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Phylogeography of the sand dollar genus *Mellita*: Cryptic speciation along the coasts of the Americas

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ABSTRACT

Sand dollars of the genus *Mellita* are members of the sandy shallow-water fauna. The genus ranges in tropical and subtropical regions on the two coasts of the Americas. To reconstruct the phylogeography of the genus we sequenced parts of the mitochondrial cytochrome oxidase I and of 16S rRNA as well as part of the nuclear 28S rRNA gene from a total of 185 specimens of all ten described morphospecies from 31 localities. Our analyses revealed the presence of eleven species, including six cryptic species. Sequences of five morphospecies do not constitute monophyletic molecular units and thus probably represent ecophenotypic variants. The fossil-calibrated phylogeny showed that the ancestor of *Mellita* diverged into a Pacific lineage and an Atlantic + Pacific lineage close to the Miocene/Pliocene boundary. Atlantic *M. tenuis*, *M. quinquiesperforata* and two undescribed species of *Mellita* have non-overlapping distributions. Pacific *Mellita* consist of two highly divergent lineages that became established at different times, resulting in sympatric *M. longifissa* and *M. notabilis*. Judged by modern day ranges, not all divergence in this genus conforms to an allopatric speciation model. Only the separation of *M. quinquiesperforata* from *M. notabilis* is clearly due to vicariance as the result of the completion of the Isthmus of Panama. The molecular phylogeny calibrated on fossil evidence estimated this event as having occurred ~3 Ma, thus providing evidence that, contrary to a recent proposal, the central American Isthmus was not completed until this date.

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1. Introduction

The interplay of factors that result in speciation, habitat specialisation and geographic distributions of marine organisms with planktonic larvae remains poorly understood. Mayr (1954) strongly advocated that speciation in shallow water echinoids occurs allopatrically. This conclusion was based on the geographic distributions of morphospecies from sixteen genera of tropical shallow water regular sea urchins, heart urchins and sand dollars, including those of the genus *Mellita* L. Agassiz, 1841. However, one species of *Mellita*, *M. grantii* Mortensen, 1948, proved problematic for Mayr (1954) and he chose to ignore it in his analysis, stating that Mortensen (1948) had described *M. grantii* based on a single specimen from the midst of the range of *M. longifissa* Michelin, 1858. Mayr's (1954) model of allopatric speciation in echinoids has been supported by molecular phylogenies of regular sea urchins (Lessios et al., 1999, 2001, 2003, 2012; McCartney et al., 2000; Zigler and Lessios, 2004; Palumbi and Lessios, 2005), but to-date this hypothesis has not been tested with a molecular phylogeny of a sand dollar genus.

Sand dollars in the genus *Mellita* are an ideal group in which to trace phylogeographic phenomena, as they have a rich fossil record. This record dates from the Early Pliocene, and is helpful in dating splitting events between lineages (Mooi and Peterson, 2000). The genus is geographically restricted to the Americas (Mooi et al., 2000), which avoids complications that can arise from species invasions out of other geographic regions.

Ten morphospecies of *Mellita* have been described, five from the Atlantic and five from the eastern Pacific. Harold and Telford (1990) conducted a morphological analysis of the genus and concluded that seven species were valid. Three of these have largely non-overlapping distributions in the Atlantic (Harold and Telford, 1990). *Mellita isometra* Harold and Telford, 1990 (type locality: Beaufort, North Carolina, USA) is distributed along the east coast of North America, from Nantucket, Massachusetts to Fort Lauderdale, Florida, and *M. tenuis* Clark, 1940 (type locality: Sanibel Island, Florida, USA) from Louisiana to west Florida in the Gulf of Mexico. Harold and Telford (1990) synonymised *M. lata* Clark, 1940 (type locality: Puerto Limòn, Costa Rica) and *M. latiambulacra* Clark, 1940 (type locality: Cumanà, Venezuela) with *M. quinquiesperforata* (Leske, 1778) (type locality: Veracruz, Mexico), and gave *M. quinquiesperforata*'s geographic range as Louisiana to Brazil, including Central America and the Greater Antilles.

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In the Pacific, Harold and Telford (1990) recognised four species: *M. longifissa* (type locality: unknown), *M. notabilis* Clark, 1947 (type locality: unknown); *M. grantii* (type locality: San Felipe, Mexico); *M. kanakoffi* Durham, 1961 (type locality: Upper Pleistocene, Newport Beach, California, USA). They synonymised *M. eduardobarrosoi* Caso, 1980 (type locality: Acapulco, Mexico) with *M. notabilis*, finding them to be morphologically identical. Pacific species have extensively overlapping distributions, from the west coast of Baja California, Mexico to Panama; the range of *M. longifissa* extends to the Galapagos Islands (Isla Santa Maria (Charles Island)). According to Mortensen (1948), *M. grantii* was only known from the Gulf of California, but Harold and Telford (1990) extended its range to Panama. Lessios (2005) suggested that specimens from the Gulf of San Miguel, Panama, belonged to this species. *Mellita grantii* is morphologically very similar to juvenile *M. longifissa*, which has led to considerable confusion in identification.

In Harold and Telford's (1990) cladistic analysis based on morphological characters the Atlantic species of *Mellita* do not form a monophyletic group. *Mellita isometra* and *M. tenuis* are sister to each other, whereas *M. quinquiesperforata* is sister to a group containing all the Pacific species. From their analysis Harold and Telford (1990) stated that they were unable to determine whether speciation in the Pacific clade had occurred allopatrically.

Experimental evidence suggests that the larvae of *M. quinquiesperforata* can settle in as little as seven days if they encounter favourable conditions, but are also able to remain in the plankton for up to four weeks (Caldwell, 1972). As *Mellita* specialise in living in terrigenous (siliceous) sands (Telford and Mooi, 1986), this flexibility in timing of settlement is vital for successful recruitment and is likely to be mediated by a chemosensitive response to either suitable terrigenous sands or adult conspecifics.

In this study we combine mitochondrial and nuclear gene sequences to reconstruct the phylogeny of *Mellita*. We attempt to answer the following questions: (1) Are species as recognised by morphology valid? (2) When did speciation events occur? (3) Does the dating of the phylogeny from fossils concur with the dating from vicariant events? (4) What were the physical barriers that resulted in speciation? (5) Does speciation in *Mellita* conform to the allopatric model?

2. Materials and methods

Specimens representing all described extant species of *Mellita* were collected throughout the range of the genus (Fig. 1). Collection sites included the type localities of *M. eduardobarrosoi* and *M. isometra*. Samples included members of the morphological variants of *M. quinquiesperforata*. Specimens of *Leodia sexiesperforata* Leske, 1778 and of *Encope grandis* L. Agassiz, 1841 were also collected for use as outgroups. The Aristotle's lantern was extracted from each specimen and preserved in 95% Ethyl Alcohol or high salt Dimethyl Sulfoxide buffer. Fossil *Mellita*, including representatives of both extant and extinct species, were examined from the collections of the Florida Museum of Natural History (FLMNH), Humbolt State University's Natural History Museum (HSU), and the Natural History Museum of Los Angeles County (LACM).

