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RESEARCH ARTICLE

Molecular relationships and species divergence among *Phragmatopoma* spp. (Polychaeta: Sabellaridae) in the Americas

Carrie A. Drake · Daniel A. McCarthy · Carol D. von Dohlen

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Abstract *Phragmatopoma* spp. are marine, reef-building polychaetes that inhabit the intertidal and shallow subtidal zones of both coasts of the Americas. *Phragmatopoma californica* is found in the Pacific Ocean along the California coast south to Mexico, while *Phragmatopoma caudata* inhabits the Caribbean islands and Atlantic Ocean from the Florida coast south to Brazil. Although apparently geographically isolated, *P. californica* and *P. caudata* have been found to interbreed (Pawlik 1988a) and thus their specific taxonomic relationship has been unclear. In this study, two genes, cytochrome c oxidase subunit I (COI) and the first internal transcribed spacer region (ITS-1), were sequenced to assess the specific status of *P. californica* and *P. caudata* as well as *Phragmatopoma virgini*. Comparison of sequences revealed that samples of *P. californica* shared no COI haplotypes or ITS-1 sequences with *P. caudata*. Phylogenetic analyses, including maximum parsimony and Bayesian methods, clustered each species in separate, well-supported clades with genetic distances between them being greater than between either contested species or the uncontested, valid species, *P. virgini*. Thus, the molecular data support that *P. californica* and *P. caudata* are separate species. However, the sample of individuals of *P. virgini* included one genetically divergent individual, whose morphology was found to match that of a species formerly recognized as *P. moerchi* but since synonymized with *P. virgini*. Divergences among lineages were dated using the COI sequences, after adjustment for non-clock-like behavior. Consequently, we suggest that *P. virgini* and *P. caudata* are sister taxons and that *P. californica* diverged from the *P. virgini*/*P. caudata* clade ~5.7 mya with *P. virgini* diverging from *P. caudata* ~3 mya.

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C. A. Drake (✉) · C. D. von Dohlen
Department of Biology, Utah State University, Logan, UT, USA
e-mail: cdrake@biology.usu.edu

D. A. McCarthy
Department of Biology and Marine Science, Jacksonville University, Jacksonville, FL, USA

Introduction

Phragmatopoma spp. are marine, reef-building polychaetes that inhabit the intertidal and shallow subtidal zones of both coasts of the Americas. They construct sand tubes on hard substrates and often form large sand mounds that significantly enhance near-shore hard-bottom habitats (Kirtley 1967; Jensen and Morse 1984, 1990; Pawlik 1986, 1988a, b; Pawlik and Faulkner 1986; Pawlik et al. 1991; McCarthy 2001). They are sessile, suspension feeders that normally live in marine hard bottom areas with moderately high turbulence. They sexually reproduce via broadcast spawning that result in trochophore larvae that passively drift in the plankton from 2 to 20 weeks until they are competent to settle. At competency, they will descend to the sea floor bottom to test for optimal substrates to eventually metamorphosise into juveniles (Eckelbarger 1976; Pawlik and Mense 1991; McCarthy et al. 2002). Once they have settled and metamorphosed, the worms become reproductively mature quickly, and can live for 1–2 years (Kirtley 1966; McCarthy et al. 2003; McCarthy 2001; Simmon et al. 2005).

The number of *Phragmatopoma* species that currently exist in North and South America is unclear because of morphological similarity (they are primarily distinguished by their opercular paleae). Hartman (1944) recognized seven species, with roughly allopatric or peripatric geographic distributions, based on an appendage attached to the outer opercular paleae. These species include: (1) *Phragmatopoma attenuata* (Ecuador to Colombia and possibly the Pacific side of Panama, (2) *Phragmatopoma californica* (California south to Enseñada, Mexico), (3) *Phragmatopoma caudata* (West Indies), (4) *Phragmatopoma lapidosa* (Brazil to Florida), (5) *Phragmatopoma moerchi* (Peru to Chile and Hawaii), (6) *Phragmatopoma peruensis* (found in Peru), and (7) *Phragmatopoma virgini* (southern Chile). Because of overlapping habitats and missing type specimens, Hartman (1944) noted that *P. caudata* might be the same species as *P. lapidosa*. Further, Pawlik (1988a) suggested that *P. californica* and *P. lapidosa* were the same species based on laboratory reciprocal matings between males and females of each species that produced healthy larvae. He suggested they be called *Phragmatopoma lapidosa lapidosa* and *Phragmatopoma lapidosa californica*. However, while the larvae settled and matured into adults they were not tested for viable gamete production.

Morphologically, the two taxa are primarily distinguished by the distal spikes attached to the outer opercular paleae (Hartmann 1944), and the presence or absence of transverse fibrous thecal bands on the middle paleae (Kirtley 1994). *P. californica* has a plumose distal spike and transverse fibrous thecal bands, while *P. caudata* has a pennate distal spike and lacks transverse fibrous thecal bands.

Although these two taxa have morphological differences, their ability to interbreed suggests that: (1) they may have been only recently separated, (2) one may have arisen through a recent founder effect, or (3) their strong dispersal ability facilitated high genetic flow. However, as their current distributions are in geographically separated oceans (northern Pacific and Caribbean-Atlantic), these possibilities seem unlikely. The Pacific and Atlantic oceans have been physically isolated in the Central American region as the complete closure of the Isthmus of Panama, approximately 3–15 mya. As a result of the gradual drop in sea level, islands formed in the region that disrupted direct currents between marine populations of these two oceans (Duque-Caro 1990; Bermingham et al. 1997).

In the 1910s, the construction of the Panama Canal opened a potential dispersal corridor between the oceans, but the large, freshwater Lake Gatun (created to fill the canal), may act as a barrier to prevent dispersal of marine organisms between the Atlantic and Pacific Oceans. Thus, barring other unknown chance dispersal events such as around the Cape Horn, it seems that *P. californica* and *P. caudata* should represent populations that have been independent and isolated for 3 million years or more.

In his revision of the Sabellariidae, Kirtley (1994) apparently examined specimens from Pawlik's study (1988a), but disagreed with the conclusions because of morphological dissimilarities between the two species. Kirtley (1994) revised the genus to contain only four species, synonymizing *P. lapidosa* with *P. caudata*, and grouping *P. moerchi*, *P. peruensis*, and *P. virgini* into one species, *P. virgini* (Fig. 1). He concluded that: (1) *P. attenuata* inhabits the Pacific Ocean along Northern Peru, Ecuador, Columbia, and Panama, (2) *P. californica* inhabits the west coast of North America from Cape Mendocino, California south to Mexico, (3) *P. caudata* occurs along the east coast of North America from Cape Canaveral, Florida to the southern coast of Brazil, and (4) *P. virgini* exists along the Pacific coast from Central America to Argentina.

