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Reconstructing a radiation: the chiton genus *Mopalia* in the north Pacific

Ryan P. Kelly[A,B,C,E] *and Douglas J. Eernisse*[D]

[A]Columbia University, New York, USA.
[B]American Museum of Natural History, New York, USA.
[C]Present address: Stanford University, Hopkins Marine Station, Pacific Grove, California, USA.
[D]California State University, Fullerton, California, USA.
[E]Corresponding author. Email: rpk@stanford.edu

Abstract. The chiton genus *Mopalia* Gray, 1847 is highly speciose despite showing little morphological differentiation. Many of the 24 extant species are conspicuous, large-bodied and ecologically important today, but pre-Pleistocene fossils for the genus are rare. Here, we use a combined analysis of four gene regions (16S and COI mtDNA, 18S and 28S rDNA) to estimate the phylogenetic relationships for *Mopalia* species and use the inferred phylogeny to analyse the group's biogeography and patterns of speciation. We then use these molecular data to distinguish between two alternative interpretations of the fossil record, as there is a large temporal gap between the oldest fossils tentatively identified as *Mopalia* and the next oldest fossils (Miocene versus Plio-Pleistocene). Based on the estimated substitution rates from a wide variety of other marine animals, we conclude that the observed rates in *Mopalia* are consistent with a Miocene origin for the genus. Given this age for the group and assuming a molecular clock, most speciation events in *Mopalia* are inferred to have occurred on average ~5 Mya. The phylogenetic results indicate that most of the speciation events leading to extant species must have occurred along the western North American coast, though there appear to have been multiple spreading events across the Pacific. When considered along with results for the many other near-shore taxa that have similar distributions to *Mopalia*, our findings suggest the emergence of a coherent historical biogeography of the northern Pacific.

Additional keywords: Mollusca, neogene, phylogeny, polyplacophora, speciation.

Introduction

The tempo and mode of speciation in the sea have long been an area of interest for biologists and paleontologists, given the perceived rarity of opportunities for marine allopatric speciation by vicariance or dispersal. This is especially true in places like the western coast of North America where there are only moderate temperature discontinuities (Dawson 2001; Dawson and Jacobs 2001; Jacobs *et al*. 2004) and a general lack of physical barriers to dispersal; under these conditions, one might expect ample gene flow, even if larval retention were relatively common (Levin 2006). However, molecular evidence suggests that speciation along this continuous coast is not uncommon in various marine animals, such as snails (Hellberg 1998; Marko 1998; Collin 2003), and older sister-species pairs might only be secondarily sympatric (Marko 1998). Such speciation events also can be associated with important climatic shifts using fossil and molecular clock calibrations to date the nodes on a phylogenetic tree (Collins *et al*. 1996; Lessios *et al*. 2001; Schroth *et al*. 2002). The correspondence between the fossil record and molecular evolutionary rates is a powerful approach to reconstructing organismal histories and clarifying the mechanisms by which marine biodiversity arises and is maintained.

The north Pacific is recognised as one of four contemporary oceanic regions that has produced an enormous diversity of endemic species (Briggs 2006). As such, it is of particular interest in understanding marine speciation. Moreover, it is perhaps the geologically youngest such region (Estes *et al*. 2005). The chiton genus *Mopalia* Gray, 1847 (Mollusca: Polyplacophora: Mopaliidae) is a useful system in which to investigate issues of historical biogeography because it has many abundant and widespread sympatric species and the genus is restricted to, but spread throughout, the northern Pacific.

There is impressive species diversity within *Mopalia*, yet only moderate morphological divergence, which could be explained in two ways. First, the morphological similarity among *Mopalia* species could reflect a recent origin and diversification. Such rapid speciation would be especially surprising because living members of *Mopalia* have widely overlapping distributions in the north Pacific (Fig. 1) and a mechanism for such diversification in sympatry is not immediately obvious. Alternatively, there has been limited and slow morphological divergence in *Mopalia* despite a more ancient history of speciation.

