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Deconstructing an assemblage of "turtle" barnacles: species assignments and fickle fidelity in *Chelonibia*

John D. Zardus · David T. Lake · Michael G. Frick · Paul D. Rawson

Abstract Barnacles in the genus *Chelonibia* are commensal with a variety of motile marine animals including sea turtles, crustaceans, and sirenians. We conducted a worldwide molecular phylogenetic survey of *Chelonibia* collected from nearly all known hosts to assess species relationships, host-fidelity, and phylogeographic structure. Using DNA sequences from a protein-coding mitochondrial gene (COI), a mitochondrial rRNA gene (12S), and one nuclear rRNA gene (28S), we found that of four species, three (*C. testudinaria*, *C. patula*, and *C. manati*) are genetically indistinguishable. In addition, we show each utilizes a rare androdioecious mode of reproduction involving complemental males. In contrast, the fourth species (*C. caretta*), which is hermaphroditic and specializes on turtles, is genetically distinct—leading to the conclusion that the three former taxa are morphotypes of the same species and should be synonymized under *C. testudinaria*. Phylogenetic analysis resulted in three geographic clades (Atlantic, Indian Ocean/western Pacific, and eastern Pacific) with haplotype parsimony networks revealing no shared haplotypes among geographic regions. Analysis of molecular variance detected significant differences among sequences by region ($p < 0.005$); conversely, there were no significant differences among sequences when grouped by host or taxonomic designation. Average pairwise genetic distances were lower between the eastern Pacific and Atlantic clades (0.053 ± 0.006) than between the eastern Pacific and Indian Ocean/western Pacific clades (0.073 ± 0.008), suggesting Atlantic and eastern Pacific populations were connected more recently, perhaps until the rise of the Isthmus of Panama. Host use by *Chelonibia* morphotypes is discussed along with speculation on possible ancestral hosts and support for a "turtle-first" hypothesis.

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Introduction

Commensal associations between species, wherein one partner in the relationship benefits and the other is unaffected, are placed somewhere between mutualism and parasitism on the symbiosis spectrum (Leung and Poulin 2008; Frick and Pfaller 2013). Flexible host partnering occurs in facultative commensalisms, whereas obligate commensalisms involve species-specific associations. Regardless of the degree of specialization in a particular host/symbiont relationship, it is increasingly recognized that symbiotic systems are important sources of evolutionary novelty (Sapp 1994; Vermeij 1994; Zook 2004) and commensalism may well be a common tipping point in the path to coevolution.

Barnacles that are obligate associates of sea turtles, the so-called turtle barnacles, present a compelling case for examining the dynamics of commensalism. Balanomorph or acorn barnacles of the superfamily Coronuloidea include approximately two dozen living species in several genera and families that are best known for living attached to turtles and whales (Pilsbry 1916; Newman and Ross 1976; Monroe and Limpus 1979; Frick and Zardus 2010; Frick et al. 2010a, b; Ross and Frick 2011; Hayashi 2013). Perhaps the most frequently encountered coronuloids are members of the genus *Chelonibia* Leach 1817, of which five living species have been described, each documented as commensal with a particular suite of hosts—ranging from crustaceans and chelicerates to sea turtles and sirenians (see Table 1). These barnacles are considered obligate commensals with narrow host specialization. Some affiliate with several host species, but no single barnacle species is reported to occur on all the documented hosts; though *Chelonibia testudinaria* (Linnaeus 1758) is reported from all species of sea turtles. The enigmatic *Chelonibia ramosa* Korschelt 1933 is known only from a written description of a single individual found on an unspecified sea turtle (Korschelt 1933) and is, therefore, excluded from our study.

The effects of chelonibiid barnacles on their hosts appear generally benign, given the paucity of records reporting negative effects and lack of correlation with turtle health (Stamper et al. 2005), but their presence in high numbers or in unusual locations (e.g., attaching in wounds and overgrowing eyes) certainly has adverse consequences for the host (Zardus et al. 2007). Unlike rhizocephalan barnacles which are parasites in the strict sense of drawing nutrition from their hosts (Høeg 1995), turtle barnacles merely use their basibont for substratum and transport. Benefits to the barnacles include increased dispersal, access to consistent feeding currents, and perhaps most importantly escape from predators (Foster 1987).

Host specificity is the distinguishing feature differentiating obligate from facultative commensalisms, and the various species of *Chelonibia* have historically been considered obligate host commensals and described according to their variation in morphology (Fig. 1). However, they do display morphological variation within species, and when shell morphologies are not entirely consistent or differ from established taxonomic characters, *Chelonibia* species are often identified by host type (Frick and Ross 2001). Usually, *C. manati* Gruvel 1903 (Fig. 1a) can be distinguished by its pleated shell plates, which produce finger-like extensions from the basal edge that aid in grasping the flexible skin of sirenians. A common diagnostic trait of *C. testudinaria* is a stellate pattern on the shell formed by open wedges at the sutures between shell plates, sculpted along their margins with indentations or "teeth" (Fig. 1b). The shell of *C. testudinaria* is thick

and of low aspect when compared to *C. patula* (Fig. 1c), which typically has wedges with smooth edges at the sutures, and is comparatively higher (taller) and thinshelled. *Chelonibia caretta* (Fig. 1d) presents a robust shell similar to, but not as thick as, *C. testudinaria* yet of higher aspect and with a uniform exterior surface lacking wedges at the sutures.

Pilsbry (1916) mentions phenotypic plasticity in *Chelonibia*, particularly in referring to *C. manati*-like forms removed from sea turtles, stating "there are certain barnacles in this series before me which, while possibly referable to *C. testudinaria* as varieties, have some of the characters of the West African species [*C. manti*]…I am giving names to these forms in order to call attention to their characters which might otherwise be overlooked by those having opportunity of seeing large numbers of turtle barnacles. Their status as races cannot yet be considered established." And he refers to some fossil forms of *Chelonibia* as "of the *testudinaria* type" that "afford no information on the phylogeny of the genus." He also remarks that these differences seem to correspond to host selection where *C. testudinaria* is "admirably adapted to the rough conditions of existence on the backs of sea turtles, the walls being enormously thickened and the stature low," whereas the relatively fragile and lighter *C. patula* is specialized for living on motile marine–estuarine animals, particularly crabs. Moreover, Henry's (1943) description of *Chelonibia patula dentata* (host unspecified) amalgamates characters of *C. patula* and *C. testudinaria* providing another indicator of phenotypic plasticity in the group. In examining large numbers of barnacles, we also have observed *Chelonibia* specimens that mingle the characters of these "species" in a variety of ways.