The goal of this study was to use molecular analyses to test whether *P. californica* and *P. caudata* are distinct species. Here we test the hypothesis that populations of *P. californica* and *P. caudata* should exhibit genetic divergence levels commensurate with their isolation time because they have been geographically (and thus reproductively) isolated for approximately 3 million years. Further, *P. californica* and *P. caudata* should be considered separate species if the genetic divergence between them is similar to that of other uncontested species pairs.

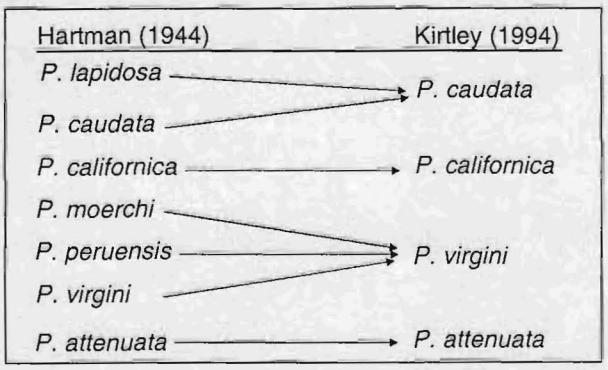

Fig. 1 Summary of the taxonomic revisions of *Phragmatopoma* based on Hartman (1944) and Kirtley (1994)

Materials and methods

Specimen collection

All specimens were collected at low tide by breaking off small chunks of the worm reef that contained ~20–30 individual worms. Worms were dissected from their sand tubes and stored in 95% ethanol for laboratory analysis. Specimens were collected from three populations of *P. caudata* along the Atlantic coast of Florida, the Virgin Islands, and Puerto Rico (12 individuals each), ten populations of *P. californica* collected along the Pacific coast of California and Baja, Mexico (2–12 individuals each), and one population of 20 individuals of *P. virgini* collected from the Pacific coast of Chile; six individuals from one population of *Idanthyrsus cretus* (also Sabellariidae), collected from the Pacific coast of Panama, were used for an outgroup (Table 1). All specimens have been deposited at the California Academy of Sciences, Department of Invertebrate Zoology and Geology, under the catalog numbers recorded in Table 1.

DNA extraction, amplification, and sequencing

Two genes were used to characterize the genetic divergence of *P. californica* and *P. caudata*. The first was a mitochondrial gene involved in the electron transport chain, cytochrome c oxidase subunit I (COI) (Rawson and Burton 2002). Because the mitochondrial genome is maternally inherited, lacks recombination, and diverges rapidly, it has been used to distinguish between closely related species and even populations (Kojima et al. 1997; Metz et al. 1998; Siddall and Burreson 1998; Burton et al. 1999; Nylander 1999; Kojima et al. 2001, 2002; Goffredi et al. 2003). The second gene was the first of two nuclear internal transcribed spacer regions (ITS-1). This region lies between the 18S and 5.8S ribosomal DNA (rDNA) subunits. After the rDNA cistron is transcribed, the ITS-1 is cleaved and removed from the transcript, so it does not contribute to the construction of the ribosome. Because ITS regions are not directly involved in the production of a ribosome, there is presumably no selective constraint on their sequences, and mutations are thought to

Table 1 Collection date, location and quantity of individuals of each *Phragmatopoma* species (and outgroup populations) collected for this study

Species[a]	ID	Number of individuals	Location	Date	Collector	Catalog number[b]
Phragmatopoma californica	PC	12	USA: California, Santa Cruz County, Santa Cruz	Nov 2002	J. Pearce	173851
P. californica	RM	5	USA: California, San Luis Obispo County, Rancho Marino	Mar 2003	M. Wilson	173852
P. californica	SB	7	USA: California, San Luis Obispo County, Shell Beach	Mar 2004	M. Wilson	173853
P. californica	ST	5	USA: California, Santa Barbara County, Stairs	Mar 2004	M. Wilson	173854
P. californica	BH	5	USA: California, Santa Barbara County, Boat House	Mar 2003–2004	M. Wilson	173855
P. californica	GP	8	USA: California, Santa Barbara County, Government Point	Mar 2003–2004	M. Wilson	173856
P. californica	LB	2	Mexico: Baja, Ensenada, La Bufadera	Mar 2003	M. Wilson	173863
P. californica	LC	5	Mexico: Baja, San Quintin, La Churera	Mar 2003	M. Wilson	173864
P. californica	PB	5	Mexico: Baja, El Rosario, Punta Baja	Mar 2003	M. Wilson	173865
P. californica	PA	5	Mexico: Baja, Guerrero Negro, Punta Abre Ojos	Mar 2003	M. Wilson	173866
Phragmatopoma caudata	PL	12	USA: Florida, Dade County, South Miami Beach	Dec 2002	D. McCarthy	173848
P. caudata	PL PR	12	USA: Puerto Rico, Punta Cangrejos	Aug 2004	C. Drake	173850
P. caudata	PL VI	12	USA: Virgin Islands, St. Croix	Jul 2004	D. McCarthy	173849
Phragmatopoma virgini	PV	20	Chile: El Morro	May 2004	V. Gallardo	173867
Idanthyrsus cretus	IC	6	Panama: Playa Chumical	Nov 2002	R. Collin	173847

All specimens were dissected from their sand tubes and preserved in 95% ETOH for molecular analysis

[a] As initially determined according to published species descriptions

[b] California Academy of Sciences Invertebrate Zoology Collection

accumulate at a neutral rate (Schlötterer et al. 1994). Thus, the ITS-1 region can be useful in distinguishing between closely related taxa (Lee and Taylor 1992; Fritz et al. 1994; Chen and Miller 1996; Chen et al. 2002; Pilgrim et al. 2002; Goffredi et al. 2003).

