ARTICLE

Genetic diversity and the discovery of a putative cryptic species within a valued crab fishery, *Portunus pelagicus* (Linnaeus 1758), in the Philippines

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The blue swimming crab, *Portunus pelagicus* (Brachyura, Portunidae), is a widely distributed crab species across much of the Indo-West Pacific. A recent taxonomic revision based on morphological and genetic divergence between populations across the species' vast range has revealed that the species is actually a four-member species complex. These species, *P. pelagicus* sensu stricto, *P. reticulatus* (Herbst 1799), *P. armatus* (A. Milne-Edwards 1861), and *P. segnis* (Forskål 1775), have juxtaposed ranges with some peripheral regions of overlap. The species *P. pelagicus* sensu stricto is believed to be the sole representative of this species-complex occurring across the entire Philippine archipelago. Here we test this assumption using the mitochondrial Cytochrome oxidase sub-unit I gene region and identify the presence of two *Portunus* species, *P. pelagicus* sensu stricto and a previously identified but un-described *Portunus* species from Japan. We describe the geographic distribution and genetic diversity of each crab, potentially two species that occur in sympatry but are genetically distinct. This study provides valuable insight into the mtDNA sequence diversity of a highly valued crab fishery in the Philippines, a fishery that is currently managed as a single species.

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INTRODUCTION

The Coral Triangle, composed of Indonesia, Malaysia, Philippines, Timor Leste, Papua New Guinea, and the Solomon Islands, is the global center of marine biodiversity (Hoeksema 2009, Veron et al. 2009) with the Philippines identified as having the highest concentration of species per unit area (Carpenter and Springer 2005, Sanciangco et al. 2013). The wider Indo-West Pacific region has also been regarded as the center of brachyuran diversity (Ng et al. 2008) as more and more species are being discovered regularly (Ahyong and Ng 2009). Furthermore, taxonomic and molecular exploration of described sister species has revealed entire species complexes, including those in abundant and economically important commodity taxa (Matsuoka and Hatanaka 1991, Knowlton 1993). For example,

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Keenan et al. (1998) used morphological and molecular features to identify four distinct species of mangrove crabs, *Scylla*, from specimens previously described as a single species. Careful scrutinizing of the broadly distributed blue swimming crab species, *Portunus pelagicus* (Linnaeus 1758), produced similar results with the taxon separated into four discrete species (Lai et al. 2010). These species include *Portunus pelagicus* sensu stricto which is widespread across Southeast and East Asia including Japan, *P. armatus* (A. Milne-Edwards 1861) distributed from Australia to New Caledonia, *P. reticulatus* (Herbst 1799) around the Bay of Bengal, and *P. segnis* (Forskål 1775) which is confined to the western Indian Ocean. Originally described as four unique taxonomic entities by authorities in the 18th and 19th century, their taxonomic classification was deemed contentious and the species were pooled under the single Latin binomial, *P. pelagicus*, by Stephenson et al. (1968). The use of molecular data from the mitochondrial Cytochrome oxidase I (COI) gene region, along with morphological and biogeographical considerations, have provided the most recent support for four discrete species (Lai et al. 2010).

Studies on *Portunus* species utilizing molecular genetic tools have not only provided valuable insights into the species delineation, but also in identifying areas of high genetic biodiversity. Allozyme variation indicated the presence of a 'cryptic' species in the Darwin region of northern Australia (Bryars and Adams 1999) and randomly amplified of polymorphic DNA (RAPD) analysis showed large genetic differentiation of *P. pelagicus* within the Gulf of Thailand and Andaman Sea (Klinbunga et al. 2010). Further, molecular data identified this same area as a zone of hybridization for *P. pelagicus* and *P. reticulatus* in the Gulf of Thailand and Andaman Sea based on shared COI haplotypes (Lai et al. 2010). Along the coast of Japan, the northern range limit of *P. pelagicus*, COI sequence data indicated the existence of an undescribed possible fifth member of the *Portunus pelagicus* species complex (Lai et al. 2010). Molecular evidence of this undescribed *Portunus* species is limited to two partial mtDNA sequences, with no available morphological or meristic data (Lai et al. 2010). Still, the use of molecular markers, such as the widely used COI barcoding gene region, provide a powerful tool for quantifying species diversity (Krishnamurthy and Francis 2012), particularly in known marine biodiversity hotspots like the Philippines (Hubert et al. 2012, Zhang and Hanner 2012, Maralit et al. 2013).