2.1. DNA extraction, sequencing and alignment

Genomic DNA was extracted from the lantern muscle of 185 specimens of *Mellita*, 9 specimens of *Leodia* and 3 specimens of *Encope* using a DNeasy tissue kit (Qiagen)®. We amplified three

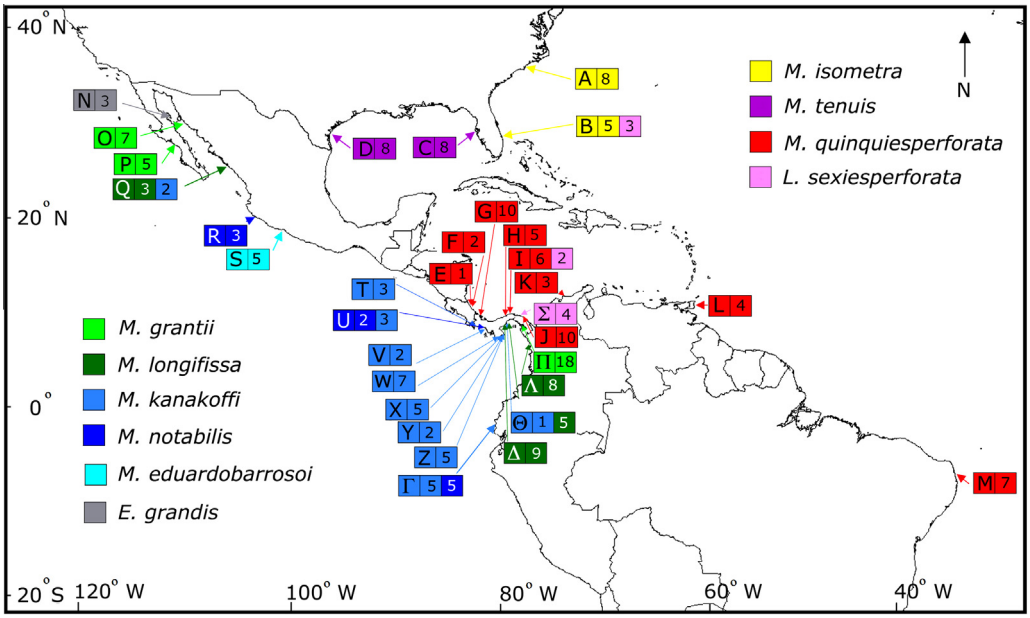

Fig. 1. Collection localities of *Mellita, Leodia* and *Encope* used in this study. Letters refer to localities, numbers to sample size, and colours to morphospecies. **A**: Beaufort, North Carolina, USA (34.6934N, 76.6981W); **B**: Fort Pierce, Florida, USA (27.4380N, 80.2779W); **C**: Mullet Key, Florida, USA (27.6216N, 82.7385W); **D**: Port Aransas, Texas, USA (27.8339N, 97.0610W); **E**: Playa Limón, Costa Rica (9.9957N, 83.0280W); **F**: Playa Cahuita, Costa Rica (9.7356N, 82.8346W); **G**: Bocas del Toro, Panama (9.3458N, 82.2519W); **H**: Palmas Bellas, Colon, Panama (9.2322N, 80.0878W); **I**: Portobelo, Panama (9.5509N, 79.6675W); **J**: Ustupo Island, San Blas, Panama (9.1374N, 77.9288W); **K**: Santa Marta, Colombia (11.2592N, 74.2050W); **L**: Cocos Bay, Trinidad (10.4152N, 61.0230W); **M**: Bessa Beach, Paraiba, Brazil (7.0656N, 34.8249W); **N**: Bahia de Los Angeles, Mexico (28.9492N, 113.5576W); **O**: Bahia de Kino, Sonora, Mexico (28.8189N, 111.9392W); **P**: Malcomb, Baja California Sur, Mexico (26.7126N, 113.2670W); **Q**: Isla de la Piedra, Mazatlan, Mexico (23.1807N, 106.3926W); **R**: Playa Azul, Michoacan, Mexico (17.9798N, 102.3497W); **S**: Playa Encantada, Acapulco, Mexico (16.6982N, 99.6652W); **T**: Puerto Armuelles, Panama (8.2223N, 82.8588W); **U**: Playa Las Lajas, Chiriqui, Panama (8.1618N, 81.8598W); **V**: Chiriqui, Panama (7.9422N, 81.6503W); **W**: Isla Caña, Azuero Peninsula, Panama (7.3753N, 80.2702W); **X**: Playa Venado, Azuero Peninsula, Panama (7.5146N, 80.1280W); **Y**: Playa Cambutal, Azuero Peninsula, Panama (7.0067N, 80.9445W); **Z**: Punta Mala, Azuero Peninsula, Panama (7.4679N, 80.0007W); **Γ**: Punta Blanca, Santa Elena, Ecuador (2.1517N, 80.7905W); **Δ**: Playa Gorgona, Panama (8.5516N, 79.8654W); **Θ**: Punta Chame, Panama (8.6446N, 79.7006W); **Λ**: Bahia Solano, Colombia (6.2304N, 77.4029W); **Π**: Gulfo de San Miguel, Panama (8.3996N, 78.2727W); **Σ**: Playón Chico, San Blas, Panama (9.3012N, 78.233W).

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regions of mitochondrial DNA (mtDNA) and one of a nuclear gene. The 5′ region of cytochorome *c* oxidase subunit I (COI) was amplified using the HCOI and LCOI primers of Folmer et al. (1994), the 3′ region of COI using the primers COIa and COIf of Lessios et al. (2001), and the 16S rRNA using the 16Sar and 16Sbr primers of Kessing et al. (1989). Each reaction contained 0.2–0.5 μl of extracted genomic DNA (approximately 10–15 ng), 12.0 μl of nuclease free H_20 (adjusted to 12.3 μl when using less genomic DNA), 5.0 μl GoTaq® Flexi Buffer (5×), 2.5 μl $MgCl_2$ (25 mM), 2.5 μl dNTPs (8 mM), 1.25 μl (10 μM) of each forward and reverse primer, and 0.6 units of Flexi-GoTaq® polymerase (Promega). These were amplified using the following protocol: (1) 96 °C for 10 s, (2) 94 °C for 30 s, (3) 50 °C for 45 s, (4) 72 °C for 1 min for 39 cycles, and a final extension at 72 °C for 5 min. The 5′ end of nuclear 28S rRNA was amplified using a HotStartTaq PCR amplification kit (Qiagen)® with the primers and protocol of Littlewood and Smith (1995). PCR products of 28S were cloned using Promega pGEM-T Easy® kits to avoid sequences with heterozygous single-nucleotide polymorphisms (double peaks on chromatograms). One clone was cycle-sequenced for each specimen using Promega M13 and M13R primers, before being sequenced in one direction using the M13 primer.

