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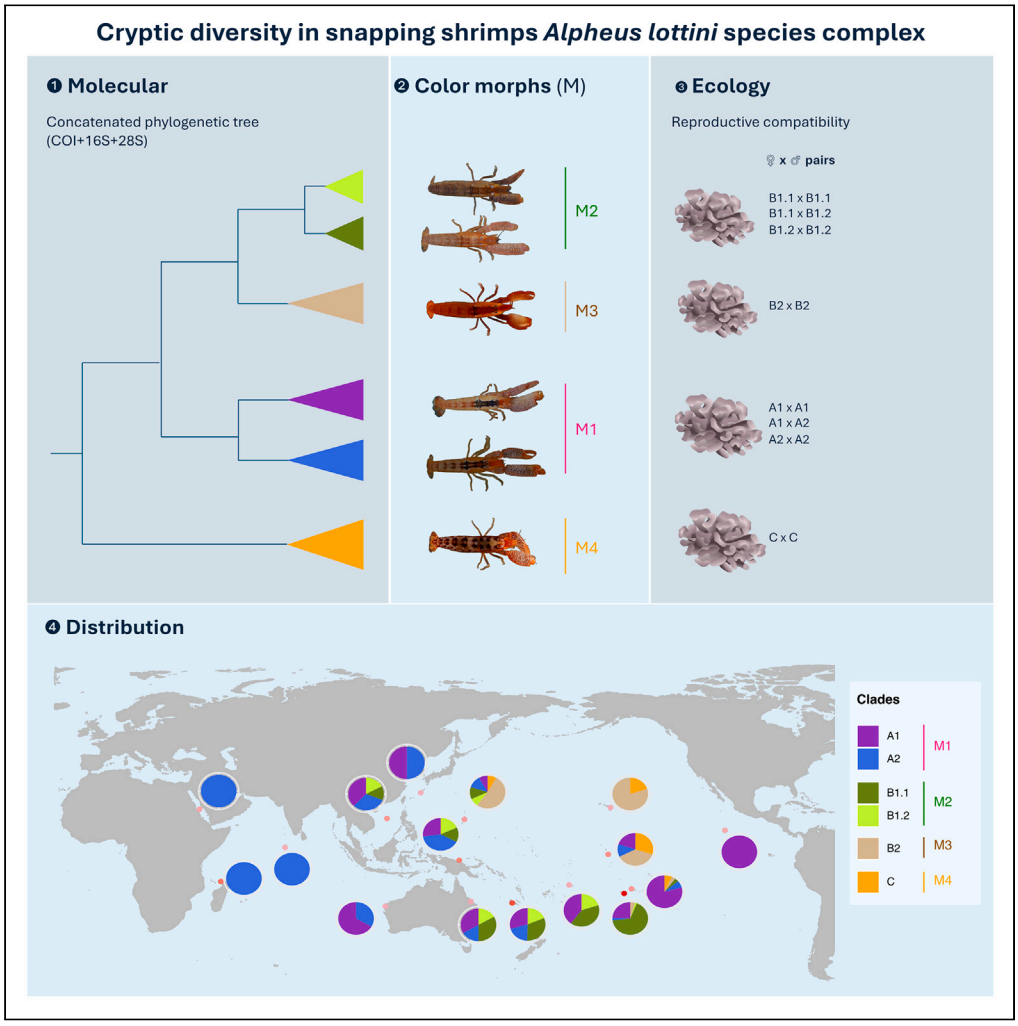

Héloïse Rouzé, Nancy Knowlton, Arthur Anker, Carla Hurt, Herman H. Wirshing, Alain Van Wormhoudt, Matthieu Leray

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Highlights

Alpheus lottini species complex is composed of at least four undescribed species

Combining molecular, color, and ecology is crucial for phylogeography reconstruction

Peak lineage diversity in Mariana and Line Islands suggests a Pacific origin beyond the Coral Triangle

Accurately identifying species matters as they may not be functionally equivalent

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An integrative phylogeography for inferring cryptic speciation in the *Alpheus lottini* species complex, an important coral mutualist

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SUMMARY

We use molecular analyses, color patterns, and records of distribution of mating pairs to reconstruct the global phylogeography of *Alpheus lottini*, a complex of cryptic coral-associated snapping shrimp species. Molecular data support the delineation of ancestral clades A, B, and C, and suggest five additional subdivisions within clades A and B. Clades A, B1, B2, and C exhibit color pattern differences and/or evidence of assortative mating, and thus merit species-level recognition. There is no evidence for assortative mating within clades A and B1, with likely reproductive compatibility (i.e., fertile clutches) in areas of sympatry. The clade diversity peaks in the Mariana Islands and the early branching clade C is restricted to the northern periphery of the Central and Western Pacific suggesting a Pacific origin of this group outside of the Coral Triangle. These findings underscore the prevalence of allopatric processes with possible ecological or microallopatric speciation in areas where clades overlap.

INTRODUCTION

High levels of biodiversity in the marine environment in the absence of obvious dispersal barriers – the marine speciation paradox – puzzled scientists for many years.[1,2] Some studies using molecular approaches have revealed genetic partitioning indicative of previously invisible geographic barriers to gene flow in many taxa,[3] while others have highlighted the contribution of processes other than vicariance, such as ecological speciation in the presence of gene flow and founder colonization. Divergent selection on traits can promote rapid speciation in sympatry, i.e., in the absence of a geographical barrier to gene flow.[4,5] The prevalence of these allopatric and sympatric mechanisms remains unknown and likely depends upon species' life history traits, behaviors, and ecology.

Numerous marine invertebrates, once thought to be widely distributed, panmictic species, are in fact composed of multiple genetically distinct entities that are very similar morphologically.[6] These cryptic/pseudo-cryptic or sibling species are often distinguishable by subtle or obvious differences in live color patterns (which typically disappear upon preservation) and sometimes also by assortative mating behaviors.[7–9] Others, however, are only delineable using molecular markers.[10,11] Cryptic species are particularly common among crustaceans and other small invertebrates,[12–17] but also occur in larger and more conspicuous animals, such as corals,[18] molluscs,[19] and starfishes.[20,21] These taxa offer many opportunities for exploring marine speciation,[22–25] for which a multi-dimensional approach, involving morphology, color pattern, behavior, genetics, and biogeography, can be particularly informative.[6,26]

Cryptic species are very common in one of the largest genera of decapod crustaceans, the snapping shrimp genus *Alpheus*.[27–33] More than 300 species of *Alpheus* have been described to date, but the true diversity of the genus appears to be much higher, judging from over 50 species described over recent decades (e.g.,[30,32,34]). These snapping shrimps live in a wide array of habitats ranging from mangroves and rocky shores to coral reefs and deep-sea muddy bottoms,[35,36] and many engage in obligate symbiotic associations, for instance, with gobies,[37] sea anemones,[26] echiurans,[31,38] and scleractinian corals.[11,39–42]

Members of the *Alpheus lottini* species complex live in obligate association with branching corals of the family Pocilloporidae, particularly *Pocillopora*, but also *Seriatopora*, throughout the tropical Indian and Pacific Oceans.[43–46] They are characterized by an overall orange-red or pale orange color pattern with distinctive darker patches and spots, which is unique within the genus *Alpheus*. As with most other symbiotic snapping shrimps,[15,47] one host (i.e., one coral colony) typically contains a single adult male-female pair of *A. lottini*. The pair is highly

