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ORIGINAL ARTICLE

Evolutionary Patterns in Pearl Oysters of the Genus *Pinctada* (Bivalvia: Pteriidae)

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Abstract Pearl oysters belonging to the genus *Pinctada* (Bivalvia: Pteriidae) are widely distributed between the Indo-Pacific and western Atlantic. The existence of both widely distributed and more restricted species makes this group a suitable model to study diversification patterns and prevailing modes of speciation. Phylogenies of eight out of the 11 currently recognised *Pinctada* species using mitochondrial (*cox1*) and nuclear (18S rRNA) data yielded two monophyletic groups that correspond to shell size and presence/absence of hinge teeth. Character trace of these morphological characters onto the molecular phylogeny revealed a strong correlation. *Pinctada margaritifera* appears polyphyletic with specimens from Mauritius grouping in a different clade from others of the French Polynesia and Japan. Hence, *P. margaritifera* might represent a species complex, and specimens from Mauritius could represent a different species. Regarding the putative species complex *Pinctada fucata*/*Pinctada martensii*/*Pinctada radiata*/*Pinctada imbricata*, our molecular analyses question the taxonomic validity of the morphological characters used to discriminate *P. fucata* and *P. martensii* that exhibited the lowest genetic divergence and are most likely conspecific as they clustered together. *P. radiata* and *P. imbricata* were recovered as monophyletic. The absence of overlapping distributions between sister lineages and the observed isolation by distance suggests that allopatry is the prevailing speciation mode in *Pinctada*. Bayesian dating analysis indicated a Miocene origin for the genus, which is consistent with the fossil record. The northward movement of the Australian plate throughout the Miocene played an important role in the diversification process within *Pinctada*.

Keywords *Pinctada* · Evolutionary patterns · Species complex · Allopatry · Biogeography

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Introduction

The observed repeated instances of speciation in marine organisms with high dispersal abilities (Taylor and Hellberg 2003; Williams and Reid 2004; Paulay and Meyer 2006) challenge the classical model for diversification in allopatry as a rare event in the sea. The analysis of present-day distribution patterns allows assessing the influence of past tectonic, climatic and oceanographic changes (Frey and Vermeij 2008) on species distribution. Biogeographic patterns may be shaped by vicariant events associated to isolation and large-scale barriers (Lessios et al. 2001) or founder dispersal, both consistent with an allopatric mode of speciation (Paulay and Meyer 2002; Williams and Reid 2004). Any attempt at unravelling the origin of ancient speciation requires first the reconstruction of a robust phylogenetic framework and accurate dating of cladogenetic events (Arbogast and Slowinski 1998; Rüber et al. 2003; McCafferty et al. 2002; Rutschmann et al. 2007).

Pearl oysters belonging to the genus *Pinctada* (Bivalvia: Pteriidae) are widely distributed between the Indo-Pacific and western Atlantic (Fig. 1) tropical and subtropical shallow-water areas, most of them associated to reef environments (Strack 2008). The relatively extended larval duration of *Pinctada* species spanning from 16 to 30 days (Gervis and Sims 1992) likely played an important role in the broad distribution of the genus. The existence of both widely distributed (e.g. *Pinctada margaritifera* in the Indo-Pacific region) and more localised *Pinctada* species (e.g. *Pinctada mazatlanica* in the Gulf of California) makes this genus a suitable model group to study diversification patterns and to assess the prevailing mode of speciation. The genus is included in the family Pteriidae, of which the origin based on the fossil record is placed in the Triassic, approximately 230 million years ago (MYA) (Hertlein and Cox 1969; Skelton and Benton 1993). The fossil record of *Pinctada* is much more recent dating back to the Miocene of Western Europe and Caucasus (Hertlein and Cox 1969; Caretto 1975; Caretto et al. 1989).

The taxonomy of pearl oyster species is complex because shells are quite similar (Masaoka and Kobayashi 2005), and there are not many morphological diagnosable characters available for species determination (Wada and Tëmkin 2008). Classification is mainly based on soft tissues and shell characters (colour and shape) (Ranson 1961). Yet, as for many bivalve species, soft tissues exhibit few informative characters, and shell morphology is difficult to distinguish in younger specimens (Wang et al. 2004). Moreover, high levels of phenotypic plasticity were detected in response to environmental heterogeneity (Hollander 2008). The current classification of species may be erroneous because previous taxonomic work on pearl oysters was exclusively based on morphological characters and did not take into account intraspecific variation (Wada and Tëmkin 2008). The use of molecular sequence data might provide valuable information regarding the taxonomic status of the species of the genus *Pinctada*. This information may be important in order to better define the status of taxa with high commercial value for management or selection programs. The pearl oyster industry is a growing segment of mollusc aquaculture spread throughout Australasia, the Middle East and South America (Bondad-Reantaso et al. 2007). The species used in aquaculture of marine pearls are the silver-lip or gold-lip pearl oyster (*Pinctada maxima*), the black-lip pearl oyster (*P. margaritifera*) and the Akoya pearl oyster (*Pinctada fucata*). The global marine cultured pearl production involves individuals, cooperatives, as well as large multinational companies, offering economic opportunities to coastal communities in less developed countries (Southgate 2007). In French Polynesia, for example, it allows maintaining a sustainable economical activity in isolated atolls, thereby maintaining local social networks and limiting exodus (Arnaud-Haond et al. 2003a). According to Ranson (1961), 11 species are recognised, while other authors agreed on the existence of more than 14 in the Indo-Pacific region (Hynd 1955; Wada and Tëmkin 2008). Due to extensive morphological variation among populations, the species *P. fucata*, *Pinctada martensii*, *Pinctada radiata* and *Pinctada imbricata*, the so-called Akoya pearl oysters, are