After purification in Sephadex columns, amplification with the same primers, and labelling with Applied Biosystems (ABI) Prism BigDye Terminators, nucleotides were sequenced in both directions using an ABI 3130 XL automatic sequencer. Pairwise sequence alignments were performed in MacClade (Maddison and Maddison, 2005). The two sequenced COI regions overlapped by 22 base pairs (bp) and after the removal of the primer regions produced a contiguous sequence of 1236 bp with no indels, covering approximately 80% of the complete coding sequence of the gene. 571 bp of 16S rRNA and 1137 bp of 28S were sequenced including several 1 bp indels. All sequences have been deposited in GenBank with the Accession numbers KF204670–KF204860 for COI, KF204861–KF205051 for 16S and KF205052–KF205242 for 28S.

2.2. Phylogenetic reconstruction

jMODELTEST v. 2.1.1 (Darriba et al., 2012) was used to determine the best model of molecular evolution for each gene based on the AIC criterion (Akaike, 1974). For COI the general time-reversible model (Tavare, 1986) was selected, with a gamma distribution shape parameter (α) of 0.1310 (GTR + G). The Hasegawa, Kishino and Yano model (Hasegawa et al., 1985) was selected for 16S (HKY + I + G; I = 0.4520 and α = 0.2350), and a transition/transversion model TIM1 + I was suggested for 28S, where I = 0.9350. As different models were selected for each gene, partitions were used in the phylogenetic analysis of the concatenated data. Saturation tests for each gene and for the concatenated data were conducted using the software package DAMBE (V.5.3.00) (Xia et al., 2003; Xia and Lemey, 2009). For COI, the index of substitution saturation I_{SS} was calculated for all sites using all codon positions, and separately using just the third position, which is prone to saturation. For the ribosomal genes, sites with indels were not included in the analysis, because they can reduce the sensitivity of this method (Xia and Lemey, 2009). In all tests I_{SS} was significantly smaller than the critical index of substitution saturation ($I_{SS.c}$) under the assumption of either a symmetrical ($I_{SS.cSym}$) or extremely asymmetrical ($I_{SS.cAsym}$) tree (p = 0 for COI, 16S, 28S and the concatenated data, p = 0.02 for the third codon position in COI).

Bayesian phylogenetic analysis was carried out on the concatenated data using MRBAYES v. 3.2.1. (Ronquist and Huelsenbeck, 2003). Each gene was analysed as a separate partition with the model suggested by jMODELTEST with parameters unlinked across partitions. A haplotype of *Encope grandis* was randomly selected as an outgroup (only one outgroup is permitted in MRBAYES). The heating parameter T of the runs was 0.15. The analysis was started with Dirichlet priors for rates and nucleotide frequencies and was run for 60 million generations, sampling every 1000th tree from two runs. Convergence was assessed according to the average standard deviation of split frequencies <0.01 and potential scale reduction factor (Gelman and Rubin, 1992) reaching 1.00 for all parameters. The runs were also visually checked in Tracer v1.5 (Rambaut and Drummond, 2007). The first 25% of trees were discarded from each run as burn-in, and a 50% majority rule tree was constructed from 90,002 trees. Clades with less than 85% support were collapsed.

Partitioned maximum likelihood analysis was also carried out in GARLI V.2.0 (Zwickl, 2006) using the model suggested by jMODELTEST for each gene. Five replicate runs, each of two million iterations, were conducted. Node support values were calculated in GARLI based on 400 bootstrap replicates, and the bootstrapped consensus tree was calculated in PAUP* (Swofford, 2002).

Genetic distances between clades, based on the appropriate models, were estimated for each gene by calculating the mean of all pairwise comparisons between species. When a clade contained more than one subclade, its mean distance from its sister clade was calculated as the average distance between clades in each group.

F_{ST} values were calculated between members of described morphospecies when the phylogeny indicated that they did not belong to separate clades in order to determine whether there was reduced gene flow between them. For this analysis, all samples of each morphospecies were pooled. F_{ST} values were calculated using the concatenated 2944 bp sequences and Tamura and Nei (1993) distances in Arlequin v. 3.1.5.3 (Excoffier and Lischer, 2010). The probability that the F_{ST} values could be due to chance was estimated with 10,000 reshufflings of sequences between morphospecies.

2.3. Timing of divergence

The COI and combined set of data were analysed in BEAST (v1.7.1) (Drummond et al., 2012). To determine time of most recent common ancestor (TMRCA), we analysed COI and the combined set of data independently using an uncorrelated lognormal relaxed clock (where the rate of substitutions per site per unit time is estimated) with the substitution models selected by jMODELTEST and the Yule speciation process. All analyses were run for 150 million generations with parameters logged every 1500 iterations and convergence assessed using Tracer. The first 15000 samples (10%) were discarded as burn-in before trees were viewed in FigTree v1.3.1. Molecular clocks were calibrated in two independent runs, one using the 3.1–2.8 million year ago (Ma) date range for the final closure of the isthmus (Coates and Obando, 1996; Coates et al., 2003), another using dates of fossils to constrain estimates of node age. An additional run used both the Isthmus of Panama and the fossils as calibration points. The oldest fossil assigned to *Mellita*, *M. caroliniana* (Ravenel, 1841) (FLMNH UF80503) from the Yorktown Formation of the middle Pliocene (Krantz, 1991), is problematic, as it is morphologically unlike all other *Mellita;* it may belong to either an ancestor of *Mellita* or to a different genus. *Mellita aclinensis* Kier, 1963 (FLMNH UF40428) from the Late Pliocene Piacenzian (3.60–2.59 Ma) Tamiami Formation (Lyons, 1991; Jones et al., 1991; Mooi and Peterson, 2000) is morphologically and geographically (Florida) very close to extant *M. tenuis* (some populations of this extant species also have six lunules (Cerame-Vivas and Gray, 1964)). We therefore constrained the minimum estimate of node age for the split of *M. tenuis* from the Atlantic + Pacific lineage of *Mellita* to 3.60–2. 59 Ma. This calibration point was used in conjunction with minimum node ages of extant *Mellita* that have well dated fossils. These were fossils of *M. grantii* (HSU, NHM943) and *M. tenuis* (FLMNH UF14478) from the Gelasian stage of the Pleistocene (2.59–1.8 Ma), and of *M. kanakoffi* (LACM 1121, 1122) from the Tarantian stage of the Pleistocene (0.13–0.01 Ma).

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3. Results

3.1. Phylogeny

Bayesian inference (BI) and Maximum likelihood (ML) analyses of the 132 unique haplotypes of *Mellita*, 9 of *Leodia* and 1 of *E. grandis* produced congruent phylogenies for all well supported clades and subclades (support >85%), with only slight differences in weakly supported subclades within species (Figs. 2a and 2b).