Past accounts indicate that *C. manati* is the most specific member of the genus—selecting manatees and occasionally loggerhead sea turtles as hosts (but see Seigel 1983)—followed by *Chelonibia caretta* (Spengler 1790) which attaches to only three of the seven extant species of sea turtles. Less selective is *C. testudinaria*, occurring on all sea turtles but also documented from saltwater crocodiles (Monroe and Garrett 1979), American alligators (Nifong and Frick 2011), terrapins (Seigel 1983), laboratory glassware (Zardus and Hadfield 2004), and slate and plastic settlement panels (Zardus, unpublished data). *Chelonibia patula* (Ranzani 1818), historically defined as a crab barnacle, is perhaps least selective and has been recorded from terrapins, (Ross and Jackson 1972), sea snakes (Badrudeen 2000), several different crustaceans (Ortiz et al. 2004; Cheang et al. 2013), and inanimate substrata (Relini 1980; Frazier and Margaritoulis 1990). A recent phylogenetic study using many individuals of *C. patula* and *C. testudinaria* collected from the vicinity of the South China Sea indicates that these two taxa are in fact the same species

Table 1 Provenance (by locality and host) of the partial gene sequences for four nominal species of *Chelonibia* barnacles and three outgroup taxa used in this study along with their assignment to species, region, and host for AMOVA analysis and GenBank accession number

Nominal species	Locality (region)	Host (host assignment)	Accession No.'s		
			CO1	12S	28S
C. caretta 1	Wassaw Is., GA (Atlantic)	Sea turtle (loggerhead)	AY174368	KF424540	KF424541
C. caretta 2	Florida Bay, FL (Atlantic)	Sea turtle (loggerhead)	JN589810	JN589834	JN589858
C. caretta 3	Mona Is., Puerto Rico (Atlantic)	Sea turtle (hawksbill)	JN589811	JN589835	JN589859
C. caretta 4	Barbados, West Indies (Atlantic)	Sea turtle (hawksbill)	JN589812	JN589836	JN589860
C. caretta 5	Milman Is., Australia (Indian/W. Pacific)	Sea turtle (hawksbill)	KF042512	KF042492	KF042472
C. caretta 6	Mon Repos, Australia (Indian/W. Pacific)	Sea turtle (loggerhead)	KF042513	KF042493	KF042473
C. manati 1	Hutchinson Is., FL (Atlantic)	Sirenian (manatee)	JN589813	JN589837	JN589861
C. manati 2	(Manatee) River, FL (Atlantic)	Sirenian (manatee)	JN589814	JN589838	JN589862
C. manati 3	Crystal River, FL (Atlantic)	Sirenian (manatee)	JN589815	JN589839	JN589863
C. patula 1	Core Sound, NC (Atlantic)	Sea turtle (loggerhead)	JN589816	JN589840	JN589864
C. patula 2	Pamlico Sound, NC (Atlantic)	Horseshoe crab (crab)	JN589817	JN589841	JN589865
C. patula 3	Charleston Harbor, SC (Atlantic)	Horseshoe crab (crab)	JN589818	JN589842	JN589866
C. patula 4	Charleston Harbor, SC (Atlantic)	Horseshoe crab (crab)	JN589819	JN589843	JN589867
C. patula 5	Charleston Harbor, SC (Atlantic)	Stone crab (crab)	JN589820	JN589844	JN589868
C. patula 6	Charleston Harbor, SC (Atlantic)	Spider crab (crab)	JN589821	JN589845	JN589869
C. patula 7	Charleston Harbor, SC (Atlantic)	Blue crab (crab)	JN589822	JN589846	JN589870
C. patula 8	Folly River, SC (Atlantic)	Buoy (inanimate)	JN589823	JN589847	JN589871
C. patula 9	Mediterranean Israel (Atlantic)	Blue crab (crab)	–	DQ777621	EU082295
C. patula 10	Hong Kong (Indian/W. Pacific)	Mantis shrimp (crab)	JF823669	JF823874	–
C. patula 11	Malaysia (Indian/W. Pacific)	Mud crab (crab)	JF823674	JF823876	–
C. patula 12	Singapore (Indian/W. Pacific)	Mud crab (crab)	JF823689	JF823891	–
C. testudinaria 1	Wellfleet Beach, MA (Atlantic)	Sea turtle (loggerhead)	JN589824	JN589848	JN589872
C. testudinaria 2	Virginia Beach, VA (Atlantic)	Sea turtle (loggerhead)	JN589825	JN589849	JN589873
C. testudinaria 3	Bulls Bay, SC (Atlantic)	Sea turtle (loggerhead)	JN589826	JN589850	JN589874
C. testudinaria 4	Pelagic SE US (Atlantic)	Sea turtle (loggerhead)	JN589827	JN589851	JN589875
C. testudinaria 5	Wassaw Is., GA (Atlantic)	Sea turtle (loggerhead)	AY174289	KF042494	KF042474
C. testudinaria 6	Keewaydin, FL (Atlantic)	Sea turtle (loggerhead)	AY174324	KF042495	KF042475
C. testudinaria 7	Kyparissia Is., Greece (Atlantic)	Sea turtle (loggerhead)	AY174354	KF042496	KF042476
C. testudinaria 8	West Indies (Atlantic)	Sea turtle (green)	JN589828	JN589852	JN589876
C. testudinaria 9	Uruguay (Atlantic)	Sea turtle (green)	JN589829	JN589853	JN589877
C. testudinaria 10	Rancho Nuevo, Mexico (Atlantic)	Sea turtle (Kemp's ridley)	JN589830	JN589854	JN589878
C. testudinaria 11	Midway Is., HI (Indian/W. Pacific)	Sea turtle (green)	KF042514	KF042497	KF042477
C. testudinaria 12	Hawaii Is., HI (Indian/W. Pacific)	Sea turtle (hawksbill)	KF042515	KF042498	KF042478
C. testudinaria 13	Galapagos Isls., Ecuador (E. Pacific)	Sea turtle (green)	KF042516	KF042499	KF042479
C. testudinaria 14	Baja, Mexico (E. Pacific)	Sea turtle (loggerhead)	AY174342	KF042500	KF042480
C. testudinaria 15	Baja, Mexico (E. Pacific)	Sea turtle (loggerhead)	AY174343	KF042501	KF042481
C. testudinaria 16	Taiwan (Indian/W. Pacific)	Sea turtle (hawksbill)	JF823740	JF823943	–
C. testudinaria 17	Taiwan (Indian/W. Pacific)	Sea turtle (loggerhead)	JF823749	JF823952	–
C. testudinaria 18	Taiwan (Indian/W. Pacific)	Sea turtle (green)	JF823751	JF823954	–
C. testudinaria 19	Hong Kong, China (Indian/W. Pacific)	Sea turtle (green)	KF042517	KF042502	KF042482
C. testudinaria 20	Ogasawara Isls., Japan (Indian/W. Pacific)	Sea turtle (green)	KF042518	KF042503	KF042483
C. testudinaria 21	Japan (Indian/W. Pacific)	Sea turtle (green)	KF042519	KF042504	KF042484
C. testudinaria 22	Senri Beach, Japan (Indian/W. Pacific)	Sea turtle (loggerhead)	AY174334	KF042505	KF042485
C. testudinaria 23	Magnetic Is., Australia (Indian/W. Pacific)	Sea turtle (green)	KF042520	KF042506	KF042486
C. testudinaria 24	Bowen, Australia (Indian/W. Pacific)	Sea turtle (green)	KF042521	KF042507	KF042487