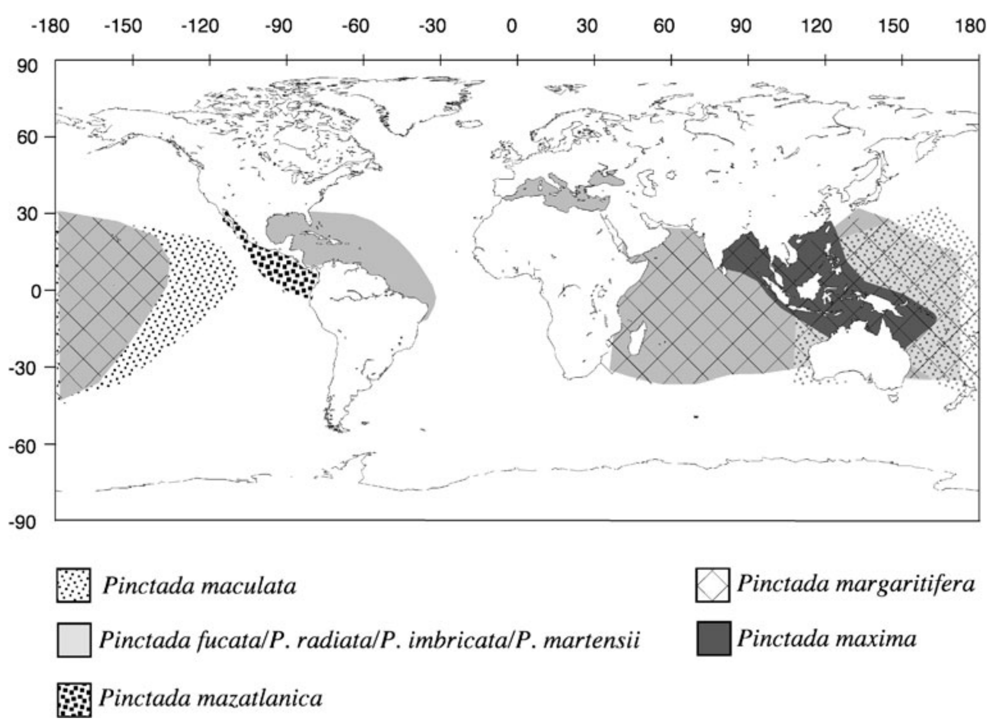

Fig. 1 Approximate distribution of the species of *Pinctada* pearl oysters used in this study

presumed to belong to a species complex. *P. radiata* has a broad geographic distribution including the Mediterranean Sea, eastern Indian Ocean and Red Sea/Persian Gulf regions, whereas *P. imbricata* is only found in the western Atlantic. *P. fucata* and *P. martensii* are found in the Indo-Pacific region (Wada and Tëmkin 2008). Jameson (1901) indicated the existence of several subspecies within *P. margaritifera* including, e.g. *Pinctada margaritifera cumingii* (Central Pacific), *Pinctada margaritifera zanzibarensis* (Mauritius) and *Pinctada margaritifera mazatlanica* (Gulf of California). Furthermore, the genetic structure found between populations of *P. margaritifera* within the Central Pacific archipelagos using mtDNA and nuclear markers also suggested the existence of an alleged species complex (Arnaud-Haond et al. 2003b, 2008).

Several phylogenetic hypotheses have been proposed for the genus. A morphology-based phylogeny analysing relationships within the superfamily Pterioidea yielded a topology in which *P. fucata* grouped with *P. mazatlanica* to the exclusion of *P. imbricata* (Tëmkin 2006). Although not relying on a phylogenetic framework, Jameson (1901) proposed the existence of two major groups defined by morphological criteria: (1) species with smaller shells and hinge teeth (*P. radiata* Leach, 1814, *P. fucata* Gould, 1850, *P. imbricata* Röding, 1798 and *P. martensii* Ranson 1961), which are structures that ensure a proper closure of shell valves, and (2) species with larger shells without hinge teeth (*P. mazatlanica* Hanley, 1855, *P. maxima* Jameson 1901 and *P. margaritifera* Linnaeus, 1758). Most of the molecular studies performed thus far analysed genetic structure of a single species within the genus (e.g. *P. margaritifera* (Arnaud-Haond et al. 2004, 2008), or *P. mazatlanica* (Arnaud-Haond et al. 2000)). The most complete attempt to resolve phylogenetic relationships within the genus was based on nuclear internal transcribed spacer markers and found a close relationship between *P. maxima* and *P. margaritifera* (Yu and Chu 2006; Yu et al. 2006). Nevertheless, these authors were unable to resolve phylogenetic relationships within the above referred species complex and only used two species from the larger shelled group recognised by Jameson (1901).

In the present study, we examined phylogenetic relationships among eight species belonging to the genus *Pinctada* based on a fragment of the mitochondrial (mt) cytochrome oxidase subunit I (*cox1*) and the complete nucleotide sequence of the nuclear 18S ribosomal RNA genes. The reconstructed phylogenies were used to: (1) analyse morphological patterns regarding shell size and presence/absence of hinge teeth testing for their correlation with molecular phylogenetic patterns; (2) infer the geographical patterns of speciation and date major cladogenetic events within the genus; and (3) shed light on the prevailing mode of speciation, i.e. allopatry or sympatry.