In the phylogeny based on the concatenated data rooted on *Encope*, *Leodia* was sister to *Mellita* with a genetic distance from

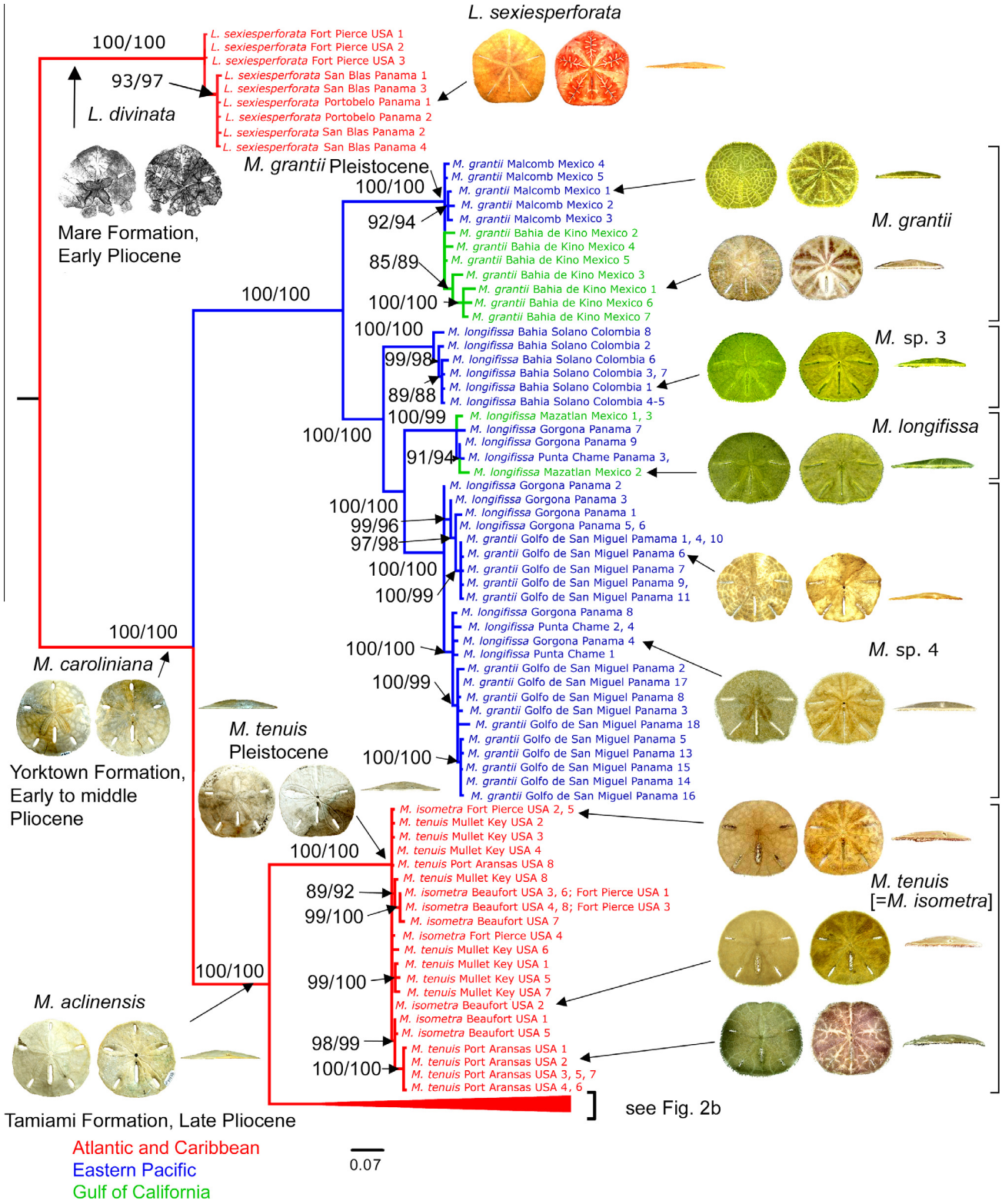

Fig. 2a. Phylogeny of *Mellita* using concatenated COI, 16S and 28S data reconstructed with MRBAYES and rooted on *Encope grandis*. Clade credibility values >85% in both the Bayesian (first number next to node) and Maximum Likelihood (second number) reconstruction are shown. Numbers after locality names indicate individuals with indistinguishable haplotypes, scale bar reflects number of changes per site. Names next to terminal branches indicate the morphology of the specimens, names to the right of the pictures are our interpretation as to species affiliation according to the molecular phylogeny.

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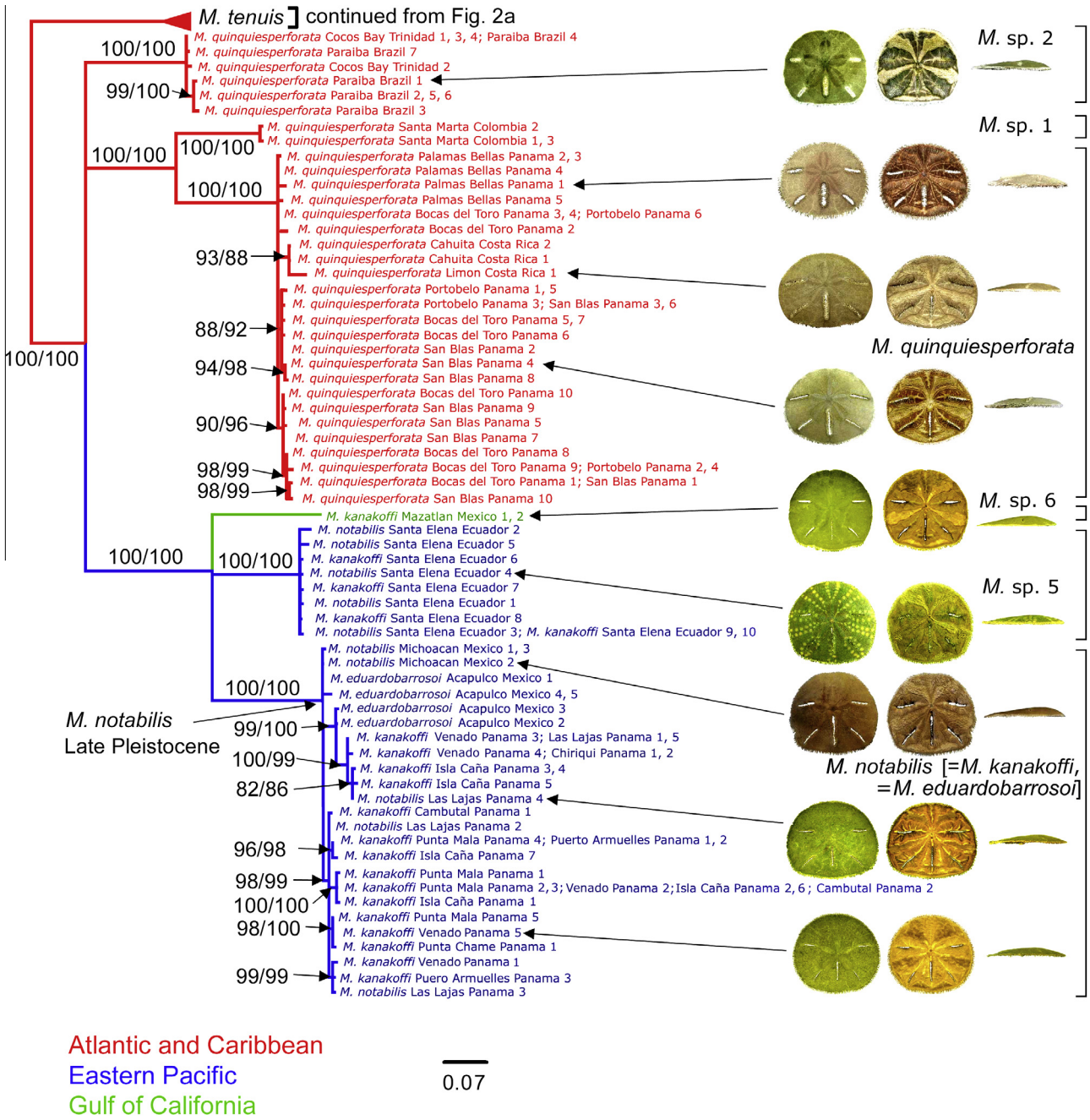