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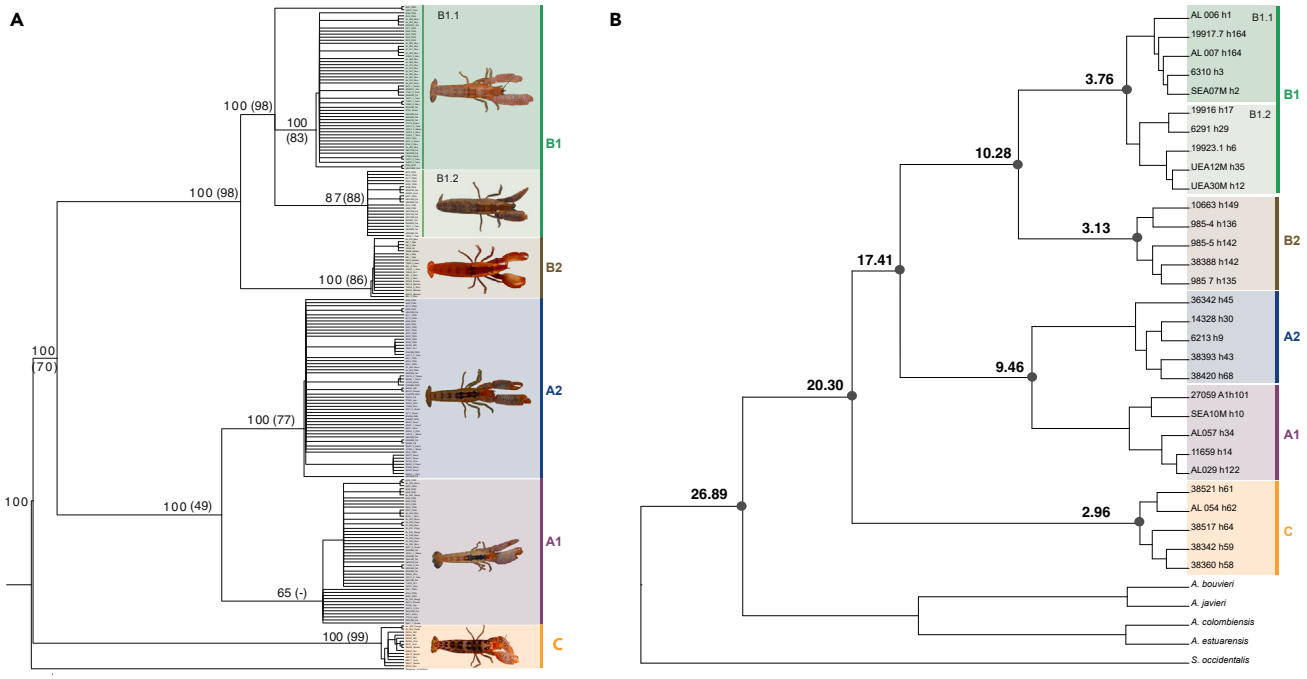

Figure 1. Phylogenetic reconstructions of *Alpheus lottini*

(A) Concatenated Bayesian phylogenetic tree of mtCOI, mt16S and 28S rRNA partial gene sequences of the *Alpheus lottini* species complex, with Bayesian posterior probabilities (first value) and ML bootstrap support values (following in parentheses).

(B) Concatenated Bayesian phylogenetic tree using a concatenated COI and 16S dataset (1144 bp) used for estimation of divergence times in million years estimated using the Bayesian MCMC method from a reduced (30 samples). Two transisthmian sister-species pairs (*Alpheus javieri* - *A. bouvieri* and *A. colombiensis* - *A. estuariensis*) were included in the analysis to calibrate the tree.

territorial, expelling all adult conspecific intruders, although tolerating small juveniles. The observation of male-female adult pairs within the same coral host in the field can be used as an assay for mating preferences and hence species boundaries.[27] They are among the best-known snapping shrimps because of the many studies that have elucidated the key ecological services they provide, such as defending their coral host against predation by the crown-of-thorns sea star, *Acanthaster planci*,[48,49] and eliminating sediments and organic material deposited on the coral surface.[42]

Preliminary studies pointed to the existence of at least two cryptic species based on genetic and color pattern differences, both observed in assortative mated pairs when found in sympatry.[50,51] Later, studies showed evidence of a genetic break between the Indo-West and the tropical Eastern Pacific[52] and described the existence of two clades living in sympatry in New Caledonia.[11] The latest research used a set of museum specimens to identify five distinct lineages based on the nuclear internal transcribed Spacers 1 (ITS1), linking at least some of them to distinctive color morphs.[53] Nevertheless, we still have a limited understanding of the distribution of these lineages because sample sizes have been small and sampling geographically limited.

In the present study, we present a detailed and comprehensive analysis of the diversity and biogeography of the *A. lottini* species complex using a large collection of specimens spanning almost the entire geographic range of this taxon. We delineate potential sibling species using a multi-locus phylogeny, live color patterns and distribution of mating pairs per host coral colony. The results highlight the remarkable range of evolutionary processes that are potentially involved in the formation of sibling species within the *A. lottini* species complex.

RESULTS

Genetics of the *A. lottini* species complex

Phylogenetic analysis

Matches to clades A and B were based on a phylogenetic analysis combining previously published COI sequences of Knowlton and Weigt[50] and sequences generated herein (Figure S1). For clades B1, B2 and C we followed previously used nomenclature of Van Wormhoudt et al. (2019) with some overlapping samples (samples starting with "AL_" in Table S1).

The concatenated phylogenetic tree (Figure 1A) identified well-supported clades A1 (BPP = 65%, ML = nd) and A2 (BPP = 100%, ML = 77%), B1.1 (BPP = 100%, ML = 83%) and B1.2 (BPP = 87%, ML = 88%), B2 (BPP = 100%, ML = 86%) and C (BPP = 100% and ML: 99%) (Figure 1A).

Trees built with the two mitochondrial markers differed in delineating genetic structure within clades A and B (Figures S2 and S3). The COI tree identified the two well-supported clades A1 (BBP = 88%, ML = 89%) and A2 (BBP = 89%, ML = 92%), whereas 16S failed to support the distinction between these two clades. Both COI and 16S trees supported B1 (COI: BPP = 99%, ML = 99%; 16S: BPP = 90%, ML = 70%) and B2