Methods

DNA Extraction, Amplification and Sequencing

To assess phylogenetic relationships of the pearl oysters, we used 43 specimens (38 from this study) belonging to the genus *Pinctada* representing eight out of the 11 currently recognised species within the genus (see Table 1 for sample locations). Three other Pteriidae, *Pteria sterna*, *Pteria hirundo* and *Pteria loveni*, are the closest sister genus to *Pinctada* and were chosen as the outgroup (Tëmkin 2006). Tissue samples were preserved in 70–100% ethanol, and total genomic DNA was isolated using sodium dodecyl sulphate/proteinase K digestion and phenol–chloroform extraction method (Sambrook et al. 1989). The specific primers LCX 5′-TCG TAT AGA GCT CCG TCG ACC TG-3′ and HCY 5′-TGG AAC AAA ACT GGA TCG CC-3′ designed in a previous study (Arnaud-Haond et al. 2000, 2003c) were used to amplify a fragment of about 600 base pairs (bp) of the mitochondrial cytochrome oxidase subunit I (*cox 1*) gene. Polymerase chain reaction (PCR) amplifications were carried out in 25 or 50 µl reactions using the following final concentrations: 10× PCR polymerase buffer (Promega), 2.5 mM of $MgCl_2$, 2 mM of each dNTP, 0.6 µM of each primer and 0.8 to 2 U of *Taq* polymerase. The following profile was used: an initial denaturing step at 94°C for 3 min; 30 cycles of denaturing at 94°C for 1 min, annealing at 45°C for 1 min and extending at 72°C for 1 min; and a final extending step at 72°C for 5 min. PCR amplicons were purified either by using the two enzymes Presequencing Kit (Eurogentec) or the Gene Clean Kit (Pharmacia Biotech.) and directly sequenced with the corresponding PCR primers. Sequencing was performed in an automated sequencer (ABI PRISM 3700) using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and following manufacturer's instructions.

Phylogenetic Reconstruction

Alignments of nucleotide sequences were constructed with Clustal X version 1.83 using default parameters (Thompson et al. 1997) and verified by eye in order to maximise positional homology. Two different data sets were analysed: (1) partial nucleotide sequences of the *cox*1 mt gene of 43 specimens representing eight species of *Pinctada* and the three outgroup (*P. sterna*, *P. hirundo* and *P. loveni*) produced an alignment of 506 bp. Of these, 209 were constant, and 248 were parsimony informative; and (2) partial nucleotide sequences of the mt *cox1* (506 bp) of seven specimens, each one representing a species of *Pinctada* (this study), and the complete nuclear 18S ribosomal RNA genes from seven *Pinctada* species retrieved from GenBank were combined into a single data

Table 1 Species used in this study, GenBank accession numbers and geographical location of the samples

Species	Haplotype	Sample location	GenBank accession no.	
			cox1	18SrRNA
Pinctada margaritifera	Pmargaritifera 101	French Polynesia	AF374320	–
Pinctada margaritifera	Pmargaritifera 102	French Polynesia	AF374321	–
Pinctada margaritifera	Pmargaritifera 103	French Polynesia	AF374322	–
Pinctada margaritifera	Pmargaritifera 104	French Polynesia	AF374323	–
Pinctada margaritifera	Pmargaritifera 105	French Polynesia	AF374324	–
Pinctada margaritifera	Pmargaritifera 106	French Polynesia	AF374325	–
Pinctada margaritifera	Pmargaritifera 107	French Polynesia	AF374326	–
Pinctada margaritifera	Pmargaritifera 108	French Polynesia	AF374327	–
Pinctada margaritifera	Pmargaritifera 109	French Polynesia	AF374328	–
Pinctada margaritifera	Pmargaritifera 110	French Polynesia	AF374329	–
Pinctada margaritifera	Pmargaritifera 111	French Polynesia	AF374330	–
Pinctada margaritifera	Pmargzanzib1	Mauritius	GQ355869	–
Pinctada margaritifera	Pmargzanzib4	Mauritius	GQ355872	–
Pinctada margaritifera	–	Okinawa, Japan	AB259166	AB214451
Pinctada mazatlanica	Pmazatlanica 101	Gulf of California	AF374307	–
Pinctada mazatlanica	Pmazatlanica 102	Gulf of California	AF374308	–
Pinctada mazatlanica	Pmazatlanica 103	Gulf of California	AF374309	–
Pinctada mazatlanica	Pmazatlanica 104	Gulf of California	AF374310	–
Pinctada mazatlanica	Pmazatlanica 105	Gulf of California	AF374311	–
Pinctada mazatlanica	Pmazatlanica 106	Gulf of California	AF374312	–
Pinctada mazatlanica	Pmazatlanica 107	Gulf of California	AF374313	–
Pinctada mazatlanica	Pmazatlanica 108	Gulf of California	AF374314	–
Pinctada mazatlanica	Pmazatlanica 109	Gulf of California	AF374315	–
Pinctada mazatlanica	Pmazatlanica 110	Gulf of California	AF374316	–
Pinctada mazatlanica	Pmazatlanica 111	Gulf of California	AF374317	–
Pinctada mazatlanica	Pmazatlanica 112	Gulf of California	AF374318	–
Pinctada mazatlanica	Pmazatlanica 113	Gulf of California	AF374319	–
Pinctada maxima	–	Philippines	–	AB214450
Pinctada maxima	–	Japan	AB259165	–
Pinctada maxima	Pmaxima6	Northern Australia	GQ355881	–
Pinctada maxima	Pmaxima7	Northern Australia	GQ355880	–
Pinctada maxima	Pmaxima 9	Northern Australia	GQ355879	–
Pinctada radiata	Pradiata1	United Arab Emirates	GQ355878	–
Pinctada radiata	Pradiata2	United Arab Emirates	GQ355877	–
Pinctada radiata	Pradiata3	United Arab Emirates	GQ355876	–
Pinctada radiata	Pradiata4	United Arab Emirates	GQ355875	–
Pinctada imbricata	Pimbricata1	Guadeloupe Island	GQ355883	–
Pinctada imbricata	Pimbricata2	Guadeloupe Island	GQ355873	–
Pinctada imbricata	Pimbricata3	Guadeloupe Island	GQ355870	–
Pinctada fucata	–	Amami, Japan	–	AB214463
Pinctada fucata	Pinctadafucata1	Japan	GQ355871	–
Pinctada martensii	–	Japan	AB076915	AB214464
Pinctada martensii	Pmartensii1	Japan	GQ355882	–
Pinctada imbricata	–	Florida, USA	–	AB214456
Pinctada maculata	–	Kagoshima, Japan	AB261166	AB214455
Pteria hirundo	–	–	AF120647	AF120532
Pteria sterna	Pteriasterna	–	AY223839	–
Pteria loveni	–	–	AB076925	–

set that produced an alignment of 2,333 bp. Of these, 2,107 were constant, and 167 were parsimony informative. *P. hirundo* was used as the outgroup.