Table 1 continued

Nominal species	Locality (region)	Host (host assignment)	Accession No.'s		
			CO1	12S	28S
C. testudinaria 25	Bundaberg, Australia (Indian/W. Pacific)	Sea turtle (flatback)	KF042522	KF042508	KF042488
C. testudinaria 26	Zululand, South Africa (Indian/W. Pacific)	Sea turtle (loggerhead)	KF042523	KF042509	KF042489
C. testudinaria 27	Ras al Jinz, Oman (Indian/W. Pacific)	Sea turtle (green)	KF042524	KF042510	KF042490
C. testudinaria 28	Masirah Is., Oman (Indian/W. Pacific)	Sea turtle (loggerhead)	KF042525	KF042511	KF042491
Outgroup taxa	Family	Locality	Accession No.'s		
			CO1	12S	28S
Chthamalus panamensis	Chthamalidae	Galeta, Panama (Caribbean)	JN589831	JN589855	JN589879
Conopea galeata	Archeobalanidae	Charleston Harbor, SC (Atlantic)	JN589832	JN589856	JN589880
Tetraclita stalactifera	Tetraclitidae	South Water Caye, Belize (Caribbean)	JN589833	JN589857	JN589881
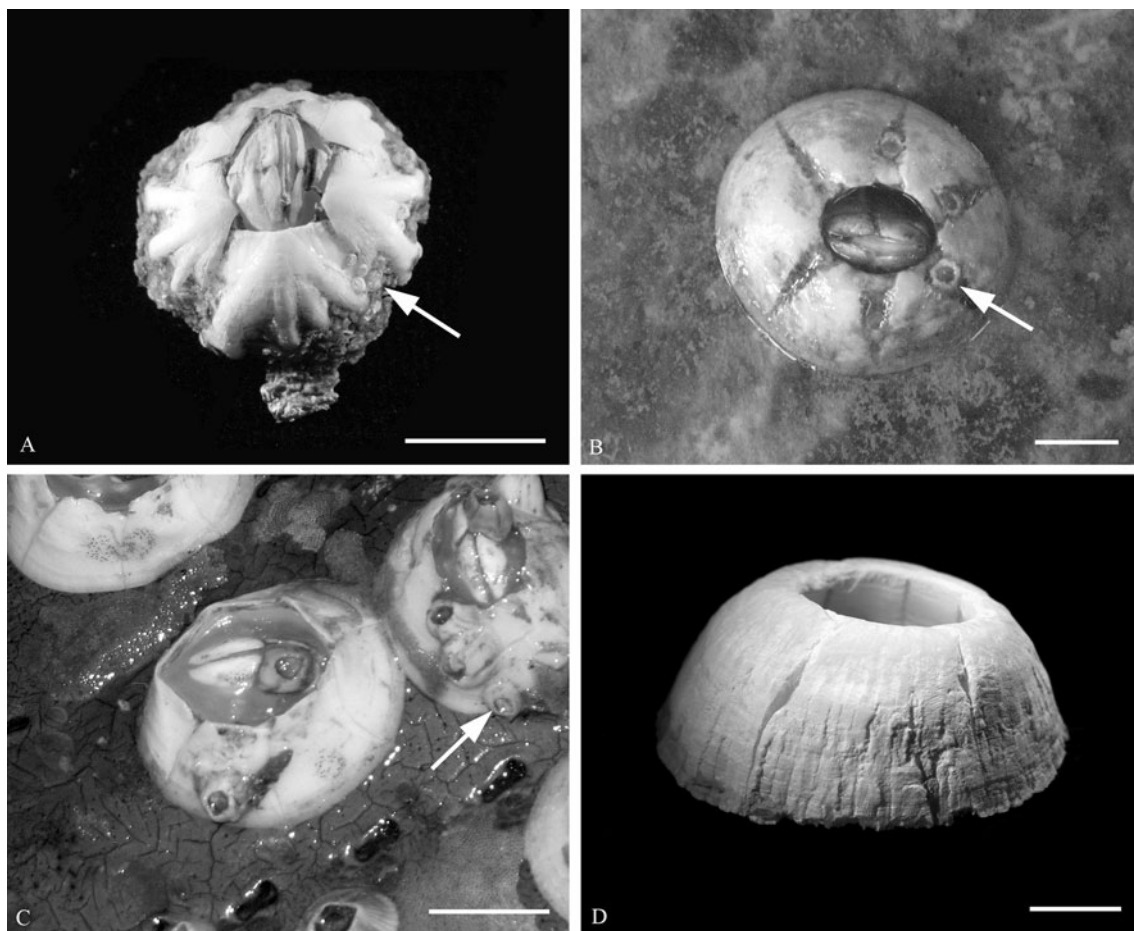
Fig. 1 The four extant described species of chelonibiid barnacles from various hosts. *Arrows* indicate attached conspecific complemental males. **a** *Chelonibia manati* removed from a manatee. **b** *Chelonibia testudinaria* on a green sea turtle. **c** *Chelonibia patula* attached to a horseshoe crab. **d** *Chelonibia caretta* taken from the carapace of a hawksbill sea turtle (*scale bar* 1 cm)

with fungible hosts and plastic morphology (Cheang et al. 2013).

The principle objective of our study was to compare host specificity among extant species of chelonibiid barnacles on a global scale using molecular phylogenetic methods. In particular, a series of analyses were performed to explicitly test barnacle species designations and fidelity to host. In addition, phylogenetic relationships and phylogeographic

patterns within the group were assessed. To this end, we undertook sampling of the genus *Chelonibia* with an emphasis on geographic diversity and host utilization.

Methods

Sample collection and DNA processing

Samples were obtained from horseshoe crabs, a variety of crustaceans, and from all common vertebrate host species—excepting olive ridley and leatherback sea turtles—from all oceans and several seas where *Chelonibia* occurs. Fully crossed sampling of host, barnacle, and locality was not possible because not all hosts and barnacles occur in all localities. For instance, *C. manati* is known only from the Caribbean, Gulf of Mexico, and western Africa (Pilsbry 1916; Stubbings 1965) where manatees occur, and in the case of olive ridley and leatherback sea turtles, reports of *C. testudinaria* with these turtle species are sparse (Rees and Walker 1993; Angulo-Lozano et al. 2007; Lazo-Wasem et al. 2011). On the other hand, some epibionts and hosts are widely present throughout the Atlantic Ocean, Mediterranean Sea, Caribbean Sea, Pacific, and Indian Oceans (Darwin 1854; Pilsbry 1916; Monroe and Limpus 1979; Frick and Slay 2000). A total of 35 specimens and 14 GenBank sequences of *Chelonibia* barnacles, the latter selected from all available to provide maximum genetic divergence and host and locality representation, were assembled from 5 species of sea turtles (out of 7 worldwide), 6 species of arthropods, the West Indian manatee, and a tethered buoy; from 11 locations in the Atlantic, 6 in the Caribbean and Gulf of Mexico, 2 in the Mediterranean Sea, 16 in the western Pacific, 2 in the eastern Pacific, and 3 in the Indian Ocean (Table 1). Three basally related barnacle species (Pérez-Losada et al. 2004; Cheang et al. 2013) were included as outgroup taxa. Samples from endangered host species were collected ethically, often from dead stranded animals, with permission and under the supervision of officers of appropriate state, federal, and international agencies listed in the acknowledgments. Upon collection, specimens were stored in 95 % ethanol for subsequent laboratory analysis. Voucher specimens were also deposited into the Peabody Museum of Natural History at Yale University, New Haven, Connecticut, and the Florida Museum of Natural History, Gainesville, Florida.