A High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) was used to extract DNA from the posterior ends of each specimen. COI and ITS-1 were amplified from each individual using the PCR. A 710-base pair (bp) fragment of the COI was amplified using the universal metazoan primers LCO1490 (5'- GGTCAACAAATGATAAA GATATTGG-3') and HCO2198 (5'-TAAACTTGA GGGTGACCAAAAAATGA-3') (Folmer et al. 1994), while the entire 450-bp section of the ITS-1 was amplified using the primers ITS-1 FOR (5'-ACGCTCAAA CTTGATCATCTAGAGG-3') and ITS-1 REV (5'-TG CGCTCTTCATCGACGCACGAGCC-3'). The ITS-1 primers were designed for this study from alignment of sequences produced from ITS-1 primers used in Pilgrim et al. (2002). Reaction volumes for both genes were 20 μL, containing 0.2 mM of dNTPs, 2 mM of $MgCl_2$, 2 μL of a 10X Promega PCR buffer, 0.25 μM of the forward primer and 0.25 μM of the reverse primer, 1 unit of *Taq* DNA polymerase, with approximately 20 ng of whole-genomic DNA. Genes were amplified in a thermocycler with an initial denaturation of 94°C for 2.5 min., 35 cycles of 94°C for 30 s, 50°C (COI) or 54°C (ITS-1) for 1 min., and 72°C for 1 min. A final elongation step of 10 min at 72°C followed the cycles, and then the reaction was cooled to 10°C.

During the course of this study, ITS-1 sequences from some individuals were found to be polymorphic (multiple sequences within an individual), presumably because of a breakdown in the process of concerted evolution that normally homogenizes the many copies of the rDNA cistron in the nuclear genome. To determine the breadth of copy variation within a single individual, the PCR products from two individuals were cloned. The ITS-1 gene was amplified from each individual using the same procedure as described. The products were then cloned with a TOPO TA cloning kit with pCR® 2.1-TOPO® vector (Invitrogen Life Technologies, Carlsbad, CA, USA). A few cells from each colony were used as templates in PCR with the same procedures as described. Twenty-five colonies from each individual were assayed.

PCR products were purified with isopropanol precipitation, using an equal volume of 4 M of ammonium acetate, vortex-mixed, then adding twice the original volume of isopropanol. Samples were vortex-mixed and left at room temperature for 1 h, then centrifuged at 12,000 rpm for 20 min to pellet the DNA. Pellets were rinsed with 500 μL of 80% ethanol, dried in a SpeedVac, and resuspended in 14–40 μL of water.

Concentrations of purified PCR products were determined on an agarose gel containing 1 μL of a quantitative ladder (Hyperladder I™, Bioline USA Inc., Randolph, MA, USA). Both Big Dye 2.0 (PE Applied Biosystems, Foster City, CA, USA) and Dynamic ET Terminator Cycle sequencing kits (Amersham Biosciences, Piscataway, NJ, USA) were used to prepare reactions for both a forward (5') and reverse (3') read of the COI and ITS-1 gene using the original PCR primers. Sequencing reactions were prepared according to manufacturer's instructions. Reaction products were visualized on an Applied Biosystems Inc. ABI 377 automated DNA sequencer. Sequences have been deposited into GenBank database [accession nos. DQ172680-DQ172764 and DQ172813-DQ172822 (COI), DQ172765-DQ172812 (ITS-1)].

Phylogenetic analyses

Sequencher v 4.1 (Gene Codes, Inc.) was used to align COI sequences from all taxa. The ITS-1 sequences were aligned with CLUSTAL W (Thompson et al. 1994) using a gap open penalty of 10.0 and a gap extension penalty of 0.05, because this program is better designed to handle the length variation found in such sequences.

In preparing the data sets for phylogenetic analysis, identical sequences of each gene were removed. The two genes were analyzed alone and in combination. NONA v 2.0 (Goloboff 1998) was used to infer relationships between unique sequences by constructing phylogenetic trees using maximum parsimony (MP). Sampling 10% of the characters in the data set, NONA was used to perform the parsimony ratchet (island hopper) analysis as a starting point to find the most parsimonious topology. NONA held a maximum of 10,000 trees for each data set while performing a heuristic search for the most parsimonious trees. After the most parsimonious trees were found and suboptimal trees were discarded, a strict consensus tree was calculated. Non-parametric bootstrapping with 1,000 replicates and ten search repetitions was then performed using NONA to evaluate support for nodes on the tree. For the ITS-1 data, all insertions and deletions were coded as a fifth character in the data matrix; if these occurred in blocks of sites, then the entire block was coded as one character. A ratchet and heuristic search was then performed using the same options as used on the COI data set, except that gaps were treated as a fifth state instead of missing data. A strict consensus tree was calculated from all the most parsimonious trees, and

non-parametric bootstrapping was performed using the same options as described for the COI data set.

A partition homogeneity test [the incongruence-length difference test of Farris et al. (1994)] was performed in PAUP* v 4.08 beta (Swofford 2001) to determine whether the two data sets should be combined. The test proved non-significant (P=0.11) and thus the ITS-1 and COI data sets were combined and subjected to the same analyses as were performed on the individual data sets.

Bayesian analyses were performed on individual and combined data sets using Mr Bayes v 3.0 (Huelsenbeck and Ronquist 2001). The best-fit model of evolution was first estimated using Modeltest version 3.06 (Posada and Crandall 1998). Searches were run using four chains (three cold, one heated) over 3 million generations, sampling trees every 100th generation. The time taken for the parameters to reach stationarity (burn-in) was estimated by plotting the log-likelihood scores of trees against generation number, and visually determining the number of generations at which the values reached an asymptote. Trees generated prior to burn-in were discarded. The consensus phylogram of remaining trees was then viewed in TREEVIEW v PPC (Page 1996) where the consensus number at each node indicated the posterior probabilities of that node as estimated by Mr Bayes. The combined data set was analyzed as partitioned by gene, with the best-fit model assigned to each gene partition, and all variables unlinked between partitions. The combined data set was not partitioned by codon position because virtually all base pair changes in the COI occurred in the third position.

Only the COI data set was employed to estimate the ages of divergence between species of *Phragmatopoma*. First, the sequences were tested for clock-like behavior of substitution rates among lineages. A likelihood score was determined for the best topology with and without the constraint of a molecular clock, and these scores were then subjected to a log-likelihood ratio test. The result indicated that there were significant differences in rates of substitution among lineages, which needed to be adjusted before divergences could be estimated. The program r8s v 1.05beta (Sanderson 2002) was used to adjust for rate heterogeneity among lineages and calculate divergence times using a non-parametric rate smoothing (NPRS) method. Rates of substitution were calibrated to absolute time using COI substitution rates from a free-spawning bivalve study (Luttikhuizen et al. 2003). The substitution rates from this bivalve study were used because these authors used COI sequences from a marine organism with a dispersal mechanism most similar to *Phragmatopoma*.