Fig. 2b. Phylogeny of *Mellita* continued from Fig. 2a.

the latter genus of 47.22% in COI. The first split within *Mellita* separated a Pacific clade consisting of *M. grantii* and *M. longifissa* from all other species, with 39.76% divergence in COI from all other extant *Mellita* (Table 1). Within this clade, *M. grantii*, from both sides of the Baja California peninsula formed a group, which had 10.48% divergence in COI from a clade containing three subclades of *M. longifissa*. *Mellita longifissa* from the Pacific side of Colombia (*M.* sp. 3) was sister to the two other clades of *M. longifissa*. Individuals of this molecular clade had no obvious morphological differences from *M. longifissa* but its COI sequences were different by 5.60%. Specimens of *M. longifissa* from Panama and from the mouth of the Gulf of California formed a separate clade. Its sister clade, *M.* sp. 4, was 3.97% different in COI and was composed exclusively of sequences from individuals collected in Panama. *Mellita* sp. 4 contained two well supported subclades, each of which included specimens that were morphologically typical of *M. longifissa*, as well as specimens from Panama identified by Lessios (2005) as *M. grantii*.

The sister clade to *M. longifissa-M. grantii* incorporated *M. isometra* from the Atlantic Coast of the United States and *M. tenuis* from the Gulf of Mexico. This clade included a well-supported subclade of *M. tenuis* from Fort Aransas, Texas. However, other specimens of *M. tenuis* were intermixed with *M. isometra* and did not form separate clades as they would if they were separate species.

Sister to the *M. tenuis-isometra* clade (18.70% divergent from it in COI) was a polytomy that included Atlantic and Pacific lineages, the separation of which corresponds with the most recent transisthmian divergence in the genus. Within this polytomy, mean genetic distance in COI between Atlantic and Pacific species was also 18.70%.

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Table 1
Mean difference and divergence times between clades and species of *Mellita* calculated using models suggested by jMODELTEST. Timing of divergence was calculated in BEAST using COI and the concatenated set of genes with a relaxed clock and the Yule speciation process. Separate molecular clock calibrations were based on the fossil record, the final closure of the Panama Isthmus, and the combined fossil/isthmus calibration.

Divergence	COI (%)	16S (%)	28S (%)	COI fossil (Ma)	COI isthmus + fossil (Ma)	COI + 16S + 28S, isthmus (Ma)	COI + 16S + 28S, fossil (Ma)	COI + 16S + 28S isthmus + fossil (Ma)
Leodia from all *Mellita*	47.22	10.65	2.05	5.96	6.02	5.95	6.14	6.01
Leodia from *M. grant.* + *M. long.* + *M.* sp. 3 and 4	45.56	10.09	2.13	5.96	6.02	5.95	6.14	6.01
Leodia from *M. tenuis* + *M. quinq.* + *M. notab.* + *M.* sp. 1, 2, 5 and 6	42.29	10.97	2.01	5.96	6.02	5.95	6.14	6.01
M. grant. + *M. long.* + *M.* sp. 3 and 4 from *M. tenuis* + *M. quinq.* + *M. notab.* + *M.* sp. 1, 2, 5 and 6	39.76	9.18	0.64	5.23	5.61	5.30	5.46	5.42
M. tenuis from *M. quinq.* + *M. notab.* + *M.* sp. 1, 2, 5 and 6	18.70	7.79	0.29	3.68	3.66	3.69	3.83	3.76
M. quinq. + *M.* sp. 1 and 2 from *M. notab.* + *M.* sp. 5 and 6	18.70	8.46	0.17	2.92	3.03	3.09	3.21	3.18
M. sp. 2 from *M. quinq.* + *M.* sp. 1	12.23	3.15	0.17	2.92	3.03	3.09	3.21	3.18
M. grant. from *M. long.* + *M.* sp. 3 nd 4	10.48	2.64	0.13	2.34	2.40	3.03	3.12	3.06
M. quinq. from *M.* sp. 1	8.64	3.12	0.09	1.78	1.81	2.09	2.23	2.12
M. notab. from *M.* sp. 5	13.12	2.74	0.02	1.59	1.63	1.82	1.88	2.03
M. notab. from *M.* sp. 6	8.64	2.63	0.01	1.59	1.63	1.82	1.88	2.03
M. sp. 5 from *M.* sp. 6	11.33	3.52	0.21	1.59	1.63	1.82	1.88	2.03
M. sp. 3 from *M. lon.* + *M.* sp. 4	5.60	1.50	0.05	1.47	1.50	2.32	2.38	2.34
M. long. from *M.* sp. 4	3.97	0.76	0.09	1.15	1.18	1.81	1.87	1.84

