansion, although deviations were not significant given the limited signal in our 16S dataset ($P = 0.20$).

Identifying invasive *Philine* spp.–*P. orientalis*

The reference sample of *P. orientalis* from Japan belonged to a highly supported clade (PP = 1.0) that otherwise comprised all specimens from San Francisco and Tomales bays, and some samples from Bodega Harbor (Fig. 1). Mean ML distance among haplotypes was 0.2 %. The Japanese haplotype was sampled four times in Tomales Bay and twice in San Francisco Bay (China Camp in the north bay, San Mateo in the south bay). A shorter version of the common haplotype was also shared by five slugs from Bodega Harbor and eight of ten slugs collected by otter trawl at ~4 m depth from south San Francisco Bay. A related haplotype was sampled three times, once each in Tomales Bay, Bodega Harbor, and San Francisco Bay; two other haplotypes differing by a single substitution were sampled once or twice from a single site. In contrast to its more widespread congener, *P. orientalis* was not sampled outside of northern California between Bodega and San Francisco (Fig. 2b,c).

The most common haplotype in *P. orientalis* was identified as the likely ancestor of the other three haplotypes sampled from invasive populations in a statistical parsimony network (Fig. 3b). The two haplotypes shared among multiple specimens of *P. orientalis* were sampled at all three sites in northern California (Fig. 3b). Mismatch distributions were unimodal and left-skewed compared to equilibrium expectations, although deviations were not significant ($P = 0.16$).

Relationships and genetic distances among *Philine* spp

The remaining sequences formed a highly supported clade that was sister to *P. orientalis* (Fig. 1). Three haplotypes from an unidentified *Philine* sp. from northern Australia formed a highly supported clade (PP = 1.0). The specimen of *P. paucipapillata* from Hong Kong was genetically distinct from all other species (1.5–5.3 % ML distance; Table 2), supporting morphological evidence that Hong Kong specimens comprise a cryptic species. The nominal *P. aperta* sequence from Spain in the NCBI database was genetically distinct (2.3 % ML distance) from the haplotype shared by South African specimens of *P. aperta*, which supports the resurrection of

Table 2 Mean within- versus between-species ML genetic distances for 16S haplotypes in the genus *Philine*

	P. angasi	*P. aperta*	*P. quadripartita*	*Philine* sp. 1	*P. paucipapillata*	*P. orientalis*	*P. auriformis*
P. angasi	**0.0035**						
P. aperta	0.0147	–					
P. quadripartita	0.0260	0.0227	–				
Philine sp. 1	0.0173	0.0214	0.0322	**0.0020**			
P. paucipapillata	0.0162	0.0210	0.0351	0.0148	–		
P. orientalis	0.0461	0.0447	0.0446	0.0525	0.0432	**0.0023**	
P. auriformis	0.0570	0.0589	0.0591	0.0529	0.0490	0.0456	**0.003**

Bold values on the diagonal are mean genetic distances among haplotypes within a species, with dashes indicating taxa with only one sampled haplotype

ML maximum likelihood

P. quadripartita as the name of the European species. No invasive haplotypes matched any of these four species, despite suggestions that one or more may have colonized the northeastern Pacific.

Five of six specimens of *P. angasi* from Wattle Bay, New Zealand, shared a haplotype that was closely related to that from the 6th specimen, and also to a clade of two haplotypes from unidentified specimens from Rottnest Island in western Australia (Fig. 1). Although New Zealand *P. angasi* and Australian *P. angasi* haplotypes did not receive significant support as a clade, mean distance between haplotypes was only 0.58 %, well under the lowest between-species divergence (Table 2); given the geographical separation of the western Australia populations, these specimens likely are a slightly divergent population of *P. angasi*.

There was no overlap in the mean genetic distance between haplotypes within a species versus the mean distance between species (Table 2). Within species, mean genetic distance among haplotypes ranged from 0.15–0.35 % (pooling New Zealand and Australian *P. angasi*). Among our study taxa, the maximum intra-specific divergence between 16S haplotypes was 1.0 % for *P. aperta* from New Zealand and western Australia; most pairwise distances among conspecific haplotypes were less than 0.5 %. In contrast, mean genetic distance between species ranged from 1.5 to 5.9 %.

Discussion

Molecular analyses confirm that *Philine auriformis*, native to New Zealand and Australia, now is a widespread invader along the US west coast. Notably, one haplotype was shared by all three slugs sampled from the native range and over half the slugs from southern California and Oregon. Allometric differences in gizzard and radular morphology reported between native and invasive *P. auriformis* may reflect the greater size of invasive slugs compared to native-range specimens (Cadien and Ranasinghe 2003), or diet-induced plasticity in radulae or allometric relationships (Jensen 1993; Trowbridge 1997). In the native range, *P. auriformis* feeds on bivalves in the genus *Nucula*, whereas introduced slugs feed on native *Transennella* and *Nutricola* spp. and introduced *Gemma gemma* in northern CA, or *Parvilucina* and *Axinopsida* in southern CA (Rudman 1972; Gosliner 1995; Chow 2001). These bivalves represent diverse groups and may induce different gizzard-plate or radular morphologies, and could also change growth trajectories, thus impeding conventional taxonomic identification.