Results

Phylogenetic analyses

Cytochrome c oxidase subunit I

The final data set for COI consisted of 540 bp from 57, 24, 17, and 6 individuals of *P. californica*, *P. caudata*, *P. virgini*, and *I. cretus* respectively. The COI sequences displayed unequal base frequencies, with an excess of A and T nucleotides (63%). As expected for a protein-coding gene, most substitutions occurred in third positions of the codon (153), with many fewer at the first position (34) and none at the second. No insertions or deletions were observed in any of the sequences.

The MP analysis yielded 10,000 most-parsimonious trees, each with a length of 686 steps, a consistency index (CI) of 0.51 and a retention index (RI) of 0.92. The strict consensus of all trees showed monophyly of each of the three *Phragmatopoma* species as determined from morphology (Fig. 2), which were also supported by the bootstrap at 99–100%. No COI haplotypes were shared among the three *Phragmatopoma* species analyzed. MP resulted in no resolution among populations within *P. californica*, while two distinct lineages were resolved for *P. caudata*—one Florida and one Caribbean population. MP resolved relationships among many individuals of *P. virgini* although most were not supported by the bootstrap except for the sister relationship of individual PV8 to the rest of *P. virgini* (99%). MP also resulted in high support for *P. virgini* as the sister clade to *P. caudata* (97%).

The best-fit model of evolution, as determined by Modeltest, was the transversion model (TVM) with variable base frequencies (A=0.32, C=0.21, G=0.11, T=0.36) and four substitution rate categories, variable transversion rates and equal transition rates, a gamma distributed rate heterogeneity model (α=0.14), and an estimated proportion of invariable sites (0.34). Bayesian analyses resulted in a topology similar to the parsimony consensus tree with a tree –ln(L) score of 3868.38 (Fig. 2). The only differences compared with the MP tree were minor rearrangements within species and less resolution within *P. virgini*.

Internal transcribed spacer region

ITS-1 sequences of 430–446 bp were obtained from a subset of samples consisting of the following number of individuals: 20 *P. californica*, 3 *I. cretus*, 24 *P. caudata*, and 19 *P. virgini*. All other *P. californica* and one *P. virgini* individuals (PV8) yielded polymorphic ITS-1

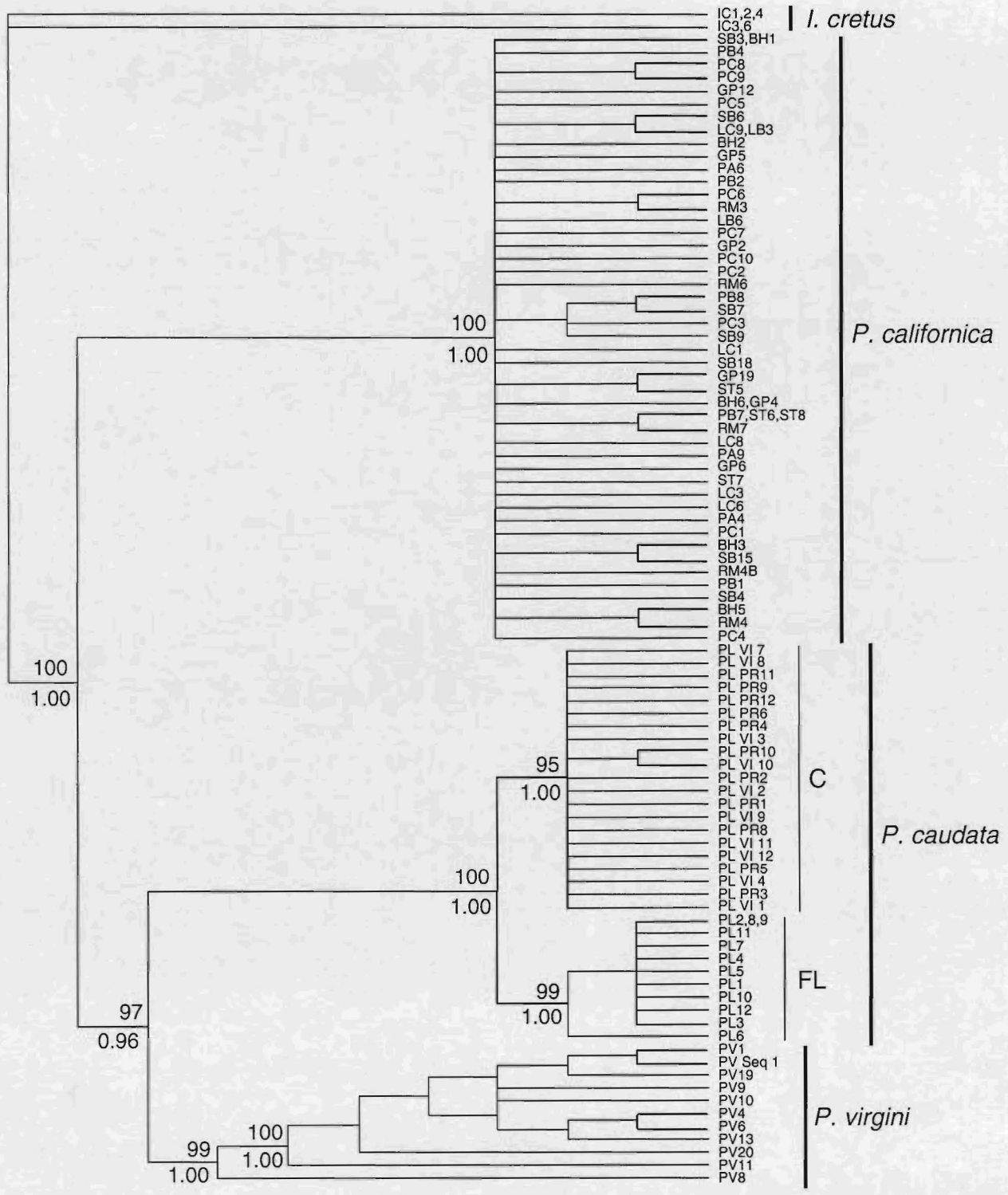

Fig. 2 The maximum parsimony/Bayesian tree from analysis of the cytochrome c oxidase subunit I gene. *Numbers above the branch* indicate bootstrap support greater than 95%. *Numbers below the branch* indicate posterior node probabilities greater than 0.90. *C* corresponds to populations from the Caribbean and *FL* corresponds to the population from Florida. *Names separated by commas* indicate identical sequences. *PV Seq1* refers to individuals PV 2, 5, 7, 12, 15, 17, 18, all with identical sequences. *Letter codes* of the terminal taxa refer to sample IDs as listed in Table 1

sequences, presumably due to multiple, non-homogeneous copies of the rDNA cistron within an individual. These polymorphic sequences were not included in analyses. Twenty-three clones from two individuals that yielded polymorphic sequences from populations PC and PA were included in the analyses of the ITS-1 region. Base frequencies were relatively even and consistent across all taxa in ITS-1. The final alignment contained multiple gaps due to length variation among sequences.