For DNA extractions, adductor muscle tissue and/or several cirri were removed from individual specimens under a stereo dissecting microscope and DNA isolated by proteinase-K lysis and centrifugation using a DNeasy Blood & Tissue Kit™ (Qiagen, Valencia, CA, USA). Three partial gene sequences were amplified from the samples by PCR for two mitochondrial genes, cytochrome *c* oxidase subunit I (COI) and ribosomal 12S (12S), and one nuclear gene, ribosomal 28S (28S). A portion of the COI marker was amplified using the primer pair LCO1490 and HCO2198 (Folmer et al. 1994). The primers 12SF and 12SR (Mokady et al. 1999) were used to amplify a segment of the 12S gene, and primers Rd4.5a and Rd6.2b (Whiting 2002) were used to amplify a region of the 28S gene. Each PCR reaction contained the following: 10–50 ng template DNA, buffer solution for a total $MgCl_2$ concentration of 1.5 mM/µL, dNTP's (200 mM/µL each), primer (0.15 pM/µL each), 1.0 unit *Taq* polymerase (Qiagen, Valencia, CA, USA), and distilled water to make a 25-µL reaction volume. Thermal cycling conditions for all three primer pairs were similar except for annealing temperatures and consisted of a one-time denaturing step of 97 °C for 1 min followed by 30–35 cycles of the following: denaturation at 95 °C for 30 s, annealing for 1–2 min, extension at 72 °C for 1–2 min, and a final extension at 72 °C for 10 min. Annealing temperatures for the three primer sets varied as follows: for COI, a two-step annealing at 50 °C for 1 min followed by 1 min at 40 °C and for 12S and 28S, a single step at 58 °C for 1 min for both.

For samples that were difficult to amplify or that resulted in ambiguous product, a methodology was employed to increase amplification specificity using tailed primers (Table 2) that anneal *and* extend at 72 °C (Weighardt et al. 1993). These primers were used in tandem with increased numbers of cycles with the same reaction mixes as above but with altered cycling conditions. For tailed COI reactions, the primer set LCO.t2 and HCO.t2 was used. Cycling began with denaturation at 95 °C for 1 min followed by 5–7 rounds of annealing and extension with the following steps: 50 °C for 30 s, 45 °C for 45 s, 40 °C for 1 min, and 72 °C for 2 min. This was followed by 35–38 rounds of denaturation at 95 °C for 30 s and annealing/extension at 72 °C for 1 min. For the tailed 12S primer set, the primer set 12SF.t2 and 12SR.t2 was used. Initially, only the primer with the lower-T_m (12SF.t2) was added to the reaction for 5–7 rounds of denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 3 min. The second primer (12SR.t2) was then added for 4–6 rounds of denaturation at 95 °C for 1 min, annealing at 66 °C for 1 min, and extension at 72 °C for 30 s. The PCR was completed with 35–38 rounds of denaturation at 95 °C for 1 min followed by annealing/extension at 72 °C for 2 min. For tailed 28S reactions, the primer set Rd6.2b.t2 and Rd4.5a.t2 was used. Similar to the previous example, at first, only the lower-T_m primer (Rd.4.5a.t2) was added to the reaction mix for 5–7 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. The higher T_m primer (Rd.4.5a.t2) was then added for 4–6 rounds of denaturation at 95 °C, annealing at

Table 2 Design of tailed PCR primers used to increase amplification specificity of difficult template

Primer name	Primer sequence 5′–3′	Mer	T_m (°C)	References
LCO.t2	**GCGTGCTGCTAGCAG**GGTCAACAAATCATAAAGATATTGG	40	78	Folmer et al. (1994)
HCO.t2	**GCATAGCTGAATGCAT**TAAACTTCAGGGTGACCAAAAAATCA	42	77	
LCO.t3	**CTAGCAG**GGTCAACAAATCATAAAGATATTGG	32	67	
HCO.t3	**CAT**TAAACTTCAGGGTGACCAAAAAATCA	29	67	
12SF.t2	**GCGTGCTGCTAGCAG**GAAACCAGGATTAGATACCC	35	77	Mokady et al. (1999)
12SR.t2	**CAT**TTTCCCGCGAGCGACGGGCG	23	80	
12SF.t3	**GCTAGCAG**GAAACCAGGATTAGATACCC	28	67	
Rd6.2b.t2	**TGCTGCTAGCAG**AATAKKAACCRGATTCCCTTTCGC	36	77	Whiting (2002)
Rd4.5a.t2	**CACGAGGTCGGCAT**AAGTTTCCCTCAGGATAGCTG	35	78	
Rd6.2b.t3	**AG**AATAKKAACCRGATTCCCTTTCGC	26	66	
Rd4.5a.t3	**GGCAT**AAGTTTCCCTCAGGATAGCTG	26	66	

The bold underlined portion of the primer sequence is a random set of nucleotides added to the original primer cited in the associated reference

Table 3 Sequence metrics and nucleotide substitution model parameters for the three partial gene sequences (individually and concatenated), obtained from the 52 taxa listed in Table 1

Locus	Sequence count	Aligned length (bp)	Variable sites	Parsimony informative sites	π[a]	Haplotype count[a]	Tr/Tv ratio	Model comparison			
								jModelTest	(AICc)	Implemented	(AICc)
CO1	51	563	201	161	0.098	30	1.50	TVM+I+Γ	(5530)	GTR+I+Γ	(5531)
12S	52	265	75	49	0.051	10	0.95	TPM3uf+Γ	(2172)	GTR+Γ	(2188)
28S	46	582	29	18	0.008	10	1.87	TrN+I	(2374)	GTR+I	(2380)
Concatenated	52	1410	305	228	0.049	36	1.38				

Nucleotide substitution models selected by jModelTest (out of 88 candidates) for the data partitions are compared by their Aikake Information Criterion correction (AICc) coefficients to the models actually implemented

[a] Sequences of outgroup taxa not included

58 °C for 1 min, and extension at 72 °C for 15 s. Amplification was completed with 35–38 cycles of denaturation at 95 °C for 1 min and annealing/extension at 72 °C for 2 min. For tailed PCR product, the following primer pairs: LCO.t3/HCO.t3; 12SF.t3/12SR.t2; and Rd6.2b.t3/Rd4.5a. t3 (Table 2) were used for sequencing the COI, 12S, and 28S gene fragments, respectively. Sequencing of purified PCR product was outsourced to GENEWIZ, Inc. (Germantown, MD, USA) or Eton Biosciences, Inc. (Research Triangle Park, NC, USA). Resulting sequences (both sense and antisense strands for most specimens) were unambiguously aligned and trimmed using Sequencher 5.1 (Gene Codes Corp., Ann Arbor, MI, USA). Nuclear 28S is a multicopy gene and potential exists for amplification of nonidentical copies within the same individual. We found no indication this occurred among our samples as there were no conflicting base calls by the alignment software between forward and reverse sequences within individuals.

Molecular analysis

The aligned sequences for each gene were concatenated into a single dataset and partitioned by locus for phylogenetic analysis. Nucleotide substitution models and parameters for phylogenetic analysis were selected for each partition using jModelTest (ver. 2.1.1) with Aikake Information Criterion corrections (AICc) (Posada 2008). Both maximum likelihood and Bayesian inference estimation methods were employed under mixed models. Online computing resources of the CIPRES (Cyberinfrastructure for Phylogenetic Research) Science Gateway (http://www.phylo.org) were used to conduct the analyses (Miller et al. 2010). Maximum likelihood estimation was implemented in RAxML-HPC BLACKBOX ver. 7.4.2 (Stamatakis 2006) with 100 bootstrap replicates. The exact nucleotide substitution model selected by jModelTest for each partition could not be implemented by the program; thus, a general time-reversible (GTR) model was applied to each

partition with or without the proportions for invariant sites and gamma shape parameters as called for by jModelTest (Table 3). The Bayesian inference analysis was implemented in MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck 2003) with model parameters for the data partitions selected by jModelTest. Two Markov chain Monte Carlo (MCMC) simulations were run from random starting points for one million generations using one cold and three heated chains. Trees were sampled every 100th generation with the first 25 % discarded for burn-in.