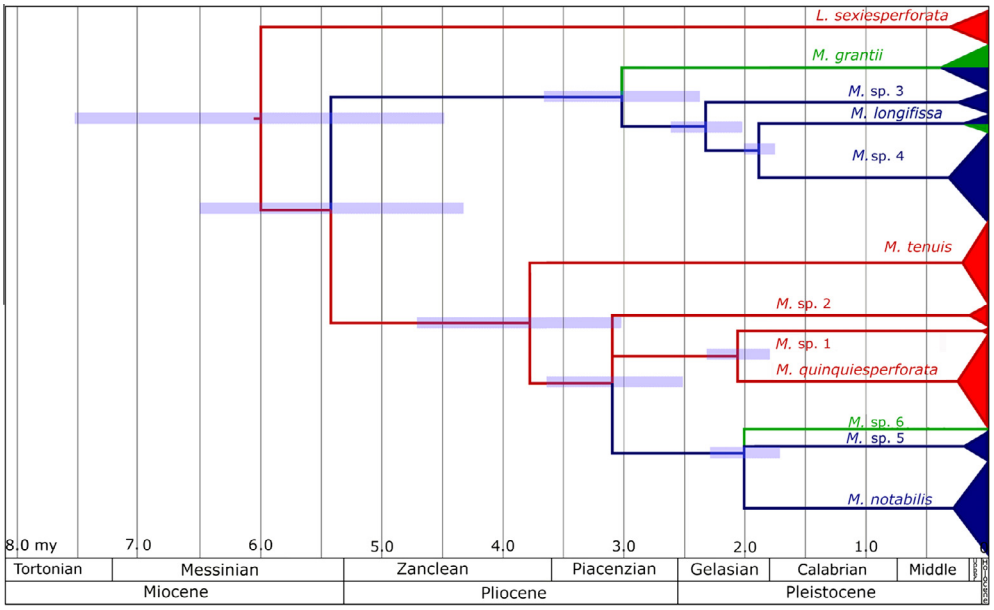

Fig. 3. Timing of cladogenesis based on concatenated COI, 16S, and 28S data, as derived from analysis on BEAST calibrated using the fossil record and the final closure of the Panama Isthmus. Ages of stages and epoch series are based on International Commission on Stratigraphy stratigraphic chart 2012. Error bars estimated by BEAST are shown in light blue. Colours of clades indicate geographic range (red = Atlantic and Caribbean, blue = eastern Pacific, green = Gulf of California).

The Atlantic lineages were formed of specimens with a range of morphologies that have previously been included in *M. quinquiesperforata*. *Mellita* sp. 2 from Trinidad and Brazil formed a well-supported clade with 12.23% divergence in COI from a lineage containing *M. quinquiesperforata* from Costa Rica and Panama as well as *M.* sp. 1 from Santa Marta, Colombia. Between the Colombian and the Central American subclades there was 8.64% divergence in COI. Specimens from Costa Rica and Panama had tests that were particularly broad relative to their length, and are therefore representative of *M. quinquiesperforata* as originally described (Klein, 1734 (Pre-Linnean) and Leske, 1778).

Within the Pacific lineage, three distinct subclades formed a polytomy. *Mellita notabilis* included adult specimens from Mexico and Panama with typical *M. notabilis* morphology, as well as *M. eduardobarrosoi* from Mexico and *M. kanakoffi* from Panama. This clade had 13.12% divergence in COI from *M.* sp. 5 from Ecuador, and 8.64% divergence in COI from *M.* sp. 6, which consisted of two specimens from Mazatlan, Mexico. The *M.* sp. 5 clade included members with some test characters of both *M. notabilis* and *M. kanakoffii*, whereas the *M.* sp. 6 was morphologically closer to *M. kanakoffii*. Genetic distance in COI between *M.* sp. 5 and *M.* sp. 6 was 11.33%.

The molecular phylogeny revealed which morphospecies corresponded with monophyletic molecular clades, but also suggested the existence of cryptic species.

An F_{ST} value of 0.09 between *M. tenuis* and *M. isometra*, ($p > 0.05$), indicated that the two putative species on either side of the Florida Peninsula interbreed freely. F_{ST} of 0.15 between *M. notabilis* (including *M. eduardobarrosoi*) and *M. kanakoffi* ($p < 0.01$) suggests that there may be some barriers to gene flow between the two sympatric morphospecies in the eastern Pacific.

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Genetic distance in COI and 16S between the Pacific *M. longifissa-grantii* lineage and all other *Mellita* is similar to that between both these *Mellita* lineages and *L. sexiesperforata*, (Table 1), suggesting that their differentiation is equivalent to that found between genera of the Mellitidae. Nevertheless, variation in COI among all species of *Mellita* consisted only of silent substitutions. Only three fixed amino acid differences differentiated *Leodia* from all *Mellita* in COI. Eleven single-bp indels were found in 16S, four of which distinguish *Leodia* from *Mellita*, but none of which differentiate the Pacific *M. longifissa-grantii* lineage from the Atlantic + Pacific lineage of *Mellita*. Two indels occurred in 28S, one insertion was unique to *Leodia*.

3.2. Timing of divergence

The calibration of divergence values based on the assumption that the completion of the Panama Isthmus 3 Ma separated *M. quinquiesperforata* from its sister lineage in the Pacific produced a mean molecular divergence rate of 6.23% per million years (my^{-1}) in COI, of 2.82% my^{-1} in 16S and of 0.06% my^{-1} in the nuclear gene 28S. This resulted in a per-lineage substitution rate of 3.12% my^{-1} in COI, 1.41% my^{-1} in 16S and 0.03% my^{-1} in 28S. When divergence was calibrated with fossils, it produced similar estimates of the timing of splitting as those obtained from the calibration based on the final closure of the Panama Isthmus (Table 1). The combined fossil-isthmus calibration using either just COI or COI + 16S + 28S also produced similar divergence dates, except in the *M. longifissa-grantii* lineage, where COI appeared to underestimate divergence times relative to those obtained by the other methods. The timing of cladogenic events using COI + 16S + 28S and a calibration based on both fossil ages and the closure of the isthmus is shown in Fig. 3. *Leodia* diverged from *Mellita* during the Messinian stage of the Miocene, and *Mellita* split into two major lineages near to the Miocene/Pliocene boundary. In the Pacific lineage, *M. grantii* diverged from *M. longifissa* in the Piacenzian stage of the Late Pliocene; splits within *M. longifissa* occurred in the Gelasian stage of the Pleistocene. In the Atlantic + Pacific lineage, *M. tenuis* diverged from the ancestor of *M. quinquiesperforata* in the Zanclean stage of the Pliocene. The final closure of the Panama Isthmus in the Piacenzian resulted in a split of the ancestor of the clades morphologically assigned to *M. quinquiesperforata* from the ancestor of the lineages assigned by morphology to *M. notabilis*. Further splits occurred between *M. quinquiesperforata* and *M.* sp. 1, and between *M. notabilis*, *M.* sp. 5 and *M.* sp. 6 during the Gelasian stage of the Pleistocene.

4. Discussion

4.1. Timing and possible causes of divergence

Our analyses revealed that *Leodia* and the ancestor of *Mellita* diverged in the Late Miocene (~6.0 Ma). Separation between *Leodia* and *Mellita* was accompanied by niche partitioning, with extant *Leodia* living only in biogenic, carbonate sands and *Mellita* specialising in terrigenous siliceous sands. Such specialisation by *Leodia* may have evolved in concert with increased carbonate deposition in the Caribbean in the Late Miocene/Early Pliocene as the result of the post-Miocene proliferation of coral reefs in the Caribbean (Johnson et al., 2007, 2008; O'Dea et al., 2007; Smith and Jackson, 2009).