The Akaike Information Criterion (Akaike 1973) implemented in Modeltest v.3.7 (Posada and Crandall 1998) was used to determine the evolutionary model that best fits the data sets.

Bayesian Analysis Bayesian inferences (BI) were conducted with MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). Four Metropolis-coupled Markov chain Monte Carlo (MCMC) analyses were run for one million generations and sampled every 100 generations. Two independent runs were performed for each data set. The mtDNA data set was analysed under the GTR+Γ, and the burn-in was 80,000 generations. The best-fit model for the nuclear data set was HKY+I, and the burn-in was 100,000 generations. The mt and nuclear partitions of the combined data set were analysed under the GTR+Γ and HKY+I models, respectively. Model parameters were estimated independently for the two data partitions using the 'unlink' command in MrBayes. The burn-in in the combined analysis was set to 60,000 generations. Robustness of the inferred trees was evaluated using Bayesian posterior probabilities (BPPs). BI analyses were carried out using the resources of the Computational Biology Service Unit from Cornell University (http://cbsuapps.tc.cornell.edu/).

Maximum Likelihood Analyses PhyML v2.4.4 (Guindon and Gascuel 2003) was used to estimate the maximum likelihood (ML) tree and to test by nonparametric bootstrap proportions the robustness of the inferred trees using 1,000 pseudoreplicates. The GTR+Γ model was selected for the mt *cox1* data set, whereas HKY+I was the best evolutionary model for the nuclear data set. The selected model for the combined data set used in ML analysis was the TrN+I+Γ. Because TrN+I+Γ model is not available in PhyML, the GTR+I+Γ (the second best-fit model, according to Modeltest) was used in the ML analysis of the combined data set. All ML analyses were carried out on the freely available Bioportal (http://www.bioportal.uio.no).

Divergence Time Estimation

Divergence times of the main cladogenetic events in the *Pinctada* phylogeny were estimated using a relaxed molecular clock Bayesian approach as implemented in Beast version 1.4.8 (Drummond and Rambaut 2007) using the mitochondrial data set because it maximises the number of analysed taxa. This methodology uses probabilistic calibration priors instead of point calibrations, allowing the incorporation of fossil uncertainties (Drummond et al. 2006). Two calibration points were provided by placing a Lognormal prior distribution on the age of the stem lineages of the genus *Pinctada* and of the species *P. mazatlanica*. The first calibration point was based on the approximate age of first occurrence of *Pinctada* in the fossil record during the Miocene between 23 and 5.3 MYA (Hertlein and Cox 1969). The second calibration point was based on the existence of a fossil of *P. mazatlanica* reported from the Pliocene of Baja California between 5.33 and 1.8 MYA (Moore 1983). We choose the Yule speciation model that assumes a constant rate of speciation, following a pure birth-dead process (Yule 1924) as suggested in (Drummond et al. 2006). This estimate assumes a constant rate of speciation but uses the phylogenetic information in the tree to estimate number of the lineages at the end and beginning of the time. The analysis was performed under the General Time Reversible (GTR) substitution model; rate variation among sites was modelled using a discrete gamma distribution with four categories, and, in addition, the proportion of invariant sites was estimated. MCMC were performed in Beast with 20,000,000 steps, following a discarded burn-in of 2,000,000 steps. The convergence of the chains to the stationary distribution was confirmed by inspection of the MCMC samples using the program Tracer v1.4 (Rambaut and Drummond 2007) that provides a measure of whether the chain has run for an adequate length.

Correlation Between Presence/Absence of Hinged Teeth and Shell Size with Molecular Phylogeny of *Pinctada*

In order to evaluate whether there is a significant clustering of *Pinctada* species and presence/absence of hinged teeth, we used MacClade v. 4.03 (Maddison and Maddison 2001). Two character states ('hinge teeth present' and 'hinge teeth absent') were mapped onto the mitochondrial tree (because it maximises the number of analysed taxa) using information from the literature (Tëmkin 2006; Wada and Tëmkin 2008). The same procedure was applied to the character shell size, with two character states ('small' and 'large') to analyse if there is correlation between shell size and the phylogenetic patterns based on the tree. See Table 2 for further information on character mapping.

Results

Mitochondrial Data Set

The BI analysis based on a fragment of the mt *cox1* gene yielded the tree (−ln L=3934.17) shown in Fig. 2. Two main clades that included smaller pearl oyster species with hinge teeth (hereafter 'smaller-toothed' clade) and larger species without hinge teeth (hereafter 'larger-toothless'

Table 2 Character list used in the analysis of correlation between the morphological characters presence/absence of hinge teeth and shell size and the reconstructed BI mt-based phylogeny

Species	Character: hinge teeth Character state: presence/absence of hinge teeth	Character: shell size Character state: small/large
Pinctada margaritifera	Absent	Large
Pinctada mazatlanica	Absent	Large
Pinctada maxima	Absent	Large
Pinctada fucata	Present	Small
Pinctada martensii	Present	Small
Pinctada radiata	Present	Small
Pinctada imbricata	Present	Small
Pinctada maculata	Present	Small
Pteria sterna	Present	Large
Pteria hirundo	Present	Large
Pteria loveni	Present	Large

clade) were evidenced, but only the latter displayed a significant BPP value (99%).