Three analyses of molecular variance (AMOVA) were conducted using Arlequin ver. 3.5.1.2 (Excoffier et al. 2005), testing for significant differences in fixation indices (*F*-statistics) calculated for the concatenated sequences grouped by (1) species designation, (2) geographic region, and (3) host utilization. Average pairwise genetic divergence (*p*-distance or mean percent substitutions per site) between the groups of sequences used for the AMOVA's was calculated using the program Mega ver. 5.1 (Tamura et al. 2011) and tabulated for comparison. Haplotype network relationships among the sequences were also examined separately for each locus via statistical parsimony implemented in TCS ver. 1.21 (Clement et al. 2000).

Results

Morphological observations

In the course of collecting barnacle samples from Florida manatees, we encountered individuals of *C. manati* with complemental males attached (Fig. 1a). Observations and measurements were made of 50 of these individuals which were situated within the narrowest portions of the wall-plate wedges of 10 adult hermaphrodites, as similarly noted in *C. testudinaria* (Zardus and Hadfield 2004). The large adults averaged 47.2 mm in diameter (SD ± 0.8) and hosted 4–15 small individuals each (mean = 7). Upon dissection, these small individuals were found with male structures only and had an average maximum basal diameter of 4.9 mm (SD ± 0.3) with an average penis length of 3.5 mm (SD ± 1.0) when preserved. The average copulation distance (measured from the center of each small male to the center of the hermaphrodite) was 12.2 mm (SD ± 2.3). This is the first report of androdioecy in this taxon, but is not surprising in light of the taxonomic conclusions reached in our study.

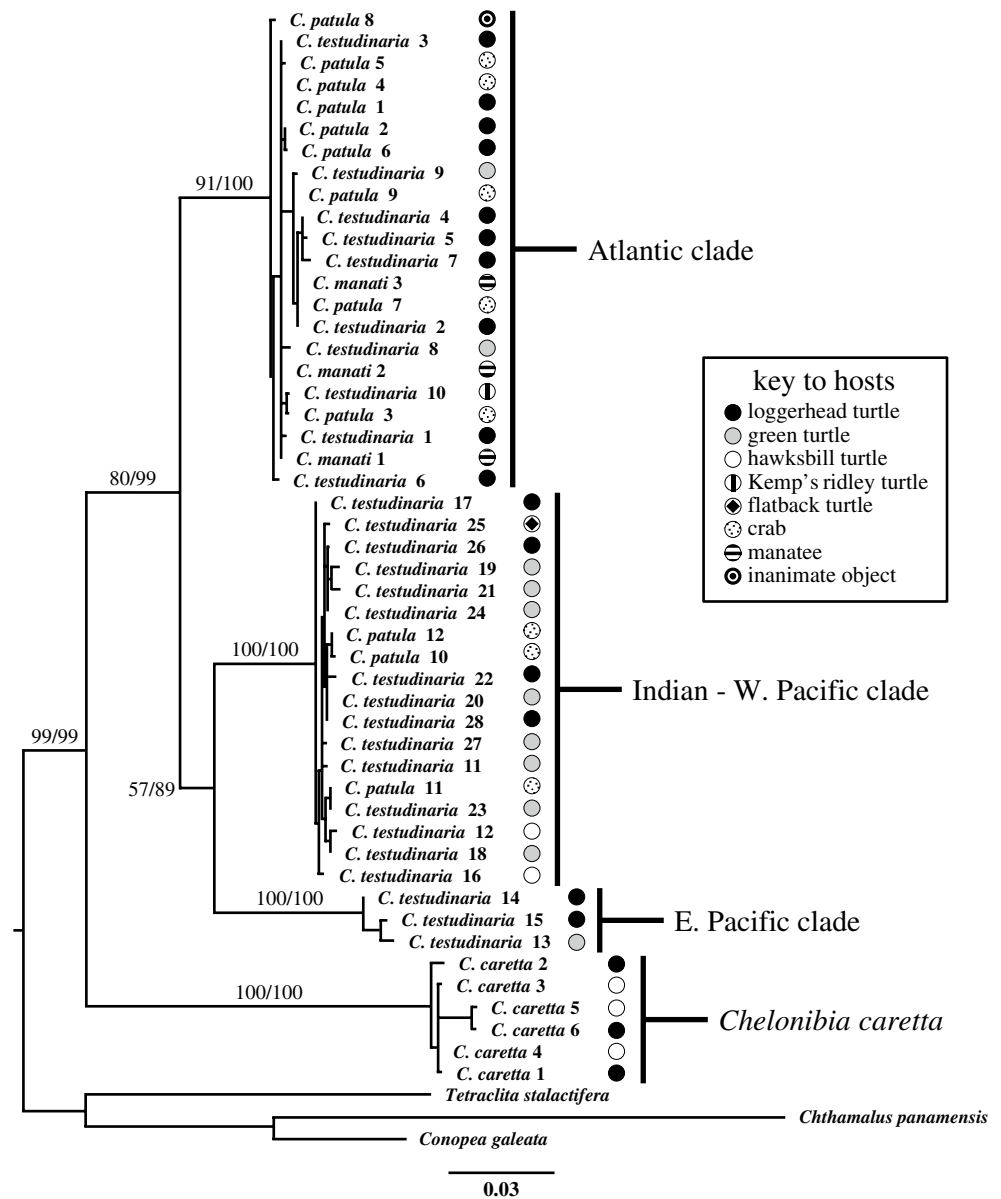### Molecular phylogenetic relationships

DNA sequence data were obtained from a total of 49 *Chelonibia* specimens. A full complement of three partial gene sequences per sample was garnered from all specimens but seven GenBank entities (Tables 1, 3). The longest sequences were obtained for 28S, but these had the fewest parsimony informative sites. Sequences of the 12S gene, while shortest in length and lowest in their ratio of transitions to transversions, had more parsimony informative sites than 28S and an equal number of haplotypes. Sequences of COI provided the most phylogenetic information; they were most variable, possessing both the highest number of informative sites and the greatest number of haplotypes. The GTR nucleotide substitution models implemented for each locus compared favorably in AICc coefficients with the models specified by jModelTest (Table 3).

For the Bayesian inference analysis, the MCMC runs reached convergence after one million generations (st. dev. of split freq. 0.0263), and a single best tree was selected by maximum likelihood estimation. Trees from both analyses were identical in gross topology with high statistical support defining four major clades (Fig. 2). In these phylogenies, six individuals identified as *C. caretta*, collected from both the Atlantic and Pacific, clustered as a unified clade, clearly separate and basal to the other *Chelonibia* species. On the other hand, forty sequences of *C. manati, C. patula,* and *C. testudinaria* were phylogenetically indistinguishable and were comingled among two geographically defined clades, one from the Atlantic and the other from the Indian Ocean/western Pacific. Three samples of *C. testudinaria* from the eastern Pacific (Baja, Mexico, and the Galapagos Islands) comprised a third clade, sister to the Indian Ocean/western Pacific clade.