The fossil record for *Mopalia* is problematic and does not allow us to distinguish between the alternative diversification scenarios for the group. One interpretation suggests that *Mopalia* had its origins as long ago as the earliest cooling of the northern Pacific during the Oligocene, with a reported Miocene first fossil appearance in the Akeya and Oldawara formations of central Japan (*c.* 17.2–15 Mya; see Itoigawa 1993). However,

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that report is inconclusive at best, as the fossils consist of only three incomplete intermediate valves and a partial head valve (Itoigawa and Nishimoto 1975; Itoigawa *et al*. 1981; Itoigawa *et al*. 1982). The next oldest known *Mopalia* fossils are four distinguishable morphospecies from the San Diego Formation of southern California at the latest Pliocene or earliest Pleistocene (*c*. 2.0–1.8 Mya; Hertlein and Grant, *c*. 1960 unpublished manuscript; M. Vendrasco and D. J. Eernisse, unpubl. data), and these are indisputably *Mopalia* spp. Other published reports are all from the Pleistocene (e.g. Valentine 1980; Ogasawar 2003). It is possible that the most recent common ancestor for *Mopalia* is not much older than these California fossils, such that the radiation has occurred since the beginning of the Pliocene.

These two alternative scenarios would place the origin and diversification of *Mopalia* in completely different ecological contexts, and supporting one or the other could provide a general case study for understanding the many other diverse taxa in the north Pacific. A Miocene to early Pliocene radiation would have taken place following a period of prolonged warming, terminated by the opening of the Bering Strait over 5 Mya (Vermeij 2001; Gladenkov *et al*. 2002; Barron 2003), after which cold water divided the north-eastern and north-western Pacific shallow-water fauna. Several molluscan groups appear to have diversified during this period (Amano and Vermeij 1998; Hellberg 1998; Amano and Vermeij 2003), in which the present patterns of upwelling and a kelp-dominated nearshore were first established (Estes *et al*. 2005). Conversely, a Pliocene diversification would have seen *Mopalia* radiating within a cold nearshore environment, interrupted by fluctuations in sea level, temperature-driven range changes, intermittent current regimes and variable glacial extent in the northern Pacific (Valentine 1980; Roy *et al*. 1995; Herbert *et al*. 2001; Jacobs *et al*. 2004).

Here, we estimate the phylogeny of the chiton genus *Mopalia* based on multiple gene regions (16S and COI mtDNA, 18S and 28S rDNA) and use this and available fossil, molecular and biogeographic data to test these alternative hypotheses for the tempo of speciation of this exclusively northern Pacific clade.

Mopalia is the most speciose chiton genus in the nearshore environment of the north-eastern Pacific Ocean. Many species are large and common intertidally and presumed to play important roles in the ecology of the western North American (West Coast) rocky shorelines. The genus encompasses at least 24 species, many of which are exceedingly difficult to distinguish from one another by morphology alone. Although morphological synapomorphies are difficult to discern within the genus, species can be distinguished on the basis of their girdle hairs, termed ‘setae’, which extend from the muscular girdle surrounding the shell plates, or ‘valves’ as well as some shell features (Eernisse *et al*. 2007). DNA barcoding methods

Fig. 1. Approximate ranges for *Mopalia* species in the eastern and (inset) western Pacific. Range number 1, *Mopalia imporcata*; 2, *M. lignosa*, *M. hindsii*, *M. phorminx*; 3, *M. cirrata*, *M. ferreirai*, *M. sinuata*, *M. swanii*, *M. vespertina*; 4, *M. muscosa*; 5, *M. kennerleyi*; 6, *M. egretta*; 7, *M. spectabilis*; 8, *M. acuta*, *M. lowei*; 9, *M. ciliata*, *M. lionota*, *M. plumosa*, *M. porifera*; 10, *M. schrencki*; 11, *M. middendorffi*, *M. seta*; 12, *M. retifera*; 13, *M*. sp. nov., distribution known only from collection in dredge off of Pta. Asunción, Baja California. All occur in the intertidal except *M. cirrata*, *M. phorminx*, *M. egretta*, and *M. sinuata*, which are almost entirely subtidal. Ranges are all estimated from published accounts and personal communications from R. Clark, and A. Draeger.

have also recently been shown to be effective for delimiting species of *Mopalia* (Kelly *et al*. 2007) and these results are congruent with distinctions based on setae. Thus, there is good morphological and molecular evidence for the validity of a large number of species and the present paper presents the first phylogenetic hypothesis for the group.