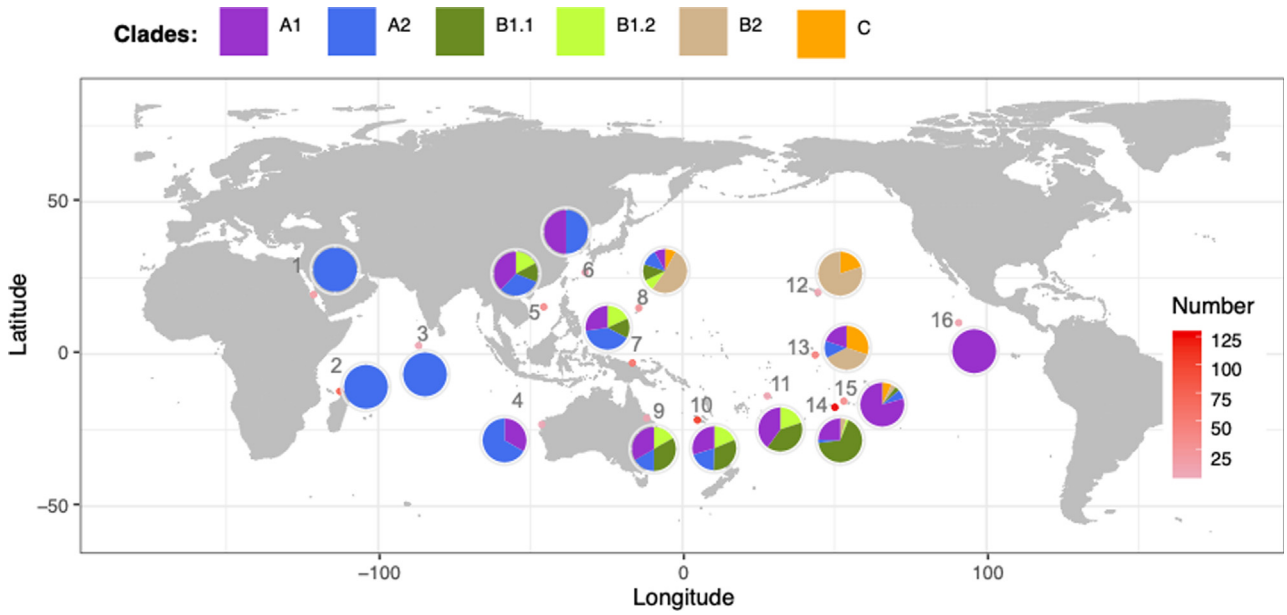

Figure 2. Geographic distribution of all genetic clades of *Alpheus lottini*

For clarity, each pie chart includes specimens from nearby localities as follows: Western Indian Ocean: Saudi Arabia + Djibouti [1]; Réunion + Madagascar + Europa Is. [2], Central Indian Ocean: Maldives [3]; Eastern Indian Ocean: W Australia (Ningaloo) [4]; Western Pacific: Vietnam + Taiwan [5], Japan (Okinawa) [6], Papua New Guinea [7], Mariana Is. [8], E Australia (GBR) [9], New Caledonia [10]; Central Pacific: Samoa [11], Hawaii [12], Line Is. [13], Society Is. [14], Tumamotu Is. [15]; Eastern Pacific: Clipperton [16].

(COI: BPP = 100%, ML = 95%; 16S: BPP = 100%, ML = nd). Finally, 16S but not COI delineated the two clades within B1 (B1.1 and B1.2, Figure S3).

Patterns of net pairwise genetic distances among clades A, B and C (Table S3) were congruent between the mitochondrial 16S and COI genes. Clade C is the most distinct genetically (Table S3; Figure 1A), with C-A or C-B mean distances of 20–22% based on COI and 11–13% based on 16S against A-B mean distances of 14–16% based on COI and 6% based on 16S. Within clades A and B, net pairwise genetic distances between clades showed similar low percentages between A1-A2 and B1-B2 based on COI (4%) and between B1.1-B2 and B1.2-B2 based on 16S (2–3%). Thus in total six groups were genetically discriminated based on COI and/or 16S genes.

Geographic distribution of genetic clades

Clades A1 and A2 have a combined distribution that stretches across the entire tropical Indo-Pacific and Eastern Pacific from the Red Sea and Madagascar to Clipperton and Panama [Figures 2, 3, and S1; data from Knowlton and Weigt[50] for Panama specimens]. A1 and A2 overlap broadly in the Eastern Indian Ocean and Western Pacific; specimens of these two clades are the only ones to co-occur in Western Australia (Ningaloo) and Southern Japan (Okinawa). A2 occurs in the Western Indian Ocean (including the Red Sea) where no other clades have been reported. Similarly, A1 is the only clade occurring in the tropical Eastern Pacific (Figure 2).

Clades B1.1, B1.2 and B2 have a combined distribution restricted to the tropical Western and Central Pacific Ocean (Figure 2). Types B1.1 and B1.2 together ranged from the South-China Sea (Vietnam) to the South-Central Pacific (French Polynesia). In contrast, clade B2 was geographically restricted to the northern part of the Central Pacific (Hawaii, Line Islands, Mariana Islands and French Polynesia). Types B1.1, B1.2 and B2 occur in sympatry in the Mariana and the Society Islands.

The genetically most distinct group, clade C, was found to be largely restricted to the Central and Central-Western Pacific (Hawaii, Line Islands, Mariana Islands, and Tuamotu Islands) and thus overlapped with clade B2 (Figures 2 and 3). Notably, B2 and C are the only clades of *A. lottini* present in Hawaii.

Overall, based on the present material, the areas with the highest clade diversity of *A. lottini* (i.e., areas with the highest number of clades in sympatry) were the Mariana Islands in the Western Pacific, with all six clades present, followed by French Polynesia with five clades (Figure 2). Most other surveyed Pacific localities had representatives from three or four clades. The lowest clade diversity was observed in the Western Indian Ocean (A2 only) and the tropical Eastern Pacific (A1 only, but see discussion below).

Haplotype diversity of ancestral clades

A total of 178 unique COI haplotypes (512 specimens sequenced) and 139 unique 16S haplotypes (513 specimens sequenced) were identified across all six delineated clades (Table 3). About 65% of the overall haplotype diversity was observed within clades A1 and A2 (Table 3), which