To assess the contributions of each marker to the phylogeny, individual gene trees were generated using maximum likelihood and Bayesian inference methods implementing the parameters given for their corresponding partitions in the concatenated dataset. Both the CO1 and 12S sequences, whether by maximum likelihood or Bayesian inference, resulted in closely congruent trees, resolving the same clades generated by the concatenated data with strong statistical support (see figs. 1–2 in supplementary materials). But clade ordering was not entirely consistent between them. The CO1 tree placed the eastern Pacific clade sister to the Indian Ocean/western Pacific clade, while the 12S tree placed the eastern Pacific and Atlantic clades sister to each other. The 12S tree also displayed finer divisions of structure within clades. On the other hand, relationships with the 28S phylogeny were largely unresolved. Maximum likelihood and Bayesian inference resulted in trees with dissimilar topologies, minimal clade structure, and mostly weak statistical support (see figs. 3–4 in supplementary materials). Despite their low reliability overall, the two 28S trees shared two important similarities. Both identified a clade made up of *C. patula, C. manati, and* Atlantic *C. testudinaria* with moderate to high support (bootstrap = 78, posterior probability = 100), strengthening the conclusion that these three taxa are in fact the same species. Both trees also

resolved a clade composed of *C. caretta* well-separated from the others with high support (bootstrap = 94; posterior probability = 98) excepting one individual (*C. caretta* 2 from Florida) which either appeared as an isolated branch from *C. testudinaria* at low support with maximum likelihood (bootstrap = 51) or isolated in a basal polytomy with Bayesian inference.

Average pairwise genetic distances among the sequences grouped by species resulted in distinct differences between *C. caretta* and the other *Chelonibia* species, on par with differences between that species and the outgroup taxa (Table 4a). On the other hand, average pairwise genetic distances among *C. testudinaria*, *C. manati*, and *C. patula* were similar to each other and almost an order of magnitude lower. For sequences of the three latter taxa, grouped into three geographic regions, the average within-group

pairwise genetic distance was low and comparable among them with low standard errors generated from 1,000 bootstrap replicates (Atlantic 0.008, SE 0.001; Indian Ocean/western Pacific 0.004, SE 0.001; and eastern Pacific 0.007, SE 0.002). But substantial differences were observed in between-group comparisons (Atlantic x Indian Ocean/western Pacific = 0.057, SE 0.006; Atlantic x eastern Pacific 0.053, SE 0.006; and eastern Pacific × Indian Ocean/western Pacific 0.073, SE 0.008). Even after subdividing the geographic regions into smaller units, the regional patterns remained largely consistent, with the greatest differences occurring between the eastern Pacific and Indian Ocean/western Pacific samples (Table 4b)—excepting the South China Sea samples which were as different or more so with the Atlantic. Values of pairwise genetic distances among sequences grouped by host were mixed (Table 4c).

Table 4 Average pairwise genetic distances (*p*-distance) between groups of barnacle sequences arranged by (A) taxonomic designation, (B) subdivision of geographic regions (see text for comparisons by region), and (C) host organism (see Table 1 for sequence assignments)

A

taxon	(n)	*C. caretta*	*C. manati*	*C. patula*	*C. testudinaria*
C. caretta	(6)	-			
C. manati	(3)	0.091	-		
C. patula	(12)	0.103	0.022	-	
C. testudinaria	(28)	0.099	0.037	0.037	-
outgroup taxa	(3)	0.114	0.107	0.119	0.114

B

ocean/sea/isl.	(n)	Med.	W. Atl.	Car/GoM	Haw.	Japan	S. China	Aus.	Ind.
Mediterranean	(2)	-							
W. Atlantic	(15)	0.004	-						
Caribbean/GoM	(5)	0.005	0.004	-					
Hawaii	(2)	0.033	0.050	0.050	-				
Japan	(3)	0.034	0.051	0.051	0.005	-			
S. China	(7)	0.055	0.077	0.077	0.006	0.005	-		
Australia	(3)	0.034	0.051	0.051	0.003	0.004	0.004	-	
Indian Ocean	(3)	0.034	0.051	0.051	0.003	0.003	0.004	0.002	-
Galapagos/Baja	(3)	0.036	0.055	0.055	0.058	0.059	0.092	0.059	0.058

C

host	(n)	manatee	loggerhead	green	hawksbill	Kemp's	flatback	arthropod
manatee	(3)	-						
loggerhead	(14)	0.027	-					
green	(11)	0.047	0.041	-				
hawksbill	(2)	0.067	0.051	0.024	-			
Kemp's	(1)	0.004	0.028	0.047	0.067	-		
flatback	(1)	0.052	0.039	0.018	0.007	0.052	-	
arthropod	(10)	0.027	0.037	0.039	0.045	0.028	0.035	-
inanimate	(1)	0.005	0.028	0.047	0.067	0.006	0.052	0.030

Sequences for outgroup taxa and *C. caretta* were excluded for B and C. Alignment gaps in sequence pairs were removed prior to comparison. For each set of comparisons, the maximum divergence value among *Chelonibia* pairs is shaded. Outlined cells within the tables identify within-group comparisons for (B) geographic regions and (C) turtle hosts only

The greatest differences were seen between hawksbill sea turtles and several other hosts, probably unduly influenced by the fact that apart from *C. caretta*, only *C. testudinaria* was collected from hawksbills and only in Australia. Otherwise, patterns of divergence were elusive among host types. In general, conclusions from the host differences may be unreliable as the results are strongly influenced by geographical bias since not all host species could be sampled for every location.

With *C. caretta* excluded, AMOVA results corroborated the patterns observed in the phylogenetic analyses. There were no statistical differences among sequences arranged by species or by host (Table 5). But a highly significant difference was found between the samples when grouped by geographic region despite the small contribution of this factor to the overall variance.

Examination of the three loci individually in parsimony network analyses revealed patterns similar to the phylogenetic trees but with differences in signal strength by gene. Analysis with COI was the clearest, resulting in three discrete haplotype clusters separated from each other by more than 50 base-pair changes and grouped by the geographic units identified in the phylogeny (Fig. 3a). No haplotypes were shared between geographic regions. The most basal haplotype, determined by the program as the sequence with the highest outgroup probability (Clement et al. 2000), was positioned in the Indian Ocean/western Pacific clade with a starburst pattern of closely similar haplotypes extending from Japan to Australia through the South China Sea to Oman and South Africa. The most distant haplotype in the cluster was found with a single individual located in Hawaii. A somewhat more diffuse but cohesive cluster formed the Atlantic clade. Our sampling was most restricted in the eastern Pacific, and the cluster of sequences from this region, represented by two individuals from Baja, Mexico, and one from the Galapagos, was most diffuse due to low sample size yet still cohesive. These same patterns were also echoed in the 12S and 28S networks (Fig. 3b, c) but with diminishing resolution and with placement of the most basal haplotype in the Atlantic clade. With the 12S marker, all the same haplotype clusters were identifiable but with reduced connection lengths, whereas with the 28S gene, haplotype clusters were not readily identifiable, but gradients of geographic position could be discerned.