Materials and methods

For molecular phylogenetic analysis, specimens were collected from the field between 2002 and 2005, with exceptions noted below, and kept in 95–100% ethanol (see Appendix). Morphological identifications were based on valve and setae characters (Eernisse *et al*. 2007) observed under a light microscope after ethanol preservation. Collection permits were obtained from the relevant agencies in Mexico, California, Oregon, Washington, British Columbia and Alaska, and each voucher specimen corresponding to a set of DNA sequences that was not obtained from another museum was deposited in the Invertebrate Zoology collection of the Santa Barbara Museum of Natural History.

In some cases, older tissues were used to supplement recent collections. Older materials had been kept at –80°C (Eernisse collections, 1986–1989) or at room temperature in 80% ethanol (1963 *M. cirrata* specimen courtesy of the Museum of Natural History of Los Angeles County and Russian specimens of *M. seta* and *M. retifera*, courtesy of B. Sirenko). Genomic DNA was extracted from specimens' muscular foot tissue using Qiagen DNeasy kits (Qiagen Corp., Valencia, CA), eluted in water and kept at –20°C for short-term use. Samples of all tissues were deposited in the Monell Cryo Collection at the American Museum of Natural History and are maintained in nitrogen vapor at –110°C.

PCR was carried out on the DNA extracts, using standard reagents, annealing at temperatures of 55–67°C (16S, 18S, 28S) and 50–51°C (COI). Published primers for these loci were used unmodified: 18S (Hillis and Dixon 1991); COI (Folmer *et al*. 1994); 16S (Palumbi 1996); and 28S primers designed by, and courtesy of, Chris Winchell at the University of California Los Angeles (UCLA): 28S-F: 5′-ACCCGCTGAATTTAAGCATAT-3′ and 28S-R: 5′-GACGATCGATTTGCACGTCAG-3′). In cases of degraded or low-concentration DNA extract, ready-made PCR beads (PuRe Taq Ready-To-Go PCR Beads, GE Healthcare, Piscataway, NJ) were used in place of batch-mixed reagents to amplify product. PCR products were cleaned using either 96-well filter plates (Millipore Corp., Billerica, MA) or AmPure beads (Agencort Corp., Beverly, MA; protocols available from manufacturer).

DNA sequencing was carried out using ABI BigDye Terminator reactions (Applied Biosystems, Foster City, CA), with an annealing temperature of 50°C. Cycle sequence products were cleaned with 70% isopropanol and ethanol, resuspended in formamide and read on an ABI 3730 automated sequencer (Applied Biosystems). The majority of the resulting DNA sequences were verified by aligning reads from both 5′ and 3′ directions where possible, using Sequencher software (GeneCodes Corp., Ann Arbor, MI). Sequences have been deposited in GenBank under the accession numbers EU406771–EU406890 (18S), EU406891–EU407017 (16S), EU407018–EU407138 (28S) and EU409064–EU409069 (COI), or were previously submitted as accession numbers EF159577 to EF159605, EF159607 to EF159616, EF159619 to EF159635, EF159637 to EF159650, EF159653 to EF159657, EF159659 to EF159680, EF159682 to EF159683, EF159685 to EF159686, EF159690 to EF159695, EF159698 to EF159701 and EF200960.

A working dataset of 134 individuals from *Mopalia* and also including 19 individuals from five closely related genera, was selected from over 500 individuals and sequenced for at least one gene region. Particular representatives from each species were selected for analysis after assessing within-species variation for one or both mitochondrial gene regions with multiple individuals sampled from multiple geographic locations for all but rare species (Table 1). Where possible, individuals from each species representing different haplotypes were included in the analysis in order to incorporate intraspecific variation. Three *Mopalia* species could not be included for lack of genetic sampling: *M. egretta* Berry, 1919, *M. middendorffii* (von Schrenck, 1861) and *M. schrencki* Thiele, 1909.