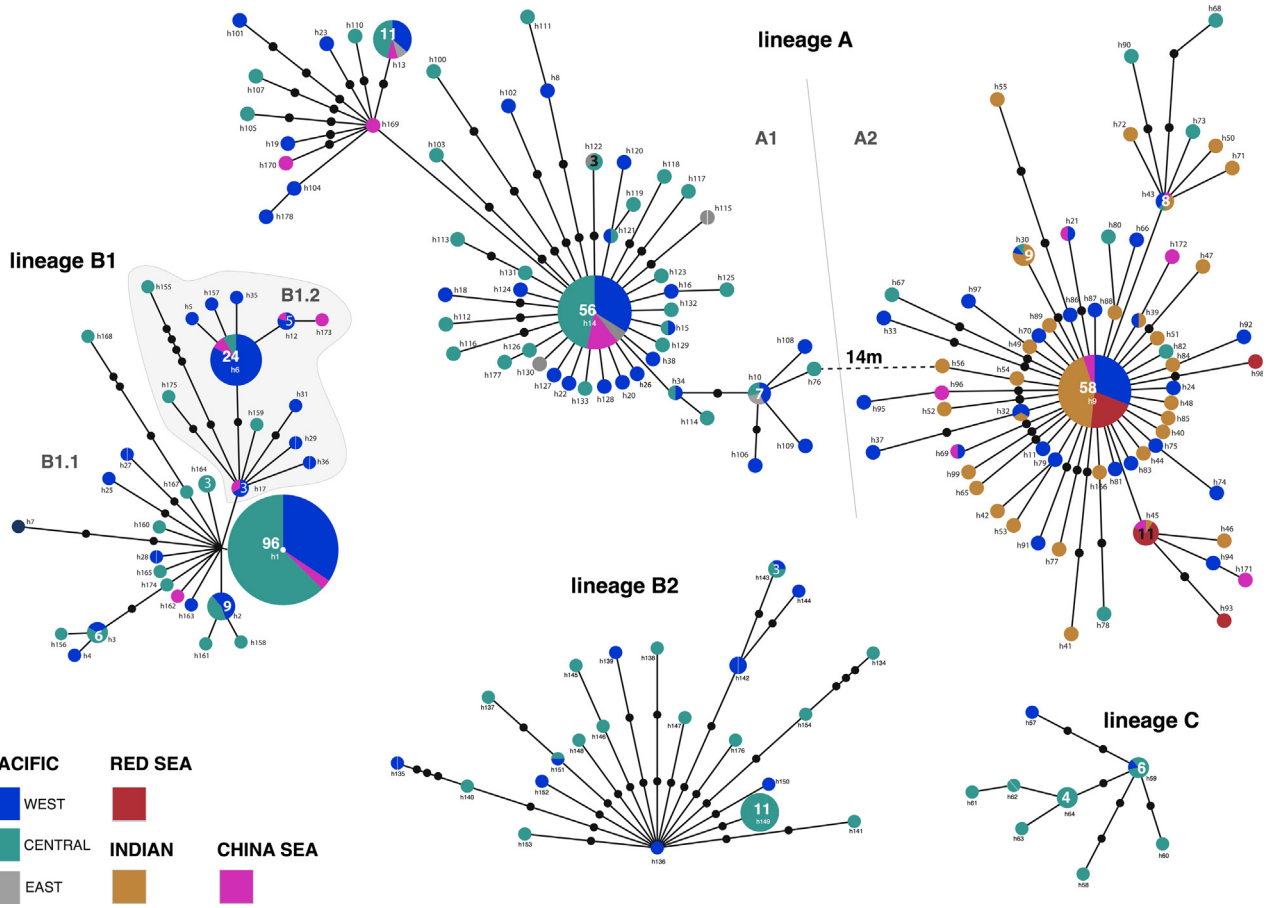

Figure 3. Minimum spanning tree of COI haplotypes from genetic clades of *Alpheus lottini*

Numbers inside circles indicate the frequency of each haplotype (when >1). The size of each circle is proportional to the frequency of each haplotype for all sites combined. Mutations are represented with black circles. Colors indicate main geographic regions, as follows: (blue) West Pacific, (green) Central Pacific, (gray) East Pacific, (red) Red Sea, (brown) Indian Ocean and (pink) China Sea.

were separated by a difference of 14 mutations in the COI minimum spanning network (Figure 3). Each of these clades was represented by a major central haplotype shared by 58 (h9) and 56 (h14) individuals for A2 and A1, respectively (Figure 3). Tajima's D tests indicated a significant deviation from the neutral model of evolution within both A1 and A2 (Table 3).

The second highest diversity was observed within clades B1.1 and B1.2. and B2; together they accounted for 30% of the total haplotype diversity in the COI and 16S datasets. The COI haplotype h1 was the predominant haplotype within clade B1 (96/178); it corresponds to specimens of clade B1.1 delineated by the 16S locus. The second most common COI haplotype, h6, was shared by 24 individuals (Figure 3) and belongs to clade B1.2 as delineated by the 16S locus (Figure 2). All COI haplotypes from the clade B2 (Figure 2) were characterized by a few (1–3) individuals, except for the haplotype h149, shared by 11 individuals. Tajima's D tests were significant for both B1.1 + B1.2 and B2 (Table 2).

Within clade C, the most common COI haplotype (h59) was shared by six individuals (Figure 3).

Color morphs and morphological diversity

The detailed examination of specimens affiliated with different genetic clades revealed the presence of four distinct color morphs (Figures 4 and S5; Table 1) that correspond to four groups delineated by the concatenated phylogenetic analysis (Figure 1A): A (both A1 and A2), B1 (both B1.1 and B1.2), B2 and C. Specimens within clades A1 and A2 are characterized by a solid black or dark red-brown mid-dorsal band on the carapace continuing onto the pleon, usually as a series of spaced rhomboid patches (Figures 1, 4, and S5). The intensity of the background color, as well as the color of the mid-dorsal band and its extension on the pleon, vary considerably among the specimens, and there seem to be no consistent difference between A1 and A2. However, most specimens from the Indian Ocean, including the Red Sea, are generally much paler, while the specimens from the Central and Tropical Eastern Pacific Ocean are typically deep-orange with a solid-black mid-dorsal band. Clades B1.1 and B1.2 have a diffuse mid-dorsal band on the carapace composed of dark red dots that does not extend to the pleon (Figures 1,

Figure 4. Live color morphs of *Alpheus lottini* belonging to each clade

4, and S5). Clade B2 is generally similar in coloration to B1.1 and B1.2, but the mid-dorsal band is solid dark-red or brown, without red spots. B2 is also unique in having a much less conspicuous dotting on the major claw, compared to all other color morphs (Figures 1, 4, and S5). Clade C is chromatically the most distinctive, with its unique double-row band on the mid-dorsal line, consisting of two pairs of large dark-red patches on the carapace and several more pairs of these on the pleon (Figures 1, 4, and S5). In summary, color pattern differences support the four clades delineated by both mitochondrial genes (A1+A2, B1.1 + B1.2, B2 and C), but fails to support the two clades defined by a single gene (A1 versus A2 with COI, B1.1 versus B1.2 with 16S).

Heterosexual pairing of *A. lottini* with respect to clade

The assortative mating in shrimp pairs observed in three regions spanning the Central Pacific (Southern Line Islands), South-Western Pacific (New Caledonia) and Western Pacific (Vietnam), is concordant with the genetic and color pattern data in supporting the distinctiveness of clades A1+A2, B1.1 + B1.2, and C (Figure 5A). Clades B1 and B2 do not occur in sympatry at any of these locations; pairing data from the Mariana Islands, where they are known to co-occur, will be needed to evaluate their reproductive compatibility.

In contrast, numerous pairings were observed between types A1 and A2 at all three locations and between clades B1.1 and B1.2 in New Caledonia (B1.1 and B1.2 did not occur at the other two locations). In the 28 coral colonies across the three locations that contained A1 or A2, the A1-A2 pairing occurred in 57% of cases compared to 29% for A1-A1 pairings and 14% for A2-A2 pairings (Figure 5A); these occurrences are

Table 1. Color morphs in the *Alpheus lottini* species complex

Clades	Cephalothorax	Pleon	Chelipeds	Color morph
A1 and A2	Solid or mostly solid mid-dorsal stripe black or dark red-brown, variably continuing from carapace onto pleon	Mid-dorsal paired dark red blotches of variable intensity	Conspicuous dark red or brown dotting on major claw	M1
B1.1 and B1.2	Mid-dorsal band primarily comprised of small dark red or brown spots	Orange with small red-brown spots	Conspicuous dark red or brown dotting on major claw	M2
B2	Mid-dorsal band dark-red or brown, mostly without spotting	Orange with no or slight paired orange blotches mid-dorsally, sometimes with pale spotting	Dotting on major claw pale and inconspicuous	M3
C	Mid-dorsal pigmentation consisting of pairs of large dark-red blotches	Paired large and conspicuous dark-red blotches along mid-line, smaller blotches laterally	Conspicuous dark red or brown dotting on major claw	M4

within expectations based on the relative abundances of A1 and A2 (binomial test: $\chi^2 = 0.57$, df = 1 and $p = 0.45$). Similarly, B1.1-B1.2 pairings occurred in 67% of corals with B1.1 or B1.2 compared to 28% of corals with B1.1-B1.1 pairings and 5% of corals with B1.2-B1.2 pairings, proportions not different from expectations based on the abundances of B1.1 and B1.2 (Figure 5A). In addition, for several females of the A1-A2 or B1.1-B1.2 pairs, it is likely that their eggs were fertile, as evidenced by the presence of eyes visible through the membrane (Figure 5B).