The Philippines is centrally positioned within the species range of *P. pelagicus* sensu stricto which is geographically disjunct from the range of other members of the species complex. This suggests the presence of only one species, *P. pelagicus*, in the Philippines. However, contention over the taxonomy of *Portunus* species (Stephenson et al. 1968), evidence of several 'cryptic' species across the Indo-Pacific region (Bryars and Adams 1999, Lai et al. 2010), and identification of localized zones of hybridization or high genetic differentiation (Klinbunga et al. 2010) warrant further investigation of this valuable harvest fishery. The Philippine crab fishery is mostly composed of portunid species and *P. pelagicus* is the most exploited species (Ingles 2004), with annual capture production reaching yields up to 40,000 tons (FAO 2011). Accurately profiling the species composition of the fishery, including identifying cryptic species, may have profound implications for management and conservation (Bickford et al. 2006).

Current species range data imply the presence of only *P. pelagicus* in the Philippines. Here, we set out to test this assumption by obtaining molecular genetic data from *Portunus* crabs sampled broadly from across the Philippine archipelago. We use validated molecular analysis to support our conclusions and integrate our results with the prevailing taxonomic framework for this genus. We conclude by highlighting how our data may be useful in the management of this Philippine crab fishery.

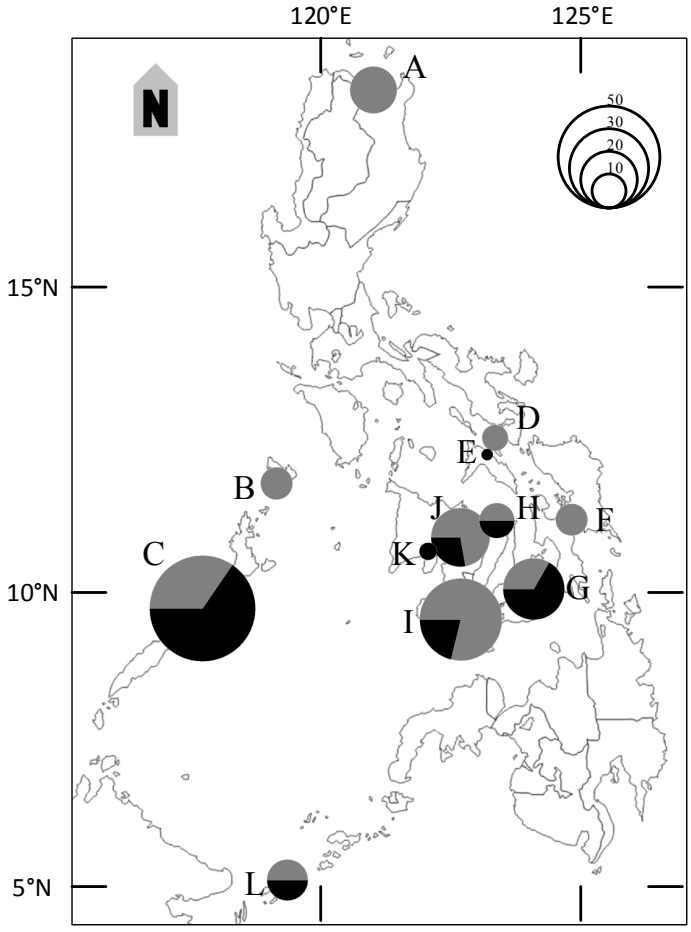

Figure 1. Map of sampling locations in the Philippines. Sampling sites of putative *Portunus pelagicus* in the Philippines indicated by letters: (A) Aparri, Cagayan; (B) Culion, Palawan; (C) Puerto Princesa, Palawan; (D) San Jacinto, Ticao Island; (E) Masbate City, Masbate Island; (F) Tacloban, Leyte; (G) Tagbilaran, Bohol; (H) Bantayan Island, Cebu; (I) Bais, Negros Oriental; (J) Manapla, Negros Occidental; (K) Iloilo, Panay Island; (L) Bongao, Tawi-Tawi. Pie charts illustrate sympatric distribution and proportion of two *Portunus* species (Clade I (black) and Clade II (grey)) sampled from each location. Size of the circle is proportional to sample size.

Table I. Sampling site and haplotype information. Site location, site letter, number of individuals sampled (N), number of haplotypes (N_H), number of unique haplotypes (H_U) (haplotypes restricted to a single sampling location), proportion of specimens in Clade I (*Portunus pelagicus*) and latitude and longitude coordinates are provided.