The 'small-toothed' clade included species belonging to the putative complex *P. fucata*/*P. martensii*/*P. imbricata*/*P. radiata* and *Pinctada maculata*. This latter species, from the central part of Ryūkyū archipelago (Okinawa Island) in SE Japan, was found in a basal position with respect to the rest of specimens belonging to the 'smaller-toothed' clade. *P. radiata* and *P. imbricata* were both monophyletic, whereas *P. martensii* and *P. fucata* clustered together in the same clade (Fig. 2). *P. imbricata* is the sister lineage of the clade (*P. fucata* + *P. martensii*) to the exclusion of *P. radiata*.

The 'larger-toothless' clade included *P. mazatlanica*, *P. margaritifera* and *P. maxima*. In this clade, only *P. mazatlanica* from the Gulf of California was monophyletic grouping with specimens of *P. margaritifera* from French Polynesia, and with a specimen from Okinawa Island. The two specimens of *P. margaritifera* from Mauritius (southwest Indian Ocean) clustered together in a basal position with respect to the remaining *P. margaritifera* specimens. *P. maxima* from Japan clustered with the Japanese specimen of *P. margaritifera*. The ML analysis showed the same topology (−ln L=3881.78) as the BI analysis.

Combined Data Set

BI analysis of the combined data set of partial mt *cox1* and complete nuclear 18S rRNA genes yielded the topology (−ln L=5366.98) depicted in the inset from Fig. 2. The two main clades ('smaller-toothed' and 'larger-toothless') were also recovered with BPP values of 87 and 100, respectively. This topology only differs from the mt-based topology in the relative phylogenetic position of *P. imbricata* that groups with *P. radiata* instead of being part of the polytomy with *P. fucata* + *P. martensii* and *P. radiata* (see Fig. 2). The reconstructed topology from the ML analysis (−ln L= 5749.24) was identical to the BI tree (not shown).

Pearl Oysters Divergence Time Estimation

The divergence time obtained with Beast between both main clades that included species with hinge teeth ('smaller-toothed' clade) and without ('larger-toothless' clade) was estimated at 13.68 [7.33, 20.09] MYA (Fig. 3). The estimated time of the most recent common ancestor (TMRCA) of 'larger-toothless' clade was 8 MYA, comparable to the TMRCA of the 'smaller-toothed'; clade origin estimated at 8.5 MYA. Within the 'larger-toothless' clade, the divergence between *P. margaritifera* from Mauritius (Indian Ocean) and the clade that grouped *P. margaritifera* from French Polynesia with *P. mazatlanica* was estimated at 6.9 MYA. Within the 'smaller-toothed' clade, the estimated TMRCAs of *P. radiata* and *P. imbricata* were 1.3 and 0.35 MYA, respectively (Fig. 3). *P. martensii* and *P. fucata* grouped in the same clade, for which TMRCA was estimated at 0.85 MYA.

Correlation Between Presence/Absence of Hinge Teeth and Shell Size with *Pinctada* Phylogeny

The projection of the two-states character 'hinge teeth present' and 'hinge teeth absent' into the *Pinctada* mitochondrial BI tree (Fig. 4a) resulted in a one-step tree, which was not in the 95% confidence interval of the null distribution. We also traced shell size with two character states 'large' and 'small' (Fig. 4b) into the BI tree, and the resultant one-step tree was not included in the 95% confidence interval of the null distribution. These results indicate that the presence/absence of hinged teeth is significantly correlated with the molecular phylogeny as well as shell size.