The ancestor of *Mellita* split into a Pacific lineage and an Atlantic lineage around the Miocene/Pliocene boundary. Origination rates of many marine taxa are reported to have peaked at this time, in response to increasing habitat heterogeneity in shallow-water marine environments (Jackson et al., 1993; Cheetham and Jackson, 1996; Collins, 1996; Knowlton and Weigt, 1998; Budd and Johnson, 1999;Marko, 2002; Smith and Jackson, 2009; Jagadeeshan and O'Dea, 2011).

Through most of the Neogene, tropical and subtropical America was biogeographically divided into two distinct regions, the Caloosahatchian province, from North Carolina through the North of the Gulf of Mexico, and the Gatunian province comprised the rest of the tropical Atlantic and the modern day tropical eastern Pacific (Petuch, 1982; Vermeij, 2005; see Fig. 4). The provinces contained unique assemblages of species, with limited overlap (Vermeij, 2005). The divergence of *M. tenuis* from the Atlantic + Pacific lineage at the end of the Zanclean stage of the Pliocene (~3.8 Ma) resulted in *M. tenuis* becoming restricted to the Caloosahatchian province, with all other Recent *Mellita* limited to the Gatunian province. These distributions are reflected in the fossil record and their present day species ranges. As the F_{ST} statistics indicate, the fine silt sediments and biogenic (carbonate) sands off the tip of the Florida Peninsula do not act as a barrier to dispersal between Atlantic and Gulf Coast *M. tenuis* as had been suggested by Harold and Telford (1990).

In the Late Pliocene, the ancestor of *M. quinquiesperforata* diverged from the ancestor of *M. notabilis* following the final closure of the Panama Isthmus. Levels of divergence between these geminate species of *Mellita* are similar to those of other echinoid species separated by the final closure of the Panama Isthmus (see Lessios, 2008). Timing of divergence of geminate species in *Mellita* using a relaxed molecular clock and the fossil record (but independent of the age of the Isthmus) produces a date of ~3 my for the final split of Atlantic and Pacific lineages, consistent with the hypothesis that it was due to the closure of the Panama Isthmus. This is congruent with the hypothesis that sea water connections between the Atlantic and the Pacific existed until the late Pliocene, as a large accumulation of palaeoceanographic (Keigwin, 1982; Collins, 1996; Haug and Tiedemann, 1998; O'Dea et al., 2007), palaeontological (Webb, 1976; Coates and Obando, 1996), and genetic (Lessios, 2008) data have indicated. Our data do not agree with the proposal of Montes et al. (2012) that the isthmus has been an uninterrupted chain above sea level since the Eocene. As no extant or fossil species of *Mellita* has ever been found outside the Americas, the possibility of post-isthmian genetic connections by stepping-stones around the world is extremely remote. The existence of dry land separating the two oceans since the Eocene, 30 my before the Pliocene, is entirely incompatible with the molecular phylogeny of *Mellita.*

Molecular phylogenies of regular echinoids (Lessios et al., 1999, 2001, 2003; McCartney et al., 2000; Zigler and Lessios, 2004; Palumbi and Lessios, 2005) have agreed with Mayr's (1954) model of allopatric speciation. However, our data revealed that not all divergence highlighted in this study fits an allopatric model.

Divergence between *Leodia* and *Mellita* occurred ~6 Ma when there was no obvious geographic isolation, but rather at a time when an increase in the interchange of transisthmian waters has been reported, following the deepening of the canal basin (Collins et al., 1996). Divergence between the Pacific *M. longifissa-grantii* lineage and the Atlantic + Pacific lineage appears to have occurred prior to the Pliocene shoaling event (which began ~4.7 Ma, Coates et al., 2003), without a clear barrier to gene flow.

Only the separation of *M. quinquiesperforata* from *M. notabilis* (and its related Pacific *M.* sp. 5 and *M.* sp.6) is clearly due to vicariance due to the completion of the Isthmus of Panama ~3 Ma.

4.2. How many species of Mellita are there?

Genetic distance in COI between echinoderm congeners is typically no larger than 15.33% (Ward et al., 2008). Our COI data

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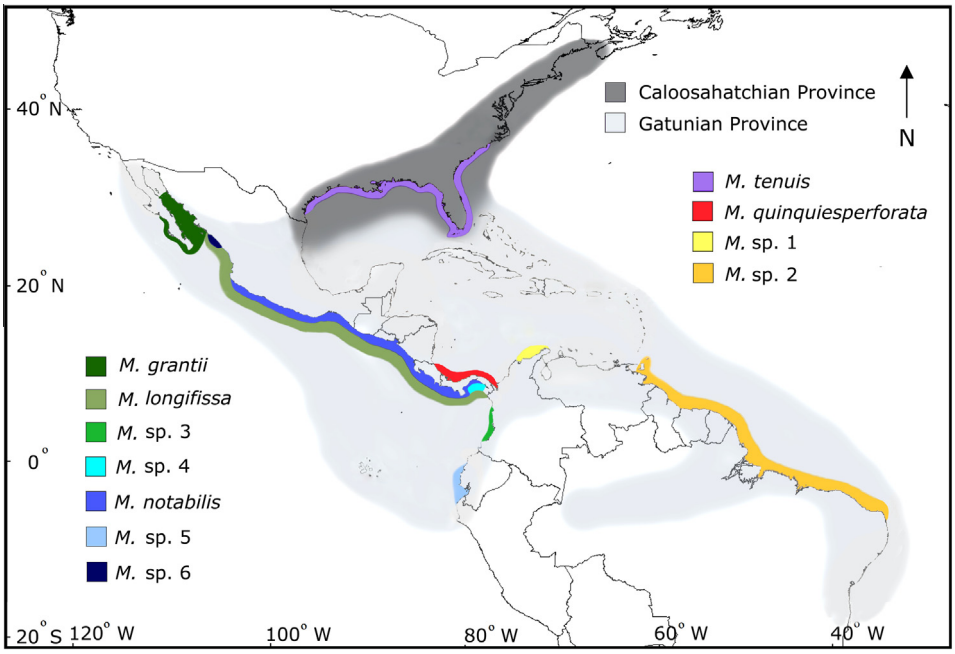

Fig. 4. Geographic distributions of extant species of *Mellita*. The figure also shows the Neogene biogeographic provinces sensu Vermeij (2005).

show that the genetic distance between *Leodia* and the Pacific *M. longifissa-grantii* lineage was 45.56%, and between *L. sexiesperforata* and the Atlantic + Pacific lineage 42.29%. L. Agassiz (1841) regarded *L. sexiesperforata* as synonymous with *Mellita*, whereas Mortensen (1948) preferred to consider *Leodia* only as a subgenus of *Mellita*. The large genetic distances we have found between *L. sexiesperforata* and *Mellita* justify Gray's (1851) transfer of the former species to a separate genus. Further subdivision of *Mellita* may well be appropriate, because the deepest split between clades within the genus, that between the *M. longifissa* + *M. grantii* lineage and the Atlantic + Pacific clade, in COI (39.67%) is suggestive of genus-level differentiation. These lineages are morphologically differentiated by the width of the ambulacral regions between the food grooves on the oral surface that surround the ambulacral lunules. Such regions are narrow in members of the *M. longifissa* + *M. grantii* lineage, and broad in members of the Atlantic + Pacific lineage.