Discussion

Site selection and attachment by barnacles can be influenced by complex chemical and physical cues from the

Table 5 AMOVA results comparing genetic variation in three nominal species of *Chelonibia* barnacles collected from seven host types and three global regions, grouped by (A) species designation, (B) host organism, and (C) geographic region

Test grouping	Source of variation	df	Sum of squares	Variance component	Percent total variance	F_{CT}	F_{SC}	F_{ST}
A. Species	Among spp.	2	0.924	−0.00023	−0.05	−0.00047		
	Among populations w/in groups (host x region)	10	4.774	−0.00788	−1.60		−0.01602	
	Within groups (host x region x spp.)	30	15.000	0.50000	101.65			−0.01650
	Total (haplotypes)	42	20.698	0.491880				
B. Host*	Among hosts	4	1.896	−0.00290	−0.59	0.00589		
	Among populations w/in groups (spp. x region)	6	2.971	−0.00005	0.01		−0.00011	
	Within groups (species x region x host)	29	14.357	0.49507	100.58			−0.00578
	Total (haplotypes)	39	19.225	0.49223				
C. Region	Among regions	2	1.228	0.01492	3.00	0.02999**		
	Among populations w/in groups (spp. x host)	10	4.470	−0.01751	−3.52		−0.03628	
	Within groups (spp. x host x region)	30	15.000	0.50000	100.52			−0.00520
	Total (haplotypes)	42	20.698	0.49741				

See assignments given in Table 1. Negative values for the variance components and fixation indices are products of imprecision by the software due to low variation and should be interpreted as effectively zero

* Flatback and Kemp's ridley turtle hosts and inanimate substratum could not be included in the analysis as they were represented by only one sequence each. ** $P < 0.005$

substratum (Standing et al. 1984; Hills and Thomason 1998; Thiyagarajan 2010), associated biofilms (Maki et al. 1992; Qian et al. 2003; Zardus et al. 2008), and the nearby environment (Rittschof et al. 1984; Larsson and Jonsson 2006). For epibiotic barnacles that associate with particular hosts, it very likely requires especially precise, though presently unknown, signals (Frick et al. 2011). Numerous obligate commensalisms have evolved between barnacles and a wide range of vertebrate and invertebrate hosts including turtles, whales, corals, and sponges, resulting in highly canalized relationships (Monroe and Limpus 1979; Scarff 1986; Anderson 1992; Ilan et al. 1999; Zardus and Balazs 2007, Tsang et al. 2009; Brickner et al. 2010). In the present study, we found a striking exception to this pattern in a genus of "turtle" barnacles and demonstrate that *Chelonibia patula,* which has been described primarily associating with crabs, *C. manati*, which occurs predominantly with sirenians, and *C. testudinaria*, which partners with sea turtles, are genetically indistinguishable. No threshold of genetic divergence exists for delineating barnacle species boundaries, but dismantling this assemblage is justified based on the fact that these three taxa comingle phylogenetically and are more genetically divergent among different geographic regions than by their taxonomic designations. Pairwise genetic differences among these taxa within the geographic regions identified in this study compare

very favorably with the average within-species sequence divergence for the COI locus measured across 255 crustacean taxa (0.023 ± 0.032) (Wares 2011). Rather than being distinct species, *C. patula*, *C. manati*, and *C. testudinaria* represent host morphotypes that should be synonymized as a single species under the more senior Linnaean epithet *testudinaria*. These findings address the appeal of Frick and Ross (2001) for taxonomic clarification of *C. testudinaria* and confirm at a global level and across many host species the results of Cheang et al. (2013); *C. testudinaria* and *C. patula* are conspecific, and with the addition of *C. manati*, this triumvirate disassembles into a single remarkable species that utilizes reptiles, mammals, and arthropods as hosts. *Chelonibia testudinaria* then is neither quintessentially a turtle barnacle nor a general fouling species, but, instead, an associate of unfixed substrata—primarily motile marine animals. On the other hand, the remaining genetically distinct species in the genus, *C. caretta*, is narrow in its affiliation with sea turtles (Torres-Pratts et al. 2009; Farrapeira 2010).

Though we make the case for high morphological and molecular variation in *C. testudinaria* globally, the possibility still remains for cryptic species in the genus. In addition to the present findings and those of Rawson et al. (2003) which show the genetic uniqueness of eastern Pacific *C. testudinaria*, Pilsbry (1916) observes that the
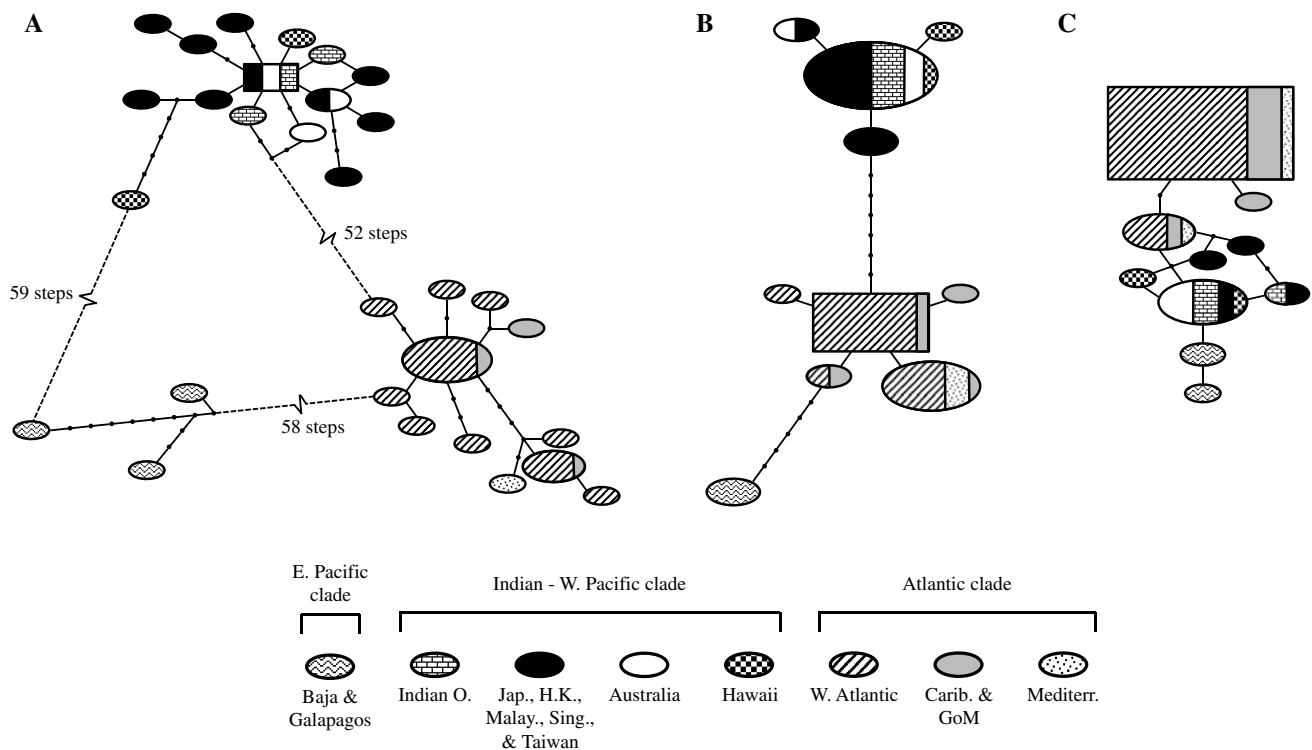
Fig. 3 Haplotype parsimony networks for each of the three partial gene sequences analyzed in this study. **a** Network for 30 haplotypes associated with the mitochondrial COI marker. **b** Network for 10 haplotypes determined for mitochondrial 12S. **c** Network for 10 haplotypes resolved for nuclear 28S. *Oval size* corresponds to haplotype count, and the *fill pattern* to geographic subunits was indicated in the key. The most basal haplotype, as estimated by the software used, is shown as a *rectangle*. Nodes on the connecting lines correspond to the number of base-pair differences between haplotypes. Multiple pathways of connection between haplotypes represent equally parsimonious scenarios of relationship

transverse ridges (teeth) in the radii of these barnacles are narrower and slightly more numerous than their counterparts in the Atlantic, and that the shell of the eastern Pacific form is much wider and lower, with the body chamber being less than half of the basal diameter. Because Indian Ocean/western Pacific, eastern Pacific, and Atlantic *C. testudinaria* differ from each other genetically by similar degrees, an argument could be made for making geographically based species assignments for these taxa. However, such a step seems premature given that haplotype distributions remain incomplete and large areas of the world ocean remain un-sampled, in particular isolated island groups and turtle populations in the Pacific.