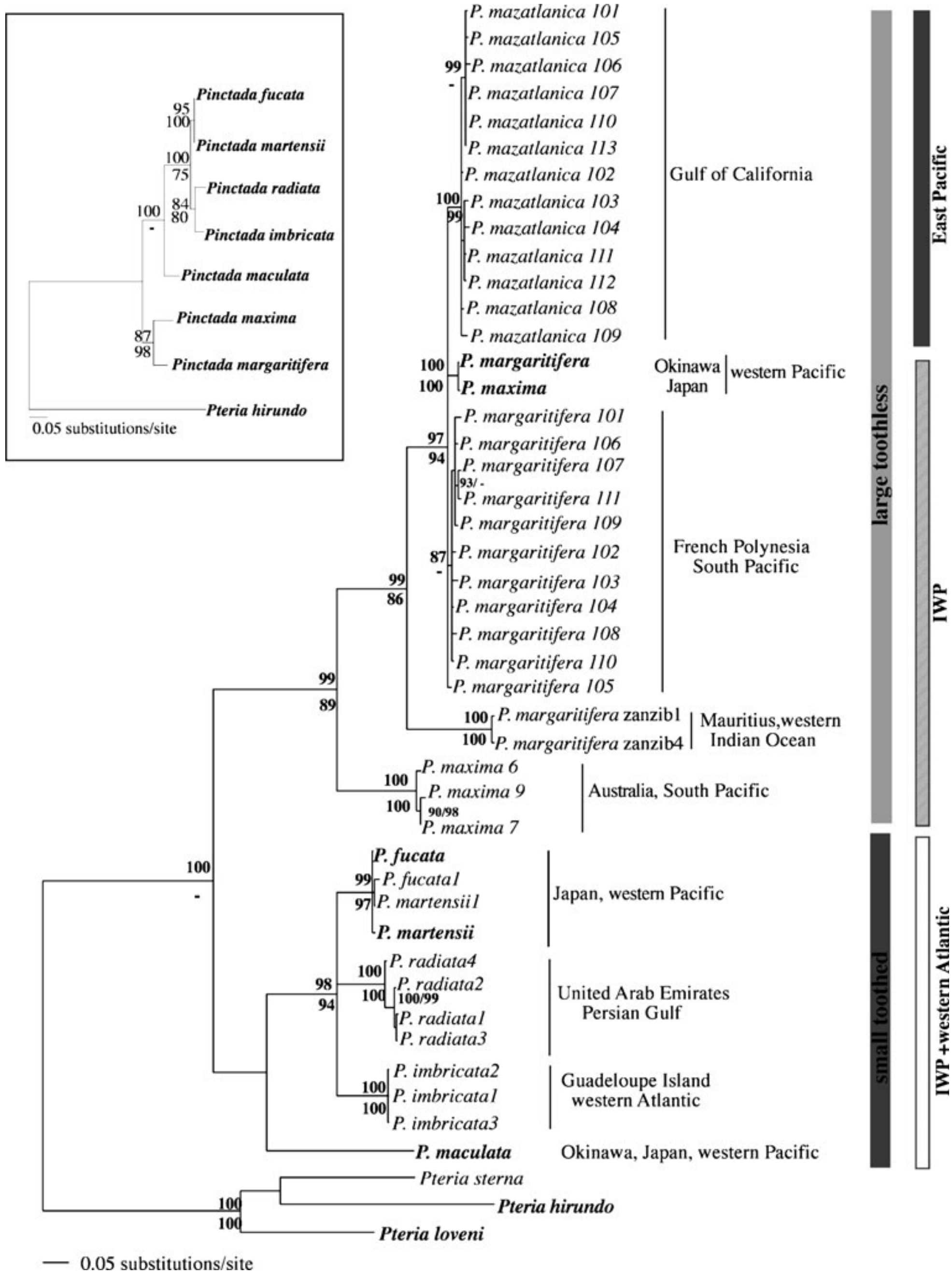

Fig. 2 Phylogenetic relationships of the genus *Pinctada* based on a Bayesian inference (BI) analysis of a partial sequence data of the mitochondrial *cox1* gene using the GTR+Γ evolutionary model. *Species in bold* were retrieved from GenBank. The *inset* shows a BI topology based on the combined data set (*cox1*+18S rRNA). *Numbers in the nodes* in both figures correspond to BI posterior probabilities (*above branches*) and maximum likelihood bootstrap proportions (*below branches*). Only values above 70% are represented

Discussion

Phylogenetic Patterns of *Pinctada* and Systematic Implications

The taxonomy of pearl oysters has been traditionally based on shell features (shape and colour) (Hertlein and Cox 1969; Oliver 1992), which are recognisably plastic characters largely influenced by environmental factors and heterogeneity among habitats (Hollander 2008). Species identification is particularly difficult in juveniles because of shell similarity (Wada and Tëmkin 2008), and the use of molecular data has shown to be quite useful to infer phylogenetic relationships in groups having insufficient, or uninformative morphological diagnosable characters (Wahlberg et al. 2005).

In this study, phylogenetic analyses based on a fragment of the mt *cox*1 gene revealed two distinct monophyletic groups according to shell size and presence/absence of hinge teeth: the ‘smaller-toothed’ and the ‘larger-toothless’ clades. The existence of these two main clades and the strong correlation found between morphological characters and the phylogeny (Fig. 4) might result either from random drift or from an early character divergence induced by natural

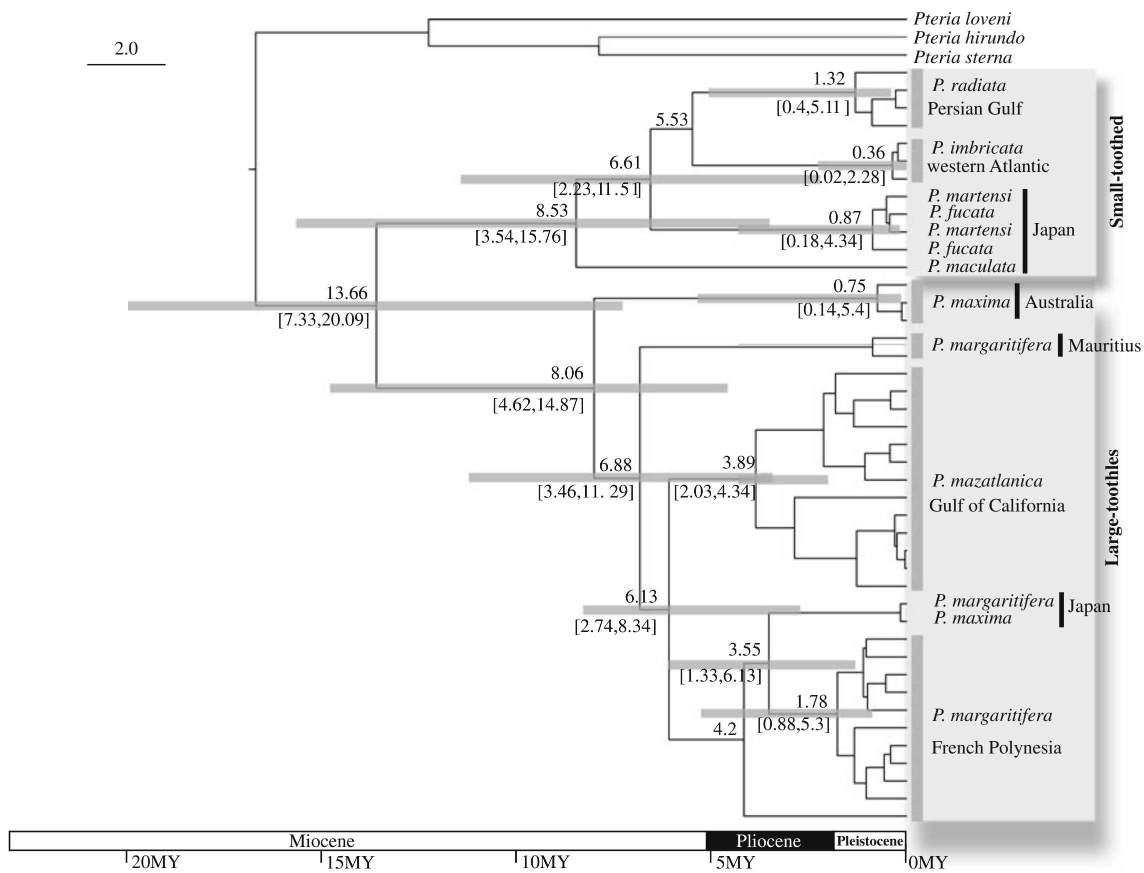

Fig. 3 Maximum clade credibility chronogram obtained with Beast showing divergence dates of main cladogenetic events within the genus *Pinctada*. Divergence dates were estimated from the mitochondrial data set. Age estimates (*above branches*; in million years) and corresponding 95% highest posterior density intervals (*bars* and *values in square brackets*, *below branches*) are depicted

selection. Two evolutionary processes induced by natural selection, i.e. character displacement and/or size assortment (Radtkey et al. 1997), might also have played a role in the divergence observed here. However, with the available data, it is not possible to distinguish between these hypotheses.