Site	Site Letter	N	N_H	H_U	Clade I	Latitude	Longitude
Aparri, Cagayan	A	12	1	0	0%	18° 21'N	121° 38'E
Culion, Palawan	B	7	1	0	0%	11° 48'N	119° 57'E
Puerto Princesa, Palawan	C	52	11	7	65%	09° 44'N	118° 43'E
San Jacinto, Ticao Island	D	5	1	0	0%	12° 34'N	123° 43'E
Masbate City, Masbate Island	E	1	1	0	100%	12° 22'N	123° 37'E
Tacloban, Leyte	F	7	2	0	0%	11° 14'N	124° 59'E
Tagbilaran, Bohol	G	21	5	2	65%	09° 38'N	123° 50'E
Bantayan Island, Cebu	H	8	6	1	50%	11° 09'N	123° 43'E
Bais, Negros Oriental	I	33	5	1	20%	09° 35'N	123° 07'E
Manapla, Negros Occidental	J	18	4	0	27%	10° 57'N	123° 07'E
Iloilo, Panay Island	K	2	2	0	100%	10° 42'N	122° 35'E
Bongao, Tawi-Tawi	L	10	5	2	50%	05° 01'N	119° 46'E

METHODS

Market samples of putative *P. pelagicus* were collected from twelve localities distributed across the archipelago (Figure 1, Table I). Specimens were identified using taxonomic keys in Lai et al. (2010) and Ng (1998). Tissue samples were taken from the cheliped manus and preserved in 95% ethanol. The tissue samples were then stored at the Institute of Environmental and Marine Sciences, Silliman University.

Partial mitochondrial DNA sequences of the COI gene region were used to examine phylogenetic relationships. Tissue samples were placed in a 1.7mL centrifuge tube with 10µL of proteinase K, 1µL of RNase, and 500µL of digest buffer, and incubated for 1 hour at 42°C. 450µL of the solution were collected from the tube and transferred to another tube with the same volume of phenol-chloroform then centrifuged at 4°C for 20 to 30 minutes at 17,000rpm. 300µL of sample were withdrawn from the surface layer to which were then added 7µL of EDTA, 30µL of NaOAc, and 660µL of 100% ETOH, and the mixture then centrifuged for 15 minutes at 15000rpm to precipitate the DNA. The aqueous phase was poured out of tube leaving the DNA pellet undisturbed. The pellet was washed with 80% ETOH, then allowed to air dry for 30 minutes or until all the ethanol had evaporated. The DNA pellet was resuspended in 50µL of Tris-HCl (10mM) at pH 8.2 and then stored at -4°C until amplification.

Amplification was carried out using Polymerase Chain Reaction (PCR) with the COIF (5'- AGAAGTGTATATTTTAATTC-3') and COIR (5'- ATGTAGAATAATATCGATAG- 3') primers (Sezmis 2004). Hotstart PCR was performed with a final volume of 25 µL containing 2.5µL of 10x PCR Buffer II and dNTPs (8mM), 2µL of $MgCl_2$, 1.25µL of both primers, 0.125µL of Hotstart *Taq* DNA polymerase (Applied Biosystems Inc. Foster City, California), and 1µL of DNA template. Thermal cycle was set as follows: initial denaturation at 95°C for 10 min; then 38 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 45 sec; then a final extension of 10 min at 72°C. All PCR products were checked for positive amplification by visualizing them in a 1% agarose gel stained with ethidium bromide. Successfully amplified PCR products were sent to the University of California, Berkeley for purification and sequencing on an ABI capillary sequencer.

After checking for disagreements, COI consensus sequence for both directions was obtained using Geneious 5.5 (Drummond et al. 2010) and alignment of the consensus sequences was conducted using the MEGA v5 (Tamura et al. 2011) Clustal W option with default settings. Aligned sequences were then exported to FaBox (Villesen 2007) for haplotype generation. To examine species relationships, generated haplotypes were compared with GenBank reference sequences of other *Portunus* species, specifically *P. armatus* [GenBank accession # EF661877, EF661887, EF661896, EF661897], *P. reticulatus* [EF661959, EF661963, EF661964, EF661973, EF661974], *P. segnis* [EF661949, EF661953, EF661955, EF661956], a *P. pelagicus* sensu stricto [EF661915, EF661944, EF661946, EF661947, EF661948] including a single sequence previously sampled from the Philippines, and the two sequences of the undescribed yet possible fifth member of the *Portunus* species complex from Japan [EF661927 and EF661931]. Sequence divergence and a Maximum Likelihood phylogenetic tree were inferred in MEGA v5 (Tamura et al. 2011) using a Three Parameter Model (TPM) as suggested by jModeltest (Guindon and Gascuel 2003, Posada 2008). Network 4.6.1 (Fluxus Technology Ltd. 2004-2012) was used for generating a haplotype network to further explore the relationship between the sequence data. Haplotype sequences obtained from the 176 specimens examined in this study were deposited in the public domain (GenBank accession numbers KJ701510-KJ701527).