DISCUSSION

In this study, we investigate the extent of cryptic speciation within one of the most ecologically important mutualists of corals in the Indo-Pacific region, the snapping shrimp *Alpheus lottini*. Using a combination of molecular analyses, color patterns, and distribution records of mating pairs from across the geographic range of *A. lottini*, we reconstructed a comprehensive and detailed phylogeography of this species complex. We find four highly divergent genetic lineages that exhibit assortative mating and distinct color patterns exist within the *A. lottini* species complex. Lineage diversity peaks in the Mariana Islands, located outside of the Coral Triangle, and gradually decreases toward the eastern Pacific and the Indian Ocean, suggesting a Pacific origin for the species complex. While there are broad differences in geographic distribution, we also find extensive overlap between the distribution ranges of these lineages indicating potential speciation primarily driven by allopatric processes with possible ecological or micro-allopatric processes playing a role as well. The discovery of unrecognized lineages in a comparatively well-studied group highlights the importance of accurately identifying, classifying and characterizing cryptic species in ecological and conservation studies, as different species may not be functionally equivalent for coral reef resistance or resilience.

Species boundaries, taxonomy, and ecological implications

Confidently delineating species often requires multiple lines of evidence.[54] Diagnostic morphological characters are not always obvious in small or colorless taxa; conversely, there is a higher risk of over-splitting larger and colorful species. Similarly, molecular data may identify population level subdivisions and ancestral polymorphisms that can be wrongly interpreted as evidence for reproductively isolated species without additional data. Our integrative approach using molecular, morphological, ecological and behavioral data strongly suggests that the *Alpheus lottini* species complex includes at least four cryptic, reproductively isolated species: (1) *A. lottini* sp. A (clades A1 and A2 combined), (2) *A. lottini* sp. B1 (clades B1.1 and B1.2 combined), (3) *A. lottini* sp. B2, and (4) *A. lottini* sp. C. These findings confirm the previous species delineations of clades A and B[50] as well as of clade B1, B2 and C[53] previously based on limited molecular data (low number of specimens, localities or molecular markers). They also highlight the split of B1 into two distinct well-supported clades. Each of these four groupings is characterized not only by well-supported genetic differences but also by a distinct color pattern, and in three cases strong evidence of assortative mating. Conversely, we found insufficient evidence to consider clades A1 and A2 or B1.1 and B1.2 as distinct species. Both A1 and A2, and B1.1 and B1.2, overlap in distribution. In addition, A1 and A2 specimens, as well as B1.1 and B1.2 specimens, had indistinguishable coloration, and mixed type heterosexual pairs were repeatedly found in the same corals, with some females carrying what appeared to be fertile clutches (observation of eyes in eggs), suggesting no mechanism of behavioral incompatibility or reproductive isolation.[55]

Competition for host space may have been an equally important mechanism for the evolution of coral mutualists in the *A. lottini* complex. Ecological processes can promote evolutionary diversification if divergent selection leads to character displacement, behavioral differences, or habitat differences in the presence of gene flow.[56] Competition-driven speciation may occur via host shift, as shown in a group of coral associated fish,[57] for which novel habitats combined with strong host fidelity promoted diversification under disruptive selection. Speciation via host selection has also been suggested in coral-associated snails[58] and anemone-associated snapping shrimps.[26] *Alpheus lottini* has been considered to use pocilloporid hosts of different species indiscriminately. However, as the taxonomy of pocilloporid corals remains largely unresolved with the presence of many cryptic lineages (e.g.,[59,60]) studies may have missed preferential associations. Also potentially

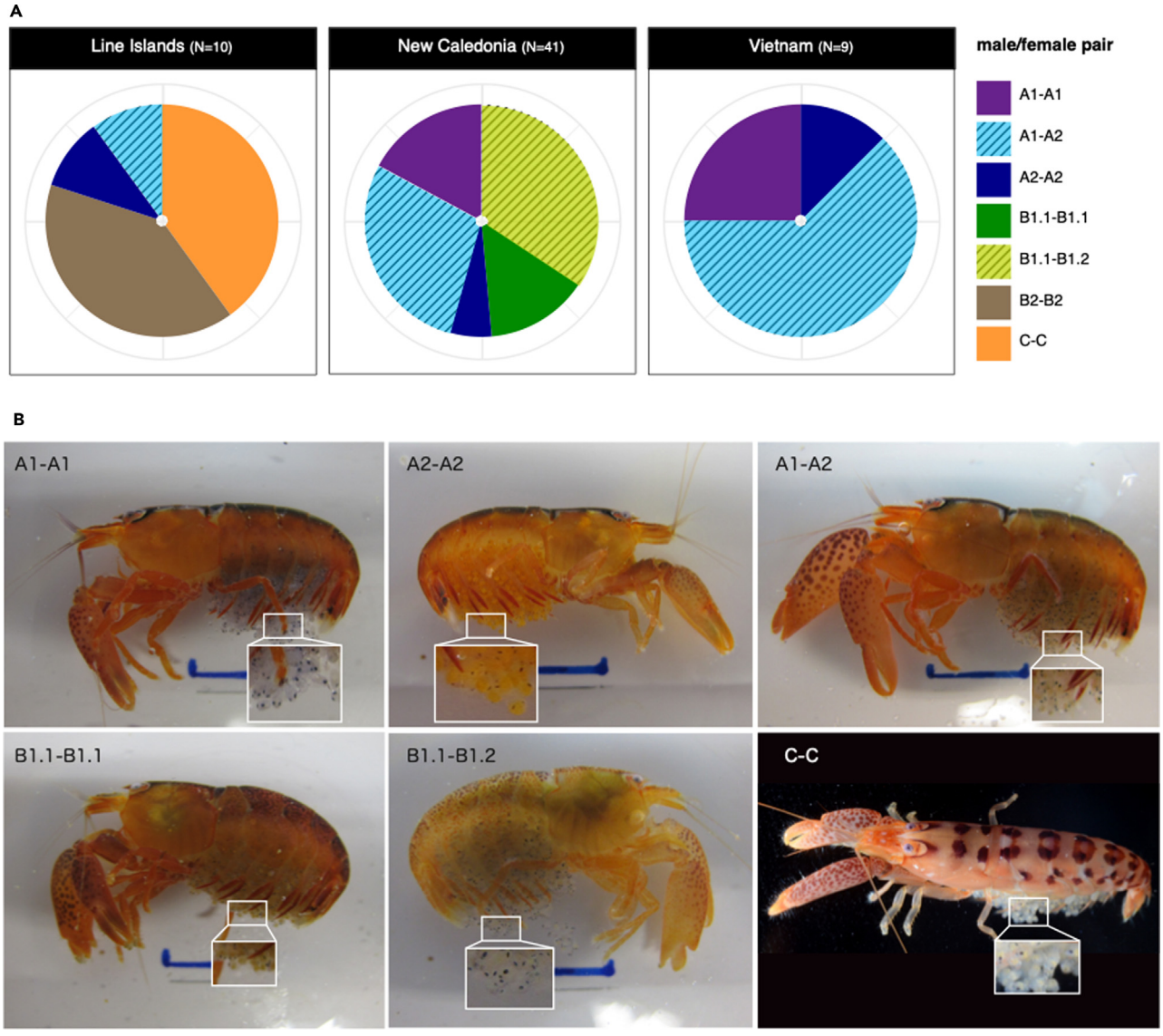

Figure 5. Heterosexual pairing of *A. lottini* with respect to clade

(A) Heterosexual pairs of *Alpheus lottini* in colonies of *Pocillopora* spp. at three locations where several genetic clades occur in sympatry. Usually, no more than one adult male and one adult female occur within each coral colony (although juveniles may be present in addition to the pair).