The MP analysis yielded 10,000 most-parsimonious trees each with a length of 303 steps, CI of 0.81 and RI of 0.92. Similar to the COI data set, each of the three *Phragmatopoma* species was monophyletic: no sequences were shared across species (Fig. 3). The monophyly of each species was supported by the bootstrap (99–100%), as was a sister relationship between *P. caudata* and *P. virgini* (95%). The resolution among populations observed with the COI data was not observed under MP of the ITS-1 sequences.

The best-fit model of evolution for ITS-1 was the K80 (Kimura 1980) with gamma-distributed rates (α=0.64). The Bayesian analysis yielded a single tree with a –ln(L) score of 1772.35 (Fig. 4). The Bayesian analysis differed from MP in that *P. virgini* was positioned as the sister to *P. californica*, although there was no statistical support for this arrangement (Bayesian posterior probability << 0.90).

From 25 clones of two individuals, 28 unique ITS-1 sequences were found. Although cloned sequences for each individual did not necessarily form a single clade within the ITS-1 phylogeny (some were more similar to sequences from other individuals than to the rest of the cloned sequences), these sequences were always part of the *P. californica* clade.

Combined analysis of COI and ITS-1

The MP analysis of combined data yielded 10,000 most-parsimonious trees, each with a length of 611 steps, CI of 0.72, and RI of 0.95. As with the analysis of individual genes, each species was monophyletic and *P. caudata* was the sister to *P. virgini*. These relationships were highly supported by the bootstrap (Fig. 5). As in the analysis of COI alone, population structure was observed in *P. caudata* but not in *P. californica*.

The Bayesian analysis resulted in a single tree with a –ln(L) score of 4133.32 (Fig. 5). *P. californica* and *P. caudata* were each monophyletic with 1.0 posterior probability. As with COI alone, *P. virgini* was the sister group to *P. caudata*, although the statistical support for this arrangement was not high (Bayesian posterior probability less than 90%). Again, population structure was seen in *P. caudata*, although not significantly so (Bayesian posterior probability less than 90%).

Divergence rates and times

Using the COI sequence data, within-species divergences (as determined by maximum-likelihood distances under the TVM model) ranged from 0.2 to 2% and between-species divergences ranged from 19 to 23% (Table 2). These numbers are within the ranges of within- and between- species differences found in similar studies at the population and species level (Hebert et al. 2003, 2004; Durish et al. 2004; Hogg and Hebert 2004; Pfeiler et al. 2005).

The log-likelihood ratio test indicated that COI sequences of this data set did not conform to a clock-like rate of substitution (P=0.001). In order to account for rate heterogeneity among lineages, divergence times were estimated using a NPRS method. Divergences were calibrated to real time using substitution rates estimated in a previous study on marine bivalves (Luttikhuizen et al. 2003), which determined rates of mutational changes in the COI gene to be between 0.07 and 0.26% per million year. These rate estimates were deemed applicable because bivalves have external fertilization (as in *Phragmatopoma*), and the study used species pairs from opposite sides of the Isthmus of Panama. These rates from Luttikhuizen et al. (2003) were divided in half from the original estimate because pair-wise distances were improperly used to obtain divergence rates in that study. Using these substitution rates, it was estimated that *P. californica* diverged from *P. caudata* and *P. virgini* approximately 140–500 million years ago. These dates appeared to be gross overestimates, because the earliest recognizable sabellariid tube fossils are from the Pennsylvanian epoch, 318 million years ago, and do not closely resemble modern sabellariid tubes (Kirtley 1994). One of the rates discarded from Luttikhuizen et al.'s (2003) study as implausible was a substitution rate of 2.1% per million year (because it was judged as too high when compared with the other rates). However, this rate is in fact comparable with other invertebrate studies using COI genes (Daniels et al. 2002; Knowlton et al. 1993; Brower 1994; Pfeiler et al. 2005). Using this rate of 0.0021 substitutions per site per million year, the node joining *P. caudata* to *P. virgini* was re-estimated to be 3 million years old, while the node joining *P. californica* and the rest of *Phragmatopoma* was estimated at 5.7 million years old. Using this substitution rate, PV8, which may represent a fourth species, *P. moerchi*, was estimated to have diverged from the rest of *P. virgini*, approximately 1.6 million years ago.