Some of the currently recognised *Pinctada* species used in this study were not monophyletic according to the mitochondrial-based analyses. Only *P. mazatlanica*, *P. radiata* and *P. imbricata* were reciprocally monophyletic and thus concordant with the taxonomy of the genus. Regarding *P. maxima*, the specimen that grouped with *P. margaritifera* was most likely misidentified because of the reduced genetic distance found between the two species (both sequences belong to Japanese specimens, retrieved from the GenBank). The species *P. margaritifera* is polyphyletic, clustering specimens from Mauritius (southwest Indian Ocean) in a different clade from other *P. margaritifera* from the French Polynesia and Japan. Additionally, specimens from French Polynesia are closer to *P. mazatlanica* rather than to *P. margaritifera* from Mauritius. *P. margaritifera* might therefore represent a species complex in which the specimens from Mauritius would represent a different species. The polyphyletic nature of *P. margaritifera* supported by the molecular analyses performed here was already intuitively speculated based on its wide distribution and intraspecific morphological variation (Allan 1959). Jameson (1901) also recognised the existence of six subspecies within *P. margaritifera* including *P. margaritifera mazatlanica* in the American Pacific coast that was later acknowledged as a distinct species (Ranson 1961). No inferences can be made about *P. maculata* on the basis of a single specimen, but it seems to be quite divergent from the remaining 'smaller-toothed' species. Regarding the putative species complex *P. fucata*/*P. martensii*/*P. radiata*/*P. imbricata*, our molecular analyses question the taxonomic validity of the morphological characters used to discriminate *P. fucata* and *P. martensii* as they clustered together (see Fig. 2) but support the taxonomic status of the species *P. radiata* and *P. imbricata*. These analyses are partially in agreement with a previous molecular study of eight species of pearl oysters based on internal transcribed spacer (ITS) nuclear markers (Yu and

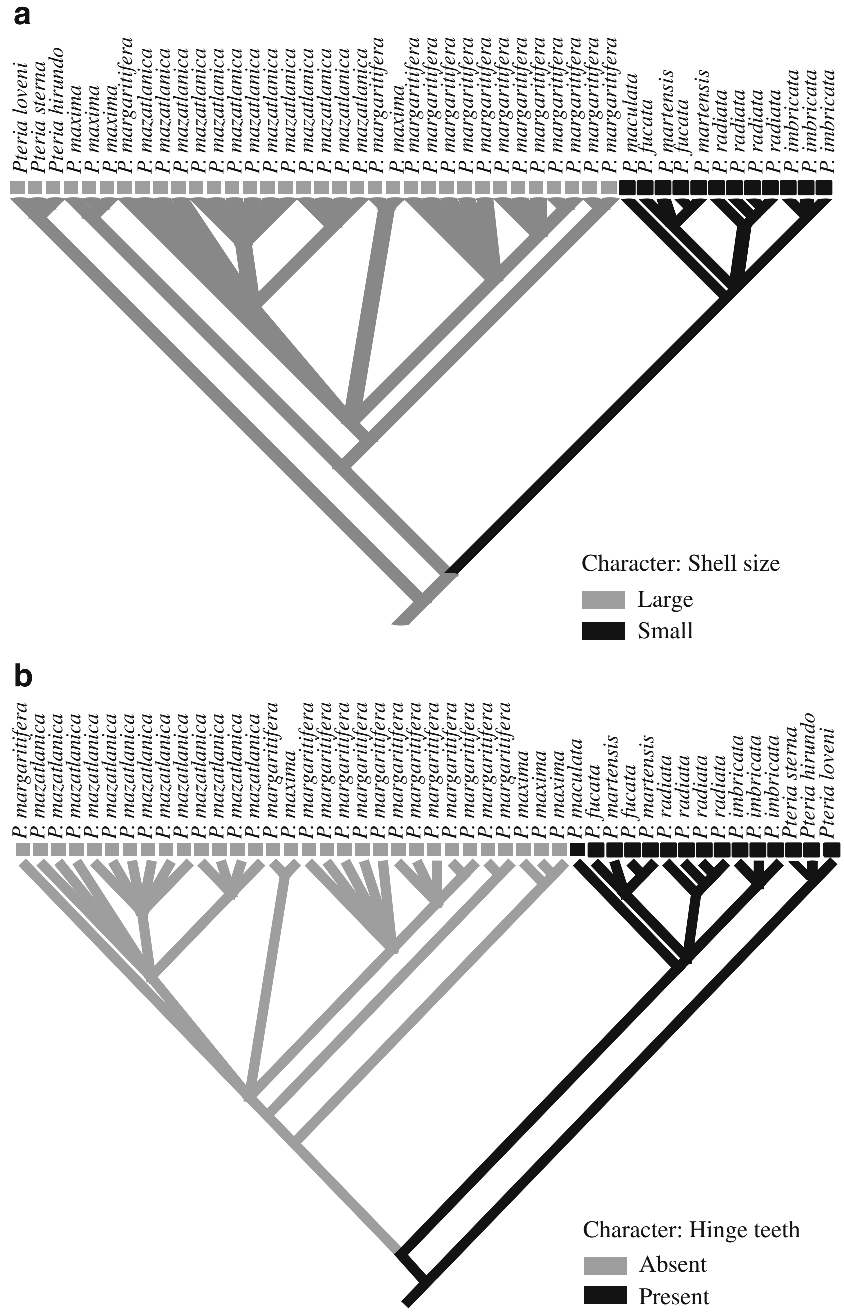

Fig. 4 Tracing evolutionary changes of the characters shell size and presence/absence of hinge teeth on the BI mitochondrial-based phylogeny. **a** Shell size; **b** presence/absence of hinge teeth