Specimens identified as *P. auriformis* were reported from as far north as Vancouver Island, British Columbia, and as far south as San Diego, California (Behrens 2004). This widespread distribution was achieved within 5 years of the first report of *P. auriformis* in San Francisco. Spread from a central point of introduction is one possible explanation for the distribution of *P. auriformis*, given the dispersal capabilities of the long-lived planktotrophic larvae produced by this species and the seasonal reversal of along-shore currents on the US west coast (Strub and James 2000). A rapid demographic and spatial expansion was consistent with the haplotype network and mismatch distribution of *P. auriformis*. Although independent introductions to northern and southern

sites could produce the appearance of post-invasion spread along a coastline (e.g., Asif and Krug 2011), the 16S network and mismatch distribution show no evidence of multiple introductions.

Based on morphology, specimens from northern California were expected to comprise 3–4 species but we detected only two species, highlighting the value of DNA-based approaches for studying invasion biology. Although *P. auriformis* initially was identified from San Francisco Bay, our recent samples from San Francisco Bay all were *P. orientalis*, including 10 trawled from shallow water in south San Francisco Bay that tentatively had been identified as *P. aperta* (Chow 2001). All five specimens from Tomales Bay and about half the slugs from Bodega Harbor were *P. orientalis*. Gosliner (1998) proposed that large *Philine* from Tomales were *P. japonica* while those from Bodega were *P. orientalis*, species that recently were synonymized (Price et al. 2011). A high level of intraspecific variability in morphological characters complicated the taxonomy of this species, but molecular data clearly indicate invasive slugs from northern California are conspecific with slugs from Japan, and rule out *P. aperta* or *P. quadripartita* as potential invaders of San Francisco Bay. Haplotype networks and mismatch distributions suggest a recent bottleneck at introduction followed by demographic expansion, but *P. orientalis* appears to be restricted spatially to northern California. Future ecological and physiological studies could test why *P. auriformis* spread so rapidly along the coast, and whether *P. orientalis* will ultimately follow a similar trajectory or remain limited to colder waters.

A phylogenetic approach can simultaneously identify unknown specimens and place actual and potential invaders in an evolutionary context. There was roughly an order of magnitude difference between mean within- and between-species divergence at the 16S locus; this gene is thus appropriate for taxon-specific bar-coding work, as there was no overlap in the range of genetic distances within versus among taxa (Meyer and Paulay 2005). The species-level divergence between "*P. aperta*" from Europe (Grande et al. 2004) and South Africa (where the type locality of *P. aperta* is located) supports reclassification of the European species as *P. quadripartita*, affirming the results of a morphological analysis by Price et al. (2011). Genetic distance between *P. quadripartita* and *P. aperta* was more than twice the maximum intra-specific divergence between 16S haplotypes for any of our sampled taxa. Further, *P. quadripartita* was as divergent from *P. aperta* as it was from the other species in its clade (*P. angassi*, *P. paucipapillata*, and *Philine* sp.). Molecular and morphological characters thus reveal congruent differences in the European material sufficient to warrant separate species status and the name *P. quadripartita*.

Our results also substantiate morphological evidence that Hong Kong specimens previously called *P. orientalis* are a distinct species, recently described as *P. paucipapillata*. Although based on only a single marker, our phylogenetic analysis also indicates *P. orientalis* is not as closely related to other members of the "*aperta* clade" (sensu Price et al. 2011) as morphological characters suggest, given the high support for a clade comprising *P. paucipapillata*, *P. aperta*, *P. quadripartita*, *P. angasi*, and the unidentified Australian species. Relationships and ecological traits of *Philine* spp. are important to resolve, given the tremendous invasion potential of at least some species in this group.

Coastal marine ecosystems in the northeastern Pacific generally have been less impacted by non-native species than estuaries (Ruiz et al. 2000; Grosholz 2002). However, the rapid spread and long-term persistence of *Philine* spp. illustrate how the benthic communities of open coasts are vulnerable to ecological disruption by a successful invader. Few invasive marine invertebrates have become abundant from the intertidal zone to depths of hundreds of meters. Maximum reported densities of *P. auriformis* were 44 slugs/m^2 intertidally in Bodega Harbor and 95/m^2 subtidally in the SCB; although abundances in the SCB declined after initial peak densities in 1995–1996, densities of $\sim$10 slugs/m^2 persist at many depths and sites across California (Chow 2001; Cadien and Ranasinghe 2003; Ranasinghe et al. 2005). *Philine* spp. thus have become an established component of benthic ecosystems and the selective landscape along the US west coast, and across a remarkable depth gradient.

The ecological impact of *P. auriformis* and *P. orientalis* is still under-appreciated. Regional monitoring in southern California correlated the arrival of *P. auriformis* with a precipitous decline in small bivalves (Cadien and Ranasinghe 2003), and there may be synergistic impacts of *Philine* spp. and the green crab *Carcinus maenas* on intertidal bivalves

that both consume (Chow 2001). In laboratory studies ~50 small bivalves per day were consumed by *P. auriformis* and *P. orientalis* (then mis-identified as "*P. japonica*"; Chow 2001). In field experiments, addition of *P. orientalis* to caged plots led to a 63 % decrease in abundance of *Nutricola* spp. bivalves over 2 weeks, but *P. auriformis* had no appreciable effect on bivalve abundance. These two species thus may have different ecological impacts, further illustrating why proper species identifications are essential to understand biological invasion, and for management efforts to deal with invaders. Our database of reference sequences will permit rapid identification of non-native *Philine* spp. in the future, and may help to detect and combat early-stage invasions and stop other coasts from being overrun by these caustic slugs.

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