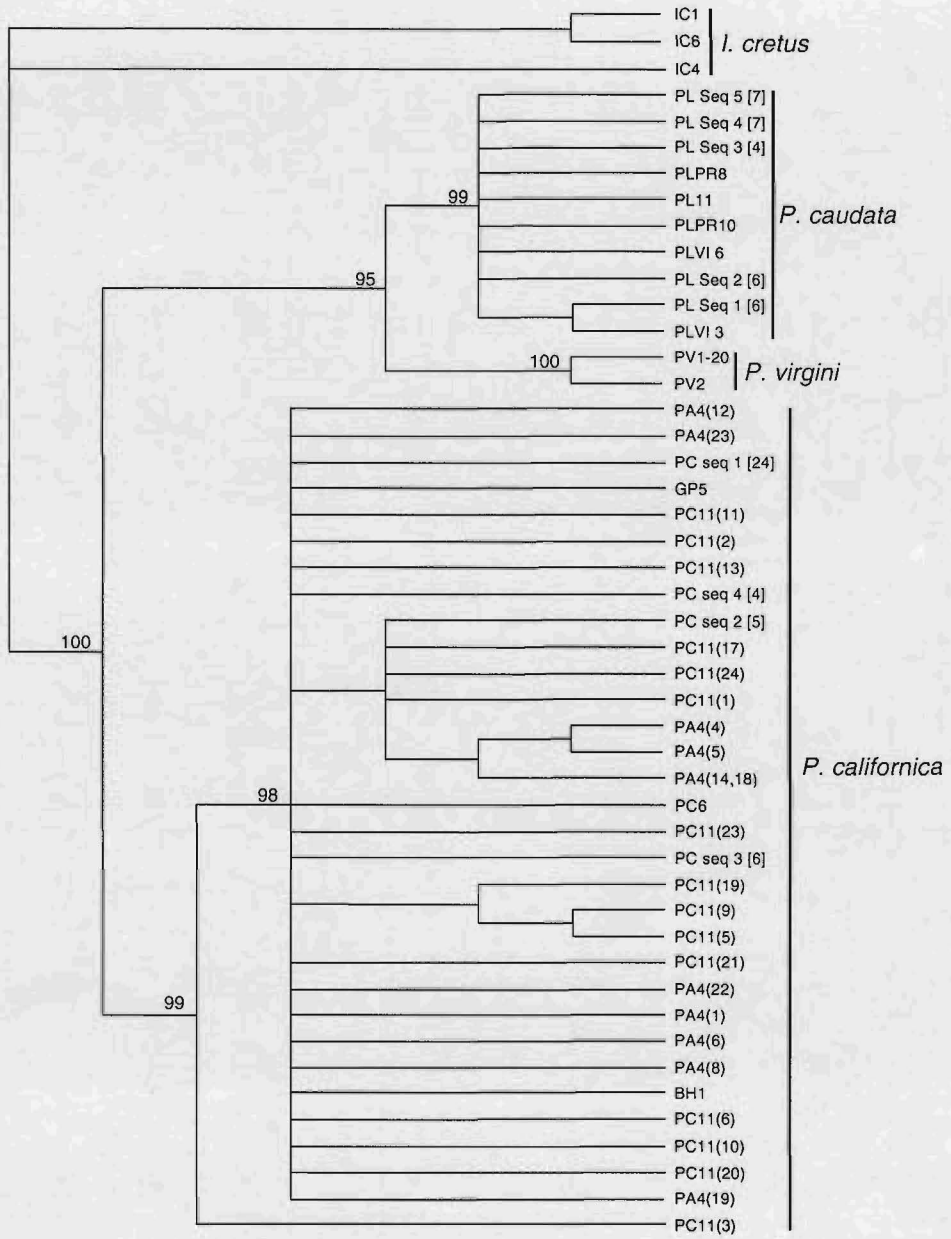

Fig. 3 Strict consensus of 10,000 maximum parsimony shortest trees of the first internal transcribed spacer region sequences using NONA. *Numbers* indicate bootstrap support for nodes greater than 50%. Identical sequences represented by more than three individuals are called 'Seq'. *Numbers in brackets* after 'Seq' indicate how many individuals possessed that sequence. *Numbers in parentheses* indicate clone identification. *Letter codes* of the terminal taxa refer to sample IDs as listed in Table 1

Discussion

All analyses supported the hypothesis that *P. californica* and *P. caudata* should be considered separate species. Individual and combined analyses of the mitochondrial and nuclear genes yielded high support for the monophyly of each of these taxa. No haplotypes or sequences of either gene were shared between these species. The level of COI sequence divergence within each species, 0.2–2%, was low compared with the level of sequence divergence between the two taxa, 19–23%. This level of divergence is comparable with those between other recognized invertebrate species pairs (Hebert et al. 2003, 2004; Durish et al. 2004; Hogg and Hebert 2004; Pfeiler et al. 2005).

The geographic distributions of *P. californica* and *P. caudata* are at present completely disjunct. *P. californica* is found on the intertidal and shallow subtidal