(B) Pictures of females' eggs showing eyes visible through the membrane observed in different heterosexual pairs.

underappreciated is the possibility of partitioning host resources by microhabitat, either related to the host morphology itself, or related to host habitat (e.g., depth zonation or wave energy).[61]

Regardless of the mechanisms, patterns of male-female pairing show that many clades in this species complex have evolved mechanisms for assortative mating and reproductive isolation where they co-occur. In socially monogamous snapping shrimps, heterosexual individuals recognize each other using a combination of olfactory and chemosensory (antennal contacts) cues.[62,63] Both male and female defend their host territory with their snapping claw.[64] Behavioral and reproductive incompatibility can occur within 3 million years in allopatry, and potentially much more rapidly in the presence of gene flow under divergent selection.[55] The presence in sympatry of different clades at some locations (e.g., Mariana Islands) strongly suggests the evolution of some mechanisms to favor reproductive isolation of derived clades.

Our results also have taxonomic implications. Among the delimited putative species, some may correspond to previously described taxa, including *A. lottini*, while others may be new to science. The existence of six additional nominal species described from across the Indo-Pacific, some lacking detailed descriptions and all currently placed under the synonymy of *A. lottini*,[36] complicates taxonomic revision. Fortunately, although Guérin-Méneville's original type material of *A. lottini* was lost,[65] the type locality for *A. lottini* s.s. is the Red Sea, where only clade A, the most wide-ranging of all the species, occurs. Thorough examination of specimens will be needed to identify morphological and

morphometric characters diagnostic for each of the four species, although truly diagnostic morphological characters are not always readily found in closely related species of *Alpheus*.[27,34] Examination of type material (if extant) of the current synonyms of *A. lottini* and consideration of type localities will be required to match existing names when possible to the four clades recovered in the present study, and for morphs where this is not possible, new names and formal descriptions will be required. In addition, species delimitation studies will be needed to test further the status of the A1 and A2, and the B1.1 and B1.2 types[26,66] and to confirm lack of inter-morph mating pairs where B1 and B2 are sympatric.

Phylogeography

It is possible to gain insights on mechanisms of divergence using comparisons of present-day distribution ranges among closely related taxa. Broadly speaking, taxa that recently diverged in allopatry with little or no gene flow are more likely to have non-overlapping distributions, whereas taxa that recently diverged in sympatry under divergent selection are more likely to have overlapping distributions. Partially overlapping or adjacent ranges (parapatric) may indicate that divergent selection and limited gene flow both drove diversification, or that range expansion or shift occurred after diversification. Our results suggest that a combination of evolutionary processes in the absence and in the presence of gene flow among the six clades is driving the diversification of the *A. lottini* species complex.

There has been particular interest in understanding patterns of divergence across the tropical Indo-Pacific. For many conspicuous species, such as fishes and corals, species diversity peaks in the Coral Triangle, otherwise known as the Indo-Malay-Philippine Archipelago.[67–69] Whether species richness is also highest in the Coral Triangle for many small invertebrates that make up the vast majority of marine diversity remains unknown for most groups. This is due to the relative lack of sampling in this area, e.g., as shown for stomatopods and decapods.[70] Our results show that clade diversity in the *A. lottini* species complex does not peak in the Coral Triangle, but rather further east in the Mariana islands, where all six clades occur. Interestingly, the Mariana Islands was also found to be the diversity hotspot for a group of hermit crabs (*Calcinus*), a pattern that the authors interpreted to be the result of a preference for oceanic islands.[71] Continental shores and oceanic habitats differ in many ways, including levels of nutrients (and hence primary productivity), sedimentation, predation and competition. *Pocillopora* corals exposed to terrigenous runoff tend to be smaller with thicker branches and hence provide less living space for associated shrimps and crabs.[11] Under these conditions, some *A. lottini* clades may not be able to sustain viable populations. More collections are needed to confirm that clade B2 and C are effectively absent from the Coral Triangle and also to determine if there are clades that remain overlooked and are unique to the Coral Triangle.

The diversity in *A. lottini* clades decreases from the Western Pacific toward the periphery of the Indian and Pacific Oceans. A single clade, A2, occurs in the Western Indian Ocean, whereas its sister clade, A1, is the only one extending its distribution to the tropical Eastern Pacific (Figure 2). However, an earlier COI-based analysis showed that at least two distinct clades occur in the waters of Clipperton Island [see van Wormhoudt in[72]], highlighting the need for additional sampling throughout the Eastern Pacific (Clipperton, Rapa Nui, Mexico, Panama).

The broad Indo-Pacific distribution of clade A2, and the Pacific-wide distribution of clade A1, suggest extensive dispersal abilities. In particular, the Eastern Pacific barrier, a 5400 km stretch of seawater that separates the islands of the Central Pacific from the islands and continental coast of the Eastern Pacific, is a barrier to gene flow for many marine species.[73,74] Thus, the distribution of clade A1 suggests high dispersal potential. Rafting (e.g., on floating wood, plastic debris or pumice) has been proposed as a one possible mechanism for the long-distance dispersal of some marine species.[75] Juvenile shrimps could easily be attracted to and cling to floating substrates which can hold scleractinian corals [e.g., pocilloporids[76]]. Pelagic larvae may also connect populations across broad geographical scales. However, current knowledge of larval durations in *Alpheus* remains limited to laboratory studies[77,78]; a larval duration of at least 25–29 days was described for *A. heterochaelis*.[79]

Also noteworthy is the presence of clades C and B2 in Hawaii, one of the most isolated island groups in the Indo-Pacific. Hawaii is made up in part of relatively young islands ranging from 25 to 0.75 Ma[80] that were progressively colonised by marine and terrestrial species as they formed. It is now home to numerous marine endemic species; 20% of mollusks,[81] 25% of red algae[82] and 24% of fish[83] that occur in Hawaii are found nowhere else. Hawaiian populations also show some level of genetic distinctiveness in species widely distributed in the Pacific.[84]

The most distinctive group, clade C (Figure 1A), occupies the narrowest geographical distribution in the species complex, being restricted to the northern periphery of the Central and Western Pacific (Figures 2 and 3). This pattern suggests a Pacific origin of this group that was dated to have diverged ~20 million years ago (Figures 1A and 1B) before spreading and diversifying throughout the Indian and Pacific Ocean (given the limited fossil record for snapping shrimps, this is almost certainly an underestimate). Three other clades restricted to the Western and Central Pacific (B1.1, B1.2 and B2) are characterized by contrasting distribution patterns that demonstrate the complex evolutionary history of this species complex.

Conclusion

Our multidisciplinary approach highlighted the complex evolutionary history of the *A. lottini* species complex. Previous ecological studies assumed that *A. lottini* represented a single species, or that the services provided by different color morphs were largely redundant (e.g.,[42]). However, a more recent survey found that sibling species interact with other members of coral-guild communities in very different ways, suggesting that cryptic species may not be functionally equivalent.[11] Additional work is needed to understand subtle differences in the ecological role of these cryptic taxa, including how they interact with foundational species and how these interactions might differ among species, particularly when they occur in sympatry. In addition, these results are a reminder that even the most iconic, distinctive, ecologically important and well-studied taxa can contain unrecognized species, posing challenges for researchers and conservation efforts.