RESULTS

COI sequence data were obtained from a total of 176 putative *P. pelagicus*. Final sequence length after aligning and trimming of primer regions was 342 base pairs (bp) of which twenty-nine polymorphic sites were identified including 2 singleton variable sites and 27 parsimony informative sites. No gaps were present after alignment and no stop codons were identified after translation using Frame 3 of the Invertebrate Mitochondrial genetic code in Geneious 5.5.

From the 176 specimens analyzed, a total of 18 haplotypes were identified (Table I). Haplotype 2 was the most common (n = 98), present in 10 out of the 12 sampling stations distributed across the Philippines. The next most common haplotypes were haplotype 1 (n = 24), haplotype 4 (n = 17), and haplotype 8 (n = 15), which together with haplotype 2 comprise 87% of the total individuals examined. Ten haplotypes were singletons and three haplotypes were shared by two or more individuals but were restricted to a single sampling station (Table I). Based on the haplotype network (Figure 2), samples sorted into two clusters separated by 16 mutational steps. All haplotypes except haplotypes 2, 6, 9, and 10 formed one cluster and will be referred to as Clade I hereafter, while the remaining four haplotypes formed a distinct cluster, Clade II. Clade I and II have a sympatric distribution (Figure 1) and no biogeographic or oceanographic barrier could be identified that separated the species.

Sequence divergence between the Philippine Clade I and Clade II was 6.4%, a difference comparable to that observed between discrete taxonomic species for the COI gene region. The sequence divergence within Clade I was 0.5% and within Clade II was 0.1%. The sequence divergence values between Clade I and Clade II are comparable to previously reported values of inter-specific divergence between *Portunus* species (3.5 and 7.6%) (Lai et al. 2010). Our clade divergence values also fall within those reported for members of the Order Decapoda for both inter-specific divergence (4.9% to 23.7%), and intra-specific divergence (0.0% to 2.6%) (Costa et al. 2007).

The Maximum Likelihood phylogenetic tree inferred using the Tamura 3 Parameter substitution model (Figure 3) generated two distinct clades, Clade I and Clade II, which are consistent with the haplotype network clusters. Clade I, together with *P. pelagicus* sensu stricto sequences from GenBank, formed a single group and was sister to the outgroup *P. segnis*. Clade II, the putative cryptic species, formed a single group with the two sequences of the undescribed *Portunus* species from Japan (Lai et al. 2010). Although categorized as a single 'cryptic species' clade, the sequence divergence between the Philippine and Japanese sequences was 1.1%. The 'cryptic species' clade was sister to a clade containing *P. armatus* and *P. reticulatus* (Figure 3).

DISCUSSION

Broad sampling of the blue swimming crab *P. pelagicus* across the Philippines confirmed the presence of *P. pelagicus* sensu stricto and revealed the comparably wide distribution of a putative cryptic *Portunus* species (Figure 1). The distinction of two *Portunus* species is supported by the high COI sequence