Phylogeographic analysis of *C. testudinaria* revealed relatively high connectivity throughout the Atlantic–Caribbean–Mediterranean region and also within the Indian Ocean–South China Sea–western Pacific area. Yet, we also recovered the great genetic divergence between eastern and western Pacific populations initially found by Rawson et al. (2003). This disjunction in the Pacific is likely explained by the greater geographic expanse of this body of water compared to that of the Atlantic or Indian oceans. From our analysis, it remains uncertain which two of the three major clades of "*testudinaria*" are most closely related and how

gene flow has proceeded historically. The eastern Pacific clade was marginally closer in terms of genetic distance with the Atlantic clade than with the Indian Ocean/western Pacific clade, but results from the individual gene trees were equivocal on this point. Atlantic and eastern Pacific populations could have been connected more recently in evolutionary time than western and eastern Pacific populations prior to the formation of the Isthmus of Panama approximately 3.5 mya (Coates et al. 1992). Interocean gene flow has recently been examined in another genus of turtle barnacle, *Stomatolepas*, and it too implies historical gene flow predating the Isthmus of Panama or possibly turtle traffic around the tip of South America (Pinou et al. 2013). Two isolated island locations in the Pacific, Hawaii, and the Galapagos gave opposing results, samples from the former clustered with the Indian Ocean/western Pacific clade and samples from the latter grouped with the eastern Pacific (Baja, Mexico) clade. This may be explained by small sample size, and potentially members of both clades could be present at each island group. Alternatively, these barnacle populations may be distinct due either to geographic proximity or to variation in migratory patterns of different hosts. If not the result of low sampling, proximity is likely the better explanation as green sea turtles were

the host species for both the Hawaii and the Galapagos specimens, whereas the hosts in Baja were loggerheads, but proximity and host behavior could both play a role. Further, detailed examinations of both turtle and barnacle phylogeographies are merited. Though, for studies of population connectivity in turtles using barnacles as markers, a barnacle other than *C. testudinaria*, one more specific to sea turtles, would likely be a better model for gaining insight on sea turtle movements.

The large host repertoire of *C. testudinaria* may be selected for and stabilized by sparse host populations, allowing the barnacle to colonize one host when others are unavailable. The androdioecious mode of reproduction described for *C. testudinaria* involving complemental males (Zardus and Hadfield 2004) is an aspect of its life history that may also be an adaptation to live on scarce substrata. Sex allocation theory suggests that limited mating opportunity favors androdioecy and dioecy (Yamaguchi et al. 2012) over the typical barnacle sexual mode of hermaphroditism (Anderson 1994). Complemental males have previously been reported only for C. *patula* (Crisp 1983) and *C. testudinaria* (Zardus and Hadfield 2004) but not for *C. caretta*. Our evidence that they are also present in *C. manati* lends further support to unifying *C. manati*, *C. patula*, and *C. testudinaria* as a single species.

Differences in the degree of host specialization by *C. testudinaria* and *C. caretta* may be associated with their modes of attachment. While both species employ adhesion as it is typical of barnacles (Walker 1978), *C. caretta* also possesses sharp, down-turned margins to its shell which are suited for cutting into the keratinous scutes of turtles and wedging the crustacean in place (Monroe 1981). The shell wall of *C. testudinaria,* on the other hand, can be particularly thick, forming a broad base with a large surface appropriate for attaching by adhesion alone to a firm substratum. When fixed to a yielding substratum such as manatee skin, this barnacle is able to grow crenulated extensions of the shell margin that grip the host epidermis like small fingers. Given the morphological plasticity displayed by *C. testudinaria*, the single reported specimen of *C. ramosa,* described by Korschelt (1933) with long host-penetrating extensions, is likely but another morphotype of *C. testudinaria*.

That morphological variability is correlated with host type in *C. testudinaria* raises the question of whether the observed phenotypes are environmentally induced or genetically predetermined. There is likely great functional significance among the different forms of *C. testudinaria* related to host association. Host lifestyles, where hosts live and feed, how their movements influence water flow to the barnacles, and what efforts hosts make in removing barnacles, may be important factors influencing the variety of forms exhibited by this species. Further investigations into the larval ecology and developmental genetics of *Chelonibia testudinaria* are needed to fully determine the forces governing morphological variation in this species.

Commensalism has arisen independently in the Cirripedia multiple times as barnacles have adapted to live with other organisms and in some instances long before vertebrate hosts were available (Lewis 1978). However, it has been argued that sea turtles were the first hosts used by chelonibiid barnacles (Harzhauser et al. 2011) as the fossils of all contemporary host species of *Chelonibia* predate the origin of this genus. Keeping in mind that a fossil's first appearance underestimates the age of its genetic divergence with a sibling species (Reisz and Müller 2004), the oldest decapods and xiphosurans date to approximately 480 mya (million years ago) (Schram et al. 1978; Dunlop and Selden 1998; Rudkin et al. 2008), whereas the oldest sea turtles appeared approximately 110 mya (Hirayama 1998). More advanced vertebrate hosts, like sirenians, first appeared during the late early Eocene 40–55 mya ago (Savage et al. 1994)—coinciding with the time period established for *Chelonibia* fossils and their related forms 35–55 mya or younger (Ross 1963; Ross and Newman 1967; Zullo 1982; Harzhauser et al. 2011). Yet, recent phylogenetic estimates place the origins of chelonibiid barnacles in the vicinity of 60–100 mya (Pérez-Losada et al. 2004; Pérez-Losada et al. 2008; Hayashi et al. 2013.

A phylogenetic answer for the ancestral host of *Chelonibia* remains elusive. The more turtle-specific *C. caretta* is ancestral to *C. testudinaria* as constituted and is basal to all other coronuloids (Hayashi et al. 2013) which lends weight to a turtle-first hypothesis. It is also important to note that the superfamily is comprised entirely of commensal species, most of which specialize on turtles (Young 1991). Nevertheless, both *C. caretta* and *C. testudinaria* share an unknown common ancestor with unknown host affiliation. Examples of extinct but related barnacles (Ross and Newman 1967) could offer further clues to the commensal origins of the genus, but information on the hosts of these fossils is presently lacking (Harzhauser et al. 2011). Cetaceans, the oldest of which dates to 35.5 mya (Bajpai and Gingerich 1998), are too recently evolved for consideration as an initial host, but besides turtles, several other now extinct reptiles could potentially have served. Ichthyosaurs, plesiosaurs, and mosasaurs all survived into the late Cretaceous 90–65 mya (O'Keefe 2002; Everhart 2005; Maisch 2010) when chelonibiid barnacles may have begun to arise (Pérez-Losada et al. 2008). Further paleontological and phylogenetic investigations are required to fully understand the evolutionary history and possibilities of this remarkable group of barnacles.

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