Limitations of the study

Although we made efforts to include specimens from across the vast distribution range of *A. lottini*, our sampling may not capture the full clade diversity within this species complex. The "Coral Triangle" and the tropical Eastern Pacific, for example, were not extensively sampled. Furthermore, our study focused on a limited number of mitochondrial and nuclear genes. A genome-wide approach with a larger number of genetic markers may provide a better understanding of the evolutionary relationships and species boundaries within the *A. lottini* complex. In addition, future work will be necessary to identify or clarify the four presumed species as part of a taxonomic revision of the *A. lottini* complex.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by the lead contact, H.R. (heloise.rouze@gmail.com).

Materials availability

Most of *Alpheus lottini* specimens used in this study have been deposited in Museum collections, under the same unique IDs as available in Table S1. Materials reported in this paper may be provided by the lead contact, H.R. (heloise.rouze@gmail.com) upon request.

Data and code availability

- Sequences data and metadata dataset are available on GenBank and as supplementary file (Table S1).
- Code required to reproduce the data analyses is available from the lead contact upon request.
- Any additional information or data required to reanalyze the data reported in this paper is available from the lead contact upon request.
- All sequences generated for this study are available on NCBI GenBank under accession numbers indicated in Table 3 and in STAR Methods.

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AUTHOR CONTRIBUTIONS

H.R., N.K., and M.L. conceived the ideas; H.R. and M.L. produced the sequence data; H.R., C.H., and H.W. conducted phylogenetic analyses; H.R. performed data analysis and prepared tables and figures; A.A. conducted morphological analyses and research on synonymy; H.R. and M.L. wrote the first draft and led the writing with the contributions of N.K., A.A., C.H., and A.V.W.; all authors reviewed the manuscript.

DECLARATION OF INTERESTS

All authors had no conflict of interest to declare.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Alpheus lottini	Available a Table S1	Available a Table S1
Critical commercial assays		
Phenol chloroform DNA extraction	Autogenprep 965	Autogen, Holliston, MA
GoTaq G2 Hot Start master mix	Promega	Cat#M7422
Deposited data		
Raw amplicon sequencing data	This study	GenBank submissions: PQ124161-PQ124672, PQ092987-PQ093499, PQ084090-PQ084543
Oligonucleotides		
LCO1490	Folmer et al.[85]	5′-GGTCAACAAATCATAAAGATATTGG-3′
HCO2198	Folmer et al.[85]	5′-GGTCAACAAATCATAAAGATATTGG -3′
H7188	Knowlton et al.[6]	5′-CATTTAGGCCTAAGAAGTGTTG-3′
COIF	Kessing et al.[86]	5′-CCAGCTGGAGGAGGAGAYCC-3′
16Sar	Palumbi[87]	5′-CGCCTGTTTATCAAAAACAT-3′
16Sbr	Palumbi[87]	5′-CCGGTCTGAACTCAGATCACGT-3′
28S-C1	Le et al.[88]	5′-ACCCGCTGAATTTAAGCAT-3′
28S-D2	Le et al.[88]	5′-TCCGTGTTTCAAGACGGG-3′
MyHC1124/MyHC1806	Williams et al.[52]	Table S2
H3F1/H3R1	Colgan et al.[89]	Table S2
Software and algorithms		
R v4.2.2	R Core Team	https://www.r-project.org
Geneious v7.0.4	Kearse et al.[90]	https://www.geneious.com
Mega11	Tamura et al.[91]	https://www.megasoftware.net/citations
Arlequin v 3.11	Excoffier et al.[92]	http://cmpg.unibe.ch/software/arlequin3/
MrBayes	Ronquist and Huelsenbeck[93]	https://nbisweden.github.io/MrBayes/index.html
Phylobayes v4.1	Lartillot et al.[94]	https://bioweb.pasteur.fr/packages/pack@phylobayes@4.1c

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The experimental procedure was performed on conserved *Alpheus lottini* specimens coming from museum collections as detailed in Table S1 and data issued from previous projects.

METHOD DETAILS

Specimen collection and DNA extraction

A total of 549 specimens of *A. lottini* collected throughout the Indian and Pacific Oceans were used in this study. Detailed collecting information is provided in Tables 2 and S1. Specimens coded with 'UF' were deposited in the Florida Museum of Natural History, University of Florida, Gainesville, FL, USA (Table S1). Specimens from the Southern Line Islands (n = 43) and French Polynesia (n = 159) in the Central-Western Pacific, New Caledonia (n = 100) in the South-Western Pacific, Vietnam (n = 18) in the South-China Sea, and Réunion Island (n = 62) from the Mascarene Islands in the South-Western Indian Ocean were collected during surveys of invertebrates associated with *Pocillopora* spp. At some of these localities (Southern Line Islands, New Caledonia and Vietnam), the occurrence of male-female pairs in corals was recorded. *Alpheus lottini* is not on the endangered species list, and hence no specific authorization was required for this study. However, approval was granted when specimens were collected in marine protected areas (see Acknowledgments). All specimens were preserved in >80% ethanol. Total genomic DNA was extracted from tissue subsamples using the automated phenol chloroform protocol (i.e., mouse tail tissue) with the Autogenprep 965 (Autogen, Holliston, MA).

Table 2. *Alpheus lottini* specimens used in this study by clade and region

Main oceanic division	Specific locality (country, territory, etc.)	A1	A2	B1.1	B1.2	B2	C
Western Indian Ocean	Djibouti (Red Sea)		4				
	Saudi Arabia (Red Sea)		18				
	Mascarene Is. (+12A unidentified)		58				
	Europa Is.		1				
Central Indian Ocean	Maldives		5				
East Indian Ocean	W Australia (Ningaloo)	2	1				
Western Pacific	Taiwan (+3A unidentified)	2	2	4	3		
	Vietnam	9	7		2		
	Japan (Okinawa)	1	1				
	Papua New Guinea (+1B1 unidentified)	15	22	8	10		
	Mariana Is.	2	2	3	2	13	2
	E Australia (Great Barrier Reef)	2	1	2	1		
	New Caledonia (+2A, +7B1 unidentified)	27	18	30	17		
Central Pacific	Samoa	2		2	1		
	Hawaii					4	1
	Line Is. (+2A unidentified)	8	5			15	13
	Society Is. (+4A, + 3B1 unidentified)	28	3	84	2	5	
	Tuamotu Is. (+4A unidentified)	23	2	1		1	2
East Pacific	Clipperton Is.	10					

The number of specimens for which the clade identification failed due to mtCOI or mt16S gene amplification issues is indicated between parentheses: (+A) no mtCOI sequence available to discriminate between clades A1 and A2, (+B1) no mt16S sequence available to discriminate between clades B1.1 and B1.2.

Molecular analyses

PCR, sequencing and sequence alignment

To delineate major clades within the *A. lottini* species complex, two hypervariable mitochondrial markers, a ~658 bp fragment of the 5′ end of the Cytochrome *c* Oxidase subunit I (COI) gene (the animal "barcode" region) and a ~520 bp fragment of the 16S gene, were amplified across all specimens using previously designed primers [COI: LCOI490/HCO2198[85]; 16S: 16Sar/16Sbr[87]] (Tables 2 and S2). Amplifications succeeded for the majority of specimens with 512 and 513 sequences obtained for COI and 16S genes, respectively (Table 3). A subset of individuals from each type (A1, A2, B1.1, B1.2, B2 and C) delineated with COI and 16S genes was then selected for amplification and sequencing of three nuclear genes commonly used in phylogenetic studies of Caridea and Decapoda[52,95]: ~304 bp of the encoding myosin heavy chain (MyHC) gene, ~344 bp of the histone H3 gene (Table S2; primers from[89]), and ~707 bp of the large 28S rRNA gene (Table S2; primers from[88]). Finally, a second fragment (~564 bp) of the mitochondrial COI gene targeted in previous studies of the *A. lottini* complex[50,51,86] was amplified for a small subset of specimens, in order to match type nomenclature between studies (comparison with 13 previously published sequences; Figure S1). This alternative COI fragment does not overlap with the 658 bp animal "barcode" region; primers H7188 and COIF (Table S2) were used following protocols described by Knowlton and Weigt.[50] Note that these primers did not amplify COI for specimens belonging to type B1 (Figure S1).