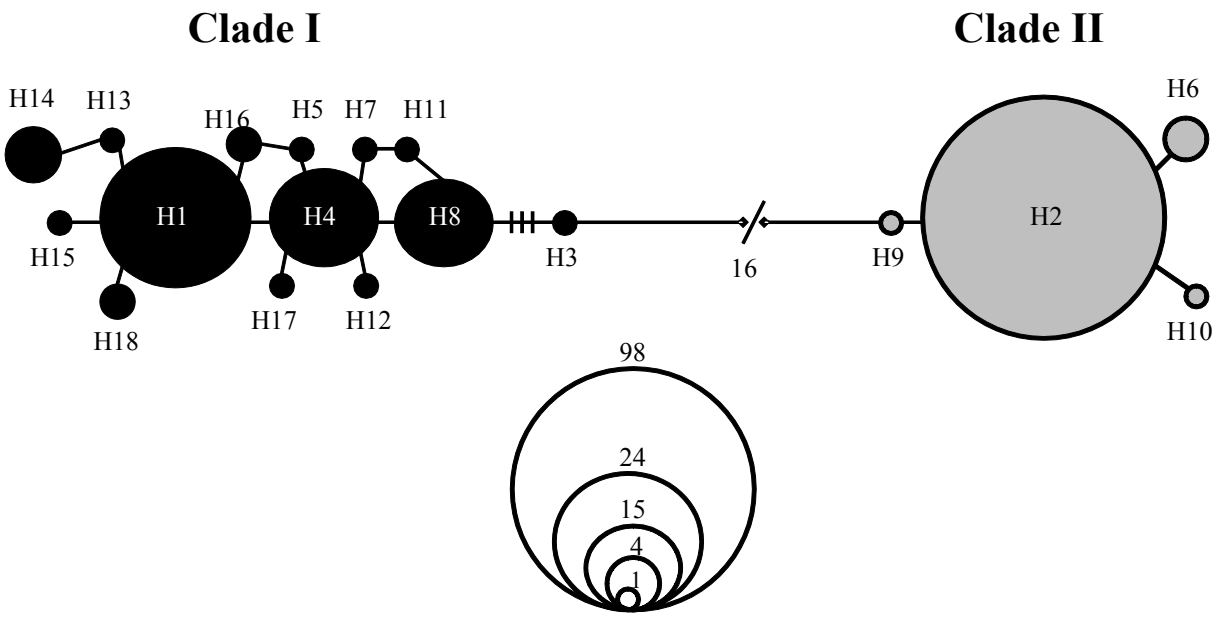

Figure 2. *Portunus pelagicus* COI haplotype network. Size of the circle is proportional to the frequency of the haplotype (Haplotype #) and colors represent the two Clades (I-black, II-grey). All haplotype are separated by one mutational step unless specified by a number or hash marks (each hash mark represents one additional mutation step).