Chu 2006). Yet, some results presented here clearly contradict this previous molecular analysis. For instance, our mitochondrial-based topology showed a polytomy with three well-supported clades including (1) *P. imbricata*, (2) *P. radiata* and (3) *P. fucata/P. martensii* (Fig. 2), whereas in the above-mentioned ITS study, all three species were included within the same clade. Many studies report incongruence between mitochondrial and nuclear-based phylogenies due to, e.g. stochastic sorting of ancestral polymorphisms, introgressive hybridisation, or different modes of inheritance (Moore 1995; Sota and Vogler 2001). To overcome these effects, it is recommended to use independent markers. Accordingly, we also included a combined data set of mt (*cox1*, 506 bp) and nuclear (complete 18S rRNA, 2,333 bp) genes that lead to identical results as the phylogeny based only on mt sequence data. The two main clades that correspond to shell size and presence/absence of hinge teeth were also recovered in the combined analyses (see the inset from Fig. 2) as well as the doubtful taxonomic status of the species *P. fucata* and *P. martensii* that exhibited the lowest genetic divergence and are most likely conspecific.

Phylogenetic Patterns and Allopatry as the Dominant Speciation Mode in *Pinctada* Pearl Oysters

Marine species with long-lived pelagic larvae usually exhibit wide geographical ranges. Populations are expected to be large due to high gene flow, and thus, speciation through allopatry would be a rare event in the sea (Palumbi 1994).

If sympatry is the dominant process of speciation, it is expected that sister species exhibit overlapping distributions, whereas complete disjunction would be expected in case of allopatry (Meyer 2003). Although this general pattern might be obscured when species range shifts subsequently to lineage sorting, the analysis at genus level may still reflect the prevailing mode of speciation.

Despite *Pinctada* species exhibiting a relatively long larval phase between 16 and 30 days (Gervis and Sims 1992), phylogeographic patterns reported here are consistent with an allopatric mode of speciation. The absence of overlapping distributions is noticeable in most of the species within the genus *Pinctada*. Only *P. fucata* and *P. martensii* sister lineages show overlapping distributions as they co-exist in sympatry. Yet, our analyses suggest that these taxa might be conspecific and their taxonomic status should be revisited. If allopatry was achieved by increasing geographical distance, it is expected that specimens separated by vast stretches of open sea would group in different clades, as is the case for *P. margaritifera* specimens from Mauritius (Indian Ocean) that group in a different clade of specimens from the French Polynesia (South Pacific) or from Japan (see Fig. 2). If the prevailing speciation mode in *Pinctada* was sympatry, we would expect *P. maculata* (from Japan) to be the sister species of the clade *P. fucata* + *P. martensii* (also from Japan), but instead, it occupies a basal position with respect to the entire 'smaller-toothed' clade.

Recent evidence suggests that allopatric speciation in marine planktonic species may be more common than previously expected (McCartney et al. 2000; Lessios et al. 2001; McCafferty et al. 2002; Williams and Reid 2004). Despite *Pinctada* extensive geographical distribution, the existence of genetic structure within the Central Pacific archipelagos was already reported in *P. margaritifera* using mtDNA and nuclear markers (Arnaud-Haond et al. 2003c) or between Australian and Indonesian populations of *P. maxima* using microsatellites (Benzie and Smith-Keune 2006). Those results suggest that differentiation can occur at the scale of several hundred kilometres regardless of larval dispersal potentially further supporting the hypothesis that allopatric speciation may be the prevailing speciation mode within this genus. Allopatry can result from vicariant events, founder dispersal or most frequently a combination of both mechanisms (Paulay and Meyer 2002). Vicariance is a possible explanation when clades show sets of species belonging to each region and species divergence is in agreement with the estimated timing of biogeographical events. Investigating present-day distribution patterns requires the understanding of how the marine realm was affected by tectonic events, oceanographic and climatic changes over evolutionary timescales (Vermeij 1987). The northward movement of the Australian plate throughout the Miocene [20.03–5.3 MYA] and following uplift of the Indian archipelago caused deep changes in the Indo-Pacific region, decreasing significantly the contact between the Pacific and Indian basins (Hodell and Vayavananda 1993). The 'smaller-toothed' clade is arranged into three monophyletic groups that embrace a broad geographic distribution. This phylogenetic pattern allows hypothesising about the geographic range occupied by the ancestral lineage that gave rise to this clade. According to our age estimates, the 'smaller-toothed' clade originated in the Miocene. The northward movement of the Australian plate throughout the Miocene might have isolated *P. radiata* of the Persian Gulf from species of the western Pacific (*P. fucata* and *P. martensii*) yielding the observed modern biogeographical pattern. Within the 'larger-toothless' clade, *P. margaritifera* shows a polyphyletic pattern in which species from Mauritius do not group in the same cluster of specimens of the French Polynesia. This divergent phylogenetic pattern might also result from the northward movement of the Australian plate during the Miocene, considering that the divergence of these two *P. margaritifera* lineages occurred during the same period. This phylogenetic reconstruction, combined with previous reports on population structure within several *Pinctada* species, supports the hypothesis that allopatric speciation may be the prevailing speciation mode within this genus.

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