Which of the clades we have observed within a morphospecies should be considered separate biological species is a difficult question, because there are no data on reproductive isolation. One approach to answering this question is the one typically used in COI barcoding. Intraspecific variation of COI in animals (except the Cnidaria) is rarely more than 2% and more typically less than 1% (Avise, 2000); similar values have also been found in echinoderms (Ward et al., 2008). Reproductive barriers between echinoid species have been found to arise in species separated for only 250,000 years (COI divergence of 0.9%), as they did between *Echinometra oblonga* (Blainville, 1825) in the central Pacific and an unnamed species from the western Pacific (Landry et al., 2003).

Mellita longifissa in the Pacific was split into three mtDNA lineages in the Pleistocene. Two of these lineages (*M. longifissa* and *M.* sp. 4) today have distributions that overlap in the Gulf of Panama. However, with 3.97% divergence in COI it is likely that they have become reproductively isolated. This is also true for *M.* sp. 3 from Pacific Colombia, which has a mean divergence of 5.67% in COI from *M. longifissa* + *M.* sp. 4. The three lineages that diverged from the ancestor of *M. notabilis* have more than 8.6% divergence in COI from one another suggesting that they are also separate species. This is also true for *M. quinquiesperforata* and *M.* sp. 1, in which divergence in COI between these two clades is also 8.6%. Short branch lengths and intermixing of morphospecies within the Gulf of Mexico and Atlantic clade suggest that *M. isometra* is not a separate species from *M. tenuis*. Seven of the eight specimens of *M. tenuis* sampled from Texas did form a geographically exclusive subclade. This is likely the result of restricted larval dispersal between east and west coast populations across the freshwater plume of the Mississippi Delta.

We therefore propose that there are four extant species of *Mellita* in the Atlantic (*M. tenuis*, *M. quinquiesperforata*, *M.* sp. 1 and *M.* sp. 2) and three extant species in the Pacific (*M. notabilis*, *M.* sp. 5 and *M.* sp. 6). A further four Pacific species (*M. grantii*, *M. longifissa*, *M.* sp. 3, *M.* sp. 4) are probably best placed in a new genus. We propose that *M. isometra* should be synonymised with *M. tenuis*, and suggest that *M. kanakoffi* and *M. eduardobarrosoi* should be considered junior synonyms of *M. notabilis*.

Considerable plasticity in test structures was encountered in the *M. longifissa-grantii* clade, particularly between those living in sheltered bays in relation to those living on exposed beaches. This was particularly evident in *M.* sp. 4 from the sheltered Gulfo de San Miguel in Panama. Specimens from this location had almost circular tests and a posterior interambulacral lunule that projected only halfway between the posterior petals, similar to *M. grantii* from the Gulf of California. Other members of the *M.* sp. 4 clade from exposed beaches outside of the Gulfo de San Miguel had pentagonal test outlines and interambulacral lunules that projected to the periproct, being more typical of *M. longifissa*. A similar pattern in posterior lunule development occurred in *M. grantii*. Members of this species from the Pacific coast of the Baja Peninsula have a longer posterior interambulacral lunule than those from within the Gulf of California.

Members of *M. notabilis* also exhibited morphological variation within subclades. Some members had very hummocky lunule margins, deep pressure drainage channels and a sub-rectangular test margin, while others of similar size had smooth lunule margins, shallow drainage channels and a sub-circular shaped test. Populations on exposed beaches at Acapulco and Michoacan in Mexico had deep pressure drainage channels and hummocky lunule

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margins, while populations in bays at Playa Venado and Punta Mala Panama had shallow pressure drainage channels and smooth lunule margins. F_{ST} statistics indicated only a moderate degree of gene flow between these ecophenotypes, suggesting that the morphological differences may, in fact be due to divergence between entities that do not interbreed freely.

A similar range of test characters occurred in *M.* sp. 5 from Santa Elena, Ecuador. However, in contrast to *M. notabilis*, the extremes of morphological variation in *M.* sp. 5 occurred in an identical haplotype (see Fig. 2b). Variation in colour pattern was also observed in this population with approximately half its members having spots down the ambulacra and interambulacra aborally (Fig. 2b), the other members being uniformly green. These colour patterns were mixed among morphotypes and both colour patterns were present in identical haplotypes. Such colour pattern variation has been observed in other populations from Ecuador (Sonneholzner Varas, pers. com.).

4.3. Species distributions

Based on our findings regarding molecular clades, we can propose ranges for each of the presumed species we have discovered (Fig. 4). *Mellita tenuis* occurs in the northern region of the Gulf of Mexico and off the US Atlantic coast. *Mellita quinquiesperforata* (name designation based on the morphology of the holotype) is distributed along the Atlantic coasts of Costa Rica and Panama. Specimens from the type locality of Veracruz, Gulf of Mexico need to be sequenced to establish whether they belong to the same clade as those from Costa Rica and Panama. *Mellita* sp. 1 occurs off Santa Marta, Colombia. This species may also occur throughout the upwelling Guajira region (Andrade and Barton, 2005), where endemic species are not uncommon (Petuch, 2004). *Mellita* sp. 2 is distributed from the Lesser Antilles through tropical Brazil.

In the Pacific, *M. grantii* occurs in the Gulf of California and along the adjacent Pacific coast of the Baja California Peninsula. *Mellita* sp. 6 occurs in the mouth of the Gulf of California, while *M. notabilis* and *M. longifissa* are sympatric from Northern Mexico to Panama. *Mellita* sp. 5 was only recorded from Pacific Colombia and *M.* sp. 5 from Ecuador.

5. Conclusions

Our molecular analyses indicate that the species designations of *Mellita* according to morphology were often erroneous due to high levels of morphological plasticity. The molecular phylogeny revealed the presence of eleven probable species in *Mellita*, including six cryptic species, whereas five described morphospecies do not include monophyletic molecular units and thus represent ecophenotypic variation within species. *Leodia sexiesperforata* diverged from the ancestor of *Mellita* in the Late Miocene. The ancestor of *Mellita* diverged into a Pacific lineage and an Atlantic + Pacific lineage close to the Miocene/Pliocene boundary. High levels of genetic differentiation occur between these lineages suggesting genus level differentiation. Pacific *Mellita,* therefore consist of two highly divergent lineages that became established in the Pacific at different times, resulting in sympatric distributions. Atlantic species, on the other hand, are alloparic with respect to one another. Judged by modern day ranges, not all divergence in this genus conforms to an allopatric model. Fossil calibration of some of the nodes of the molecular phylogeny dated the separation of *M. quinquiesperforata* and *M. notabilis* event at ~3 Ma in the Late Pliocene, a date consistent with a great deal of evidence regarding the final closure of the Panamanian Isthmus, but at odds with a recent suggestion (Montes et al., 2012) that uninterrupted land was present as early as the Eocene.

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