Table 3. Statistics of mitochondrial (mtCOI, mt16S) and nuclear (28S rRNA) gene sequences for each genetic clade of *Alpheus lottini*

Loci/species		N	Haplo	S	Theta (π)	Tajima's D	GenBank nos. (all clades)
COI	(Sites: 582)	–	–	–	–	–	–
	A1	131	53	72	0.004	−2.557**	*PQ124161-PQ124672*
	A2	151	63	78	0.003	−2.646**	–
	B1	176	32	54	0.014	−2.304**	–
	B2	37	22	33	0.0045	−2.199**	–
	C	17	8	10	0.003	−1.095	–
	Total	**512**	**178**	–	–	–	–

(*Continued on next page*)

Table 3. *Continued*

Loci/species		N	Haplo	S	Theta (π)	Tajima's D	GenBank nos. (all clades)
16S	(Sites: 519)	–	–	–	–	–	–
	A	284	90	73	0.007	−2.099**	*PQ092987-PQ093499*
	B1.1	135	17	16	0.001	−1.991	–
	B1.2	38	7	5	0.001	−1.187	–
	B2	38	16	17	0.002	−2.236**	–
	C	18	9	9	0.005	−0.755	–
	Total	**513**	**139**	–	–	–	–
28S	(Sites: 666)	–	–	–	–	–	–
	A	245	2	NA	NA	NA	*PQ084090-PQ084543*
	B	191	1	NA	NA	NA	–
	C	17	1	NA	NA	NA	–
	Total	**453**	**4**	–	–	–	–

Sites, length of the final alignment (bp); N, number of alleles used in the analysis; Haplo, number of haplotypes; S, number of segregating sites; Theta (π), nucleotide diversity; Tajima's D (*p*-value) and Genbank Accession numbers. Tajima's D values with (**) indicate significant deviation from the neutral model of evolution (when <-2 or >+2).

Polymerase Chain Reaction (PCR) was performed in a total of 19 μL including 6.6 μL of nuclease-free water, 10 μL of GoTaq G2 hotstart master mix, 0.2 μL of BSA (100X), 0.6 μL (10 μM) of each forward and reverse primer, and 1 μL of genomic DNA. Cycling conditions for each gene and primer set are detailed in Table S2. The PCR product was visualized on a 1.5% agarose TBE gel. Successful amplifications were purified with ExoSAP and sequenced in both directions on an ABI 3730cL 96-well capillary sequencer at the Laboratory of Analytical Biology at the Smithsonian National Museum of Natural History.

All DNA sequences were trimmed and aligned using ClustalW implemented in Geneious version 7.0.4.[90] Given the lack of variability within MyHC and H3, these markers were not used in downstream analysis. All sequences were submitted to GenBank under accession numbers indicated in Table 3.

Phylogenetic reconstruction

We first computed separate analyses for each target marker gene (COI, 16S and 28S; Figures S2–S4), using the best-fit model of nucleotide substitution selected using both the Bayesian (BIC; Schwarz, 1974) and Akaike[96] information criteria (AIC), as implemented in jModelTest v.2.1.1[97,98]. Bayesian inferences (BI) of phylogenetic relationships were performed using MrBayes version 3.1.2.[93] Markov Chain Monte Carlo (MCMC) analyses included two independent runs of 5M generations, with tree sampling every 1000 generations. Convergence for each run was determined when the average standard deviation of split frequencies was <0.01. Maximum Likelihood (ML) phylogenetic analyses were performed in MEGA version 11.[91] The ML analysis was performed with 1000 bootstrap replicates to evaluate node support.

Then, a combined phylogenetic analysis was conducted using 16S, COI (the "animal" barcode region) and 28S sequences from 218 specimens. The concatenated dataset was partitioned by gene (1849 bp in total), and the best-fit model of nucleotide substitution was selected statistically for each partition by AIC calculation using PartitionFinder v.1.1.0[99] and applied to each partition (COI: HKY + I (frame 1), GTR + I (frame 2), and GTR + G (frame 3); 16S and 28S: GTR + I + G). For the protein-coding COI gene, the intra-partition of three blocks in the corresponding alignment was dissociated in order to separate out codon positions. The phylogenetic reconstruction of the concatenated tree was performed on each locus using Bayesian Inference with MrBayes as detailed above. In addition, Maximum Likelihood (ML) reconstruction of the concatenated tree was conducted using RaxML v.8[100] with 1000 bootstrap replicates and partitioned according to the results of Partition Finder. Sequences of *Alpheus lobidens* were used as an outgroup. Nodal support for Bayesian and ML phylogenetic trees (single and concatenated) was estimated by Bayesian posterior probabilities (BPP) and ML bootstrap (BS) values, respectively, and visualized with FigTree v. 1.4.4..[101]

Divergence times were estimated using the Bayesian MCMC method as implemented in Phylobayes v. 4.1.[94] A concatenated COI and 16S sequence alignment was used for the input dataset. This alignment included a subset of 30 individuals belonging to each one of the different clades delineated using Bayesian and ML phylogenetic analyses. Two transisthmian sister-species pairs (*Alpheus javieri* - *Alpheus bouvieri* and *Alpheus colombiensis* - *Alpheus estuariensis*) were included to calibrate the tree, and *Synalpheus occidentalis* was used as an outgroup. We used three calibration intervals for dating nodes. Divergence times for transisthmian sister-species were based on the final closure of the Isthmus of Panama at ~3 Ma.[50,102] Calibration of the crown node for the entire genus was set at 18 Ma, based on the earliest fossilized claw morphotypes identified as *Alpheus*.[103,104] We used an informative age-root prior (30 Ma), as recommended by the program developer, corresponding to the oldest fossil record for *Alpheus*.[103]

Distances, evolution models and geographical distribution

MEGA11 and Arlequin v. 3.11[92] were used to examine summary statistics within each type. Haplotype diversity, nucleotide diversity and Tajima's D test of neutrality[105] were calculated for each discriminated type. A minimum spanning tree was drawn among COI haplotypes from 548 specimens using HapStar v. 0.7.[106] The distribution of each genetically delineated type was plotted on a world map.

Color and morphology

Photographs of living shrimps were available for 209 specimens used in the study. They were sorted by type identified by the concatenated phylogenetic analysis (COI, 16S and 28S). We classified color patterns, which have been shown to be species-diagnostic in many other species of *Alpheus*,[27,31,107] using the following criteria: (1) the overall coloration and pigmentation pattern of the dorsal carapace and abdomen and (2) the coloration and pigmentation pattern of the major chela.

Mate choice: Male-female pairs in coral host

We observed 75 male-female mating pairs from the Central Pacific (Southern Line Islands: $N = 10$ pairs), South-Western Pacific (New Caledonia: $N = 41$ pairs; from[11]) and Western Pacific (Vietnam: $N = 9$ pairs). The hypothesis that pairs of heterospecific individuals and pairs of conspecific individuals (based on genetic delineation) are in equal proportions at each site was tested with binomial tests using R software.