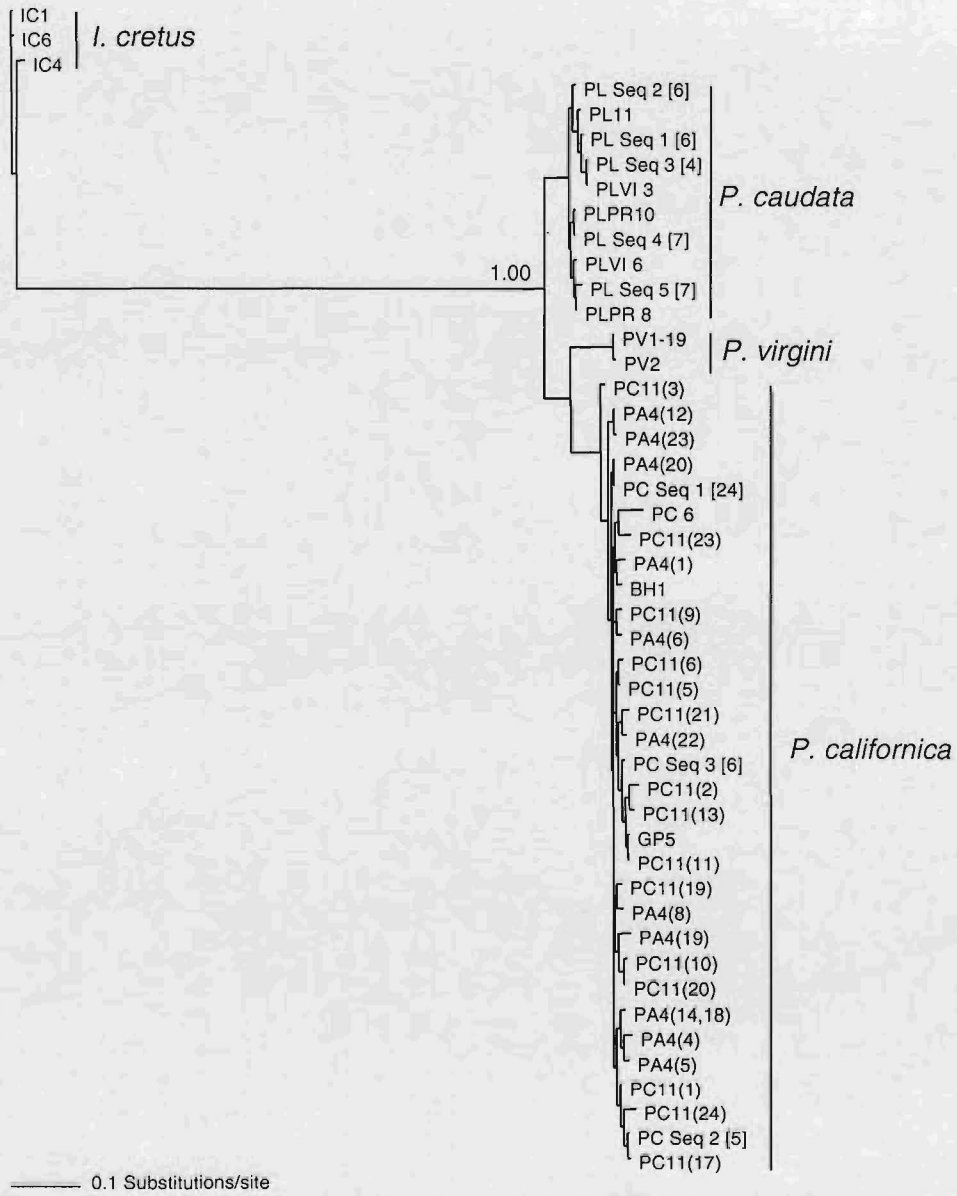

Fig. 4 Topology produced from the Bayesian analysis of the first internal transcribed spacer region sequences with branch lengths proportional to substitutions per site. The *number 1.00* above the branch indicates high Bayesian posterior probability for this clade. Identical sequences represented by more than three individuals are called 'Seq'. *Numbers in brackets* after 'Seq' indicate how many individuals possessed that sequence. *Numbers in parentheses* denote clone identification. *Letter codes* of the terminal taxa refer to sample IDs as listed in Table 1

zones of the western coast of North America, while *P. caudata* is found in similar habitats of the Caribbean and on the eastern shores of North, Central, and South America (Kirtley 1994). No ocean current presently exists to directly connect these two ranges and allow gametes from each of the two species to have contact. Presumably, this has been the case for 3 million years since the Isthmus of Panama arose in the late Pliocene, and possibly earlier if ocean currents were disrupted by island formation 11–12 million years (Duque-Caro 1990; Coates et al. 1992; Collins et al. 1996; Bermingham et al. 1997). At present, the man-made Panama Canal is a passageway between the Atlantic and Pacific Oceans, but the large, freshwater Gatun Lake most likely interrupts dispersal by marine species. Consequently, *P. californica* and *P. caudata* have probably been geographically and reproductively isolated for at least 3 million years.

Because *P. californica* and *P. caudata* presumably have been physically isolated with no range overlap,

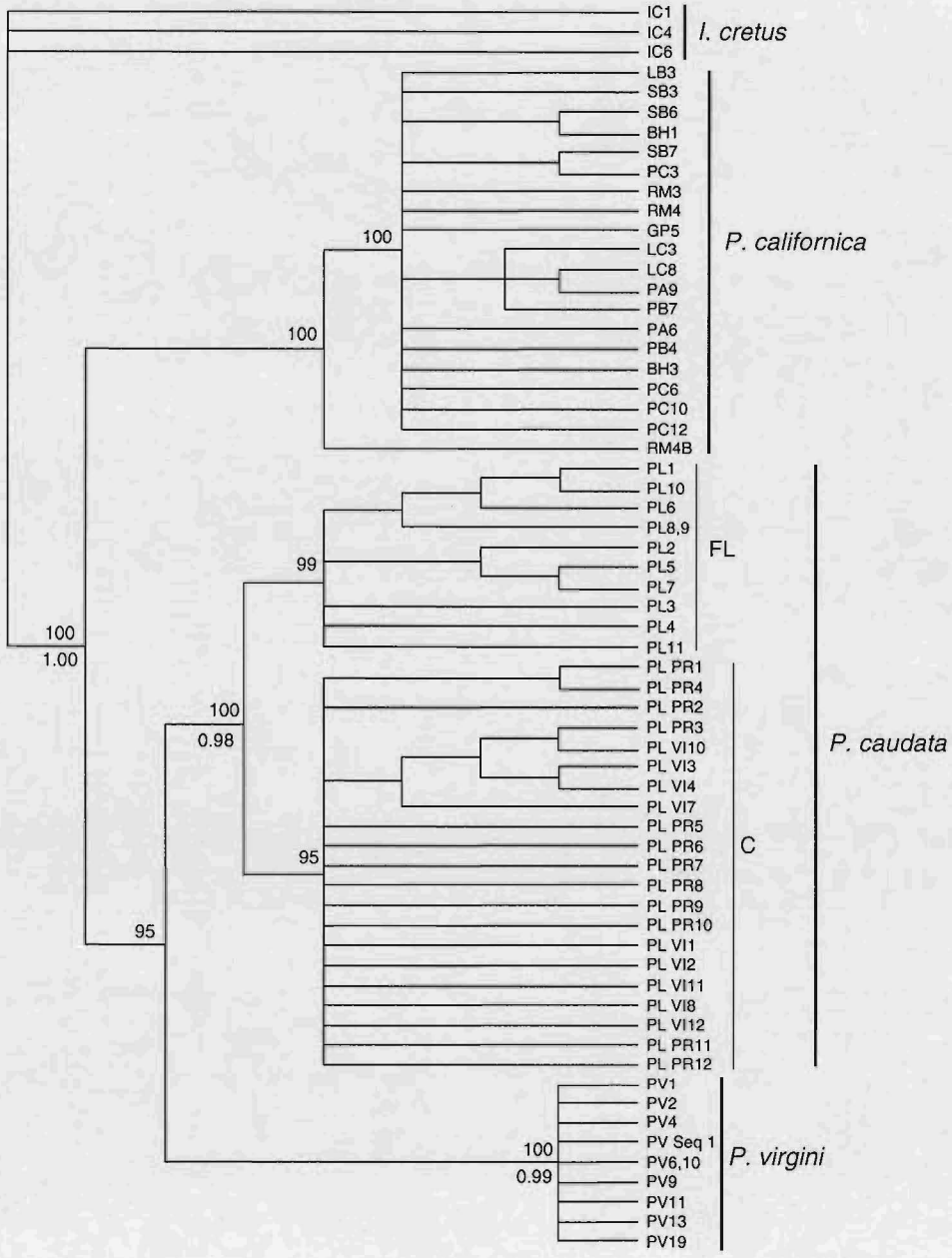

Fig. 5 Topology produced from maximum parsimony and Bayesian analysis of combined cytochrome c oxidase subunit I and the first internal transcribed spacer region. *Numbers above branches* indicate bootstrap support for nodes greater than 90%, while *numbers below branches* correspond to Bayesian posterior probabilities greater than 0.90. *FL* corresponds to individuals from the Florida population, while *C* corresponds to individuals from populations in the Caribbean. *Names separated by commas* indicate identical sequences. *Letter codes* of the terminal taxa refer to sample IDs as listed in Table 1

there has been no selective pressure to drive reproductive isolation between these taxa. This may explain why Pawlik's (1988a) cross-mating experiments were successful. Pawlik (1988a) carried out this experiment in a laboratory setting that overlooked many pre-zygotic reproductive barriers. First, the experiment did not account for any pre-zygotic barriers such as differences in temperature (Atlantic being warmer than the Pacific). Second, there could be a temporal difference when these two species spawn their gametes into the water column. Third, the experiment was not continued to determine if the larvae produced from reciprocal matings could produce viable eggs and sperm, and thus viable F2 larvae.