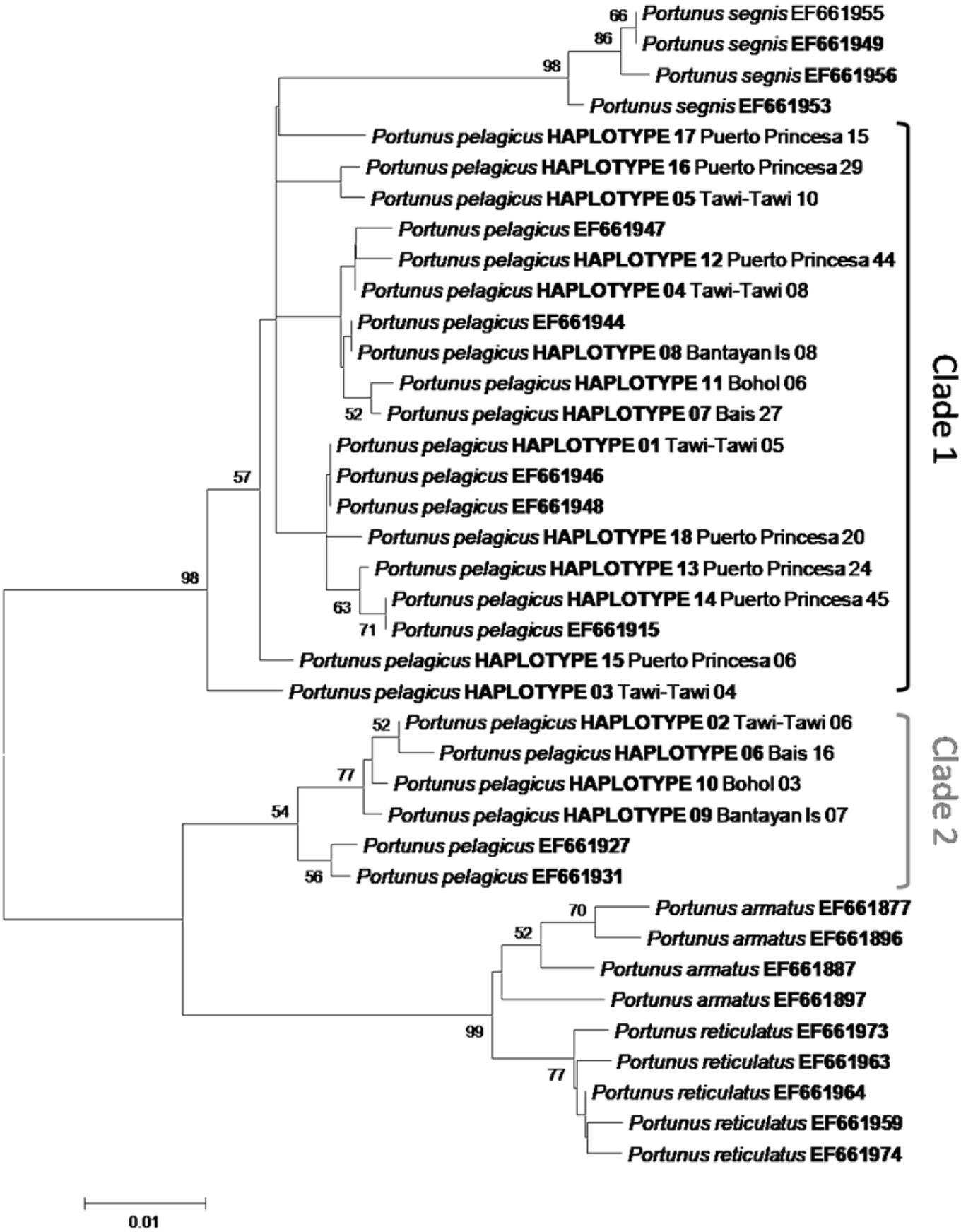

Figure 3. Maximum Likelihood tree topology of haplotypes (species/haplotype #/sampling location/sample number) together with GenBank sequences (accession no.). Distances were computed using the Tamura 3-parameter Model as suggested by jModeltest. Bootstrap consensus tree for all tree topologies and was inferred from 1,000 replicates and indicated at tree nodes.

divergence between the two clades equal to the genetic divergence observed among distinct species (Costa et al. 2007, Lai et al. 2010).

This study is the first molecular assessment of the commercially valued *P. pelagicus* in the Philippines and the first Philippine report of a possible 'cryptic' *Portunus* species previously only documented along the coast of Japan. This signifies a substantial broadening of its known range. Its broad distribution might suggest this to be a case of an undocumented population present in a region where mitochondrial DNA studies are just beginning to fully realize the area's wealth of marine biodiversity. Further, molecular data do not indicate whether the putative 'cryptic' species has an evolutionary origin in the Philippines and then extended to Japan, or originated in Japan and then extended to the Philippines; only that it is wide-spread in the Philippines and co-occurs with the species *P. pelagicus*. It is noteworthy that 59% of the 176 specimens sampled were members of the 'cryptic' *Portunus* species, a testament to the degree of how little is known about the biodiversity of this genus and commodity species.

Balancing the conservation and the exploitation of marine fisheries is a difficult challenge facing resource managers and policymakers (Francis et al. 2010). Meeting this challenge will require fundamental baseline data like the data presented here that provide: (a) a genetic species assessment of the biodiversity of the Philippine blue swimming crab fishery, and (b) an assessment of the genetic diversity within each of the identified crab species from multiple localities across the archipelago. Currently, the approved administrative order on the conservation of blue swimming crab in the Philippines focuses on the fishery as a single commodity differentiating the species based on morphological characters (DA-DILG 2014). Our results identifying a putative cryptic *Portunus* species caution the sole use of morphologically based approaches to discriminate between species, as the fishery may be more diverse than previously believed. Catch rates of *Portunus* in the Philippines have been on the decline in recent years in part due to unregulated increased fishing efforts (Ingles 2004), and a slow progress in developing alternative technologies to wild-caught seeds for rearing blue crabs in captivity (Soundarapandian et al. 2007). By unraveling the species composition of the crab fishery and quantifying the species genetic diversity, opportunities arise to use emergent management tools such as phylogenetic diversity, a genetic metric tool for prioritizing species in biodiversity conservation problems (Hartmann and Andre 2013), and to provide managers and policymakers with more management options for the conservation and exploitation of *Portunus* crabs.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest in this study.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

This manuscript was conceived and developed by PM Sienes and DA Willette. Field sampling and laboratory work were performed by PM Sienes, C Alvior, L Romena, J Estacion, and DA Willette. The data were analyzed and the manuscript was written by PM Sienes and DA Willette, with all the authors providing substantial contributions during the editing and revision phases.

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