Morphologically, *P. californica* and *P. caudata* can be distinguished by the distal spikes attached to the

Table 2 Pairwise maximum-likelihood distances of COI sequences under the TVM model between and among the four species analyzed in this study

Species	*P. californica*	*P. caudata*	*P. virgini*	PV8
P. californica	0.004–0.02	0.19–0.22	0.22–0.23	0.21
P. caudata		0.002–0.02	0.21–0.22	0.19
P. virgini			0.002–0.02	0.14

PV8 is an individual collected from the Chilean population, determined in this study to be a separate species, *P. moerchi*

outer opercular paleae, and the presence or absence of transverse fibrous thecal bands on the middle paleae. However, morphological determinations of these species can be difficult because the distal spikes can be destroyed or distorted when worms are removed from their sand tubes. Further, the fibrous bands can only be visualized using electron microscopy and can be affected by the preservative used to fix the worms. Consequently, geographical ranges and molecular characters seem to be the best way, at present, of positively identifying these species unless specimens are handled carefully and examined with proper methods.

In all but one analysis, *P. caudata* and *P. virgini* were found to be more closely related to each other than either was to *P. californica*. This result supported the idea that not only are *P. californica* and *P. caudata* separate species, but that they may be more distantly related than previously expected. *P. caudata* is found throughout the Caribbean and on the Atlantic shores of North and Central America, while *P. virgini* is found on the Pacific coast of Mexico south of Argentina. A close relationship between a Southern Pacific Ocean species and a Caribbean species is entirely plausible, given the pattern of ocean currents before the rise of the Isthmus of Panama in the late Pliocene. In the middle Miocene (~15.1–12.9 million years), oceanic waters traveled westward from the Caribbean and then southward to the southern Pacific Ocean (Duque-Caro 1990). Consequently, gametes of *Phragmatopoma* may have dispersed via these ocean currents between Caribbean/Atlantic and Southern Pacific Oceans, maintaining gene flow between populations of these two species. At the same time, the California current flowed south meeting the Atlantic current along the Equator and was deflected westward towards Asia, thereby never coming directly in contact with Southern Pacific Ocean water (Duque-Caro 1990). Duque-Caro's work (1990) suggests that ocean currents flowed in this pattern until ~11.8 million years when islands began to rise in the Isthmus of Panama. Even in the presence of these islands, ocean currents still flowed from the Atlantic to the Pacific until the full closure of the Isthmus approximately 3 million years. This late Tertiary history of ocean currents is consistent with the pattern and estimated ages of lineage splitting in these three *Phragmatopoma* species.

One major issue regarding *Phragmatopoma* is just how many species should be recognized. The molecular phylogenetic results reported here support the recognition of *P. californica*, *P. caudata*, and *P. virgini* as distinct species. In addition, the individual coded as PV8 may represent a fourth species (*P. moerchi*) distinct from the rest of the *P. virgini* population. The 14% level of the COI sequence divergence between PV8 and other *P. virgini*, was much larger than levels within the remaining *P. virgini* population (0.2–2%), but less than the divergence between *P. californica* and *P. caudata* (19–23%). Consistent with the molecular divergence, morphological differences were apparent between PV8 and the rest of the *P. virgini* population. PV8 had distinctly different distal spikes attached to the outer opercular paleae when compared to other individuals in the *P. virgini* population. PV8 had a frayed distal appendage while the other *P. virgini* had no distal appendage. According to Hartman's (1944) key, PV8 would be identified as *P. moerchi*, while according to Kirtley's (1994) key, PV8 would be identified as *P. virgini*. Kirtley (1994) did not use plumage on the outer opercular paleae to distinguish species within *Phragmatopoma*. He used the shape of the median plume together with middle opercular paleae shape. While greater sampling is clearly needed to thoroughly test the status of all proposed species in the southern Pacific, our molecular data and re-examination of morphology strongly support the idea that *P. virgini* and *P. moerchi* should be recognized as separate species.

One unexpected finding in this study was the detection of multiple different sequences of the ITS-1 gene within individuals of *P. californica, I. cretus*, and PV8. Of the 58 *P. californica* individuals used in this study, 38 had polymorphic ITS-1 sequences. Multiple copies (up to hundreds) of the rDNA cistron, including ITS-1 and 2, occur throughout the eukaryotic genome, but concerted evolution is thought to homogenize sequence differences throughout these reiterated copies within a species (Álvarez and Wendel 2003). The polymorphic ITS1 sequences found in this study suggests that complete concerted evolution is not occurring for this gene in these species. Such evidence of an incomplete process or breakdown in concerted evolution has been accumulating in many diverse organisms since the widespread application of PCR in the past 15 years (Vogler and DeSalle 1994; Ko and Jung 2002; Mes and Cornelissen 2004; Parkin and Butlin 2004; Wörheide et al. 2004). Currently, little is known about

what may interfere with the process of concerted evolution (Álvarez and Wendel 2003).

Evidence from the COI and the combined gene results indicated that population structure did not correlate with geographical location in *P. californica*. In *P. caudata*, however, there appeared to be two separate populations, one in Florida and one in the Caribbean. These populations did not exhibit any noticeable morphological differences. This population structure associated with geography in *P. caudata* could indicate the possibility of speciation in the future if gene flow between them is severed. In contrast to the COI results, the ITS-1 gene yielded no pattern of population structure by geography within any species. This may be indicative of the evolutionary rates of the two genes studied: based on a comparison of genetic distances, COI is evolving at a faster evolutionary rate than the ITS-1 region in these species.

Further studies of the morphological differences between *P. californica* and *P. caudata* should be undertaken to determine whether additional characteristics might be found that better distinguish between these two species. Additional studies to validate other species proposed for *Phragmatopoma* should include sampling of *P. attenuata* populations and greater sampling within *P. virgini* and *P. moerchi*. Sampling throughout the entire habitat of *Phragmatopoma* and performing the necessary molecular work would also help reveal if other, previously undetected, species exist within *Phragmatopoma*. The addition of other mitochondrial genes, the addition of more taxa, or other molecular techniques, such as amplified fragment length polymorphisms (AFLPs), could help to resolve the broader species relationships within *Phragmatopoma*.

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