Alignment generally was not problematic and was carried out by eye with the aid of MANIA alignment editor (D. Swofford and D. Eernisse, unpubl. data). Though some gaps were present in the 16S, 18S and 28S alignment, the sequences could be aligned with relatively few ambiguities. Excluding these sites had little effect on the resulting tree topology; for the final analysis, 13 sites out of 1510 were excluded. MacClade (Maddison and Maddison 2003) and DNA Stacks software package ver. 1.3.4 (available at http://biology.fullerton.edu/deernisse/dnastacks.html) were used for data management.

A combined dataset alignment of ~2387 bp was analysed for the four fragments (16S, 524bp; 18S, 857bp; 28S, 351bp; COI, 655bp), including 463 parsimony-informative characters, over half of which were from the COI dataset. We estimated phylogenetic relationships using maximum likelihood analysis, Bayesian inference and parsimony.

Maximum likelihood analysis was carried out with GARLI (genetic algorithm for rapid likelihood inference) ver. 0.951 (Zwickl 2006), which allows for likelihood estimates with searching speed largely independent of the number of taxa. Multiple searches were conducted, each resulting in a single best tree. The resulting likelihood values were compared, selecting from among these the tree with the highest likelihood score. Bootstrap proportions were calculated from 1000 bootstrap replicates.

For Bayesian analysis, the models of sequence evolution were calculated using ModelTest software (Posada and Crandall 1998). These were determined to be as follows: 16S and COI: GTR (generalised time reversible) + I (proportion of invariable sites) + G (gamma distribution); 18S: GTR+I; 28S: SYM (symmetric model) +I+G. Bayesian phylogenetic analysis was carried out with MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) using the specified models for each corresponding data partition subset and beginning with the default prior. The resulting likelihood values of two Markov chain Monte Carlo (MCMC) chains of 1 000 000 generations each were graphically checked to determine when the chains had sufficiently burned in, and the combined sampled trees from the last 900 000 generations were used for estimating posterior probabilities for each node (Huelsenbeck *et al*. 2002).

A parsimony-based phylogenetic analysis of equally weighted DNA characters was carried out using PAUP* ver. 4.0b10 software (Swofford 2003), with a heuristic search employing TBR branch swapping, with 10 random stepwise-addition replicate searches each to determine starting trees, and results based on the first 1000 equally parsimonious trees found. An alternative and generally more effective and efficient two-part searching strategy was also conducted and used for the results reported here. The first part involved 1000 replicate searches with only 10 trees held per replicate, keeping all minimum length trees found. Then, the second part involved swapping on all minimum length trees found with the trees-held restriction removed, starting with trees already in memory and with maxtrees set to 1000. A strict consensus of these trees preserved the dichotomy of all interior nodes, collapsing only intraspecific nodes. Bootstrap support was evaluated using TBR branch swapping, with 10 random stepwise-addition replicate searches each, for 5000 replicates. Bremer support indices (Bremer 1994) were calculated by using the same two-part robust search method specified above, with a separate converse constraint search (automated with DNA Stacks software) corresponding to each internal node of the strict consensus tree, except for intraspecific nodes. Less robust searching would have artificially inflated the Bremer support indices.

The open-source software r8s ver. 1.71 (by M. J. Sanderson; available online at http://ginger.ucdavis.edu/r8s/) was used to calculate an estimate of the time of divergence for lineages emerging from each internal interspecific node of the ingroup (*Mopalia*), relative to the basal node of the ingroup. The absolute age of the basal *Mopalia* node was fixed to one of two alternative minimum ages, either 16 Mya, or 3 Mya, in order to contrast the implied substitution rates for the two alternative hypotheses of the origin of the genus. Because r8s is not effective at finding optimal solutions with large numbers of taxa owing to escalating computational complexities, we used a version of our best tree, as calculated by GARLI and imported into PAUP*, retaining branch lengths, and then pruned to one sequence per species, for 27 taxa. The exemplar sequences were selected to avoid those with missing data or those having long branches relative to other members of the species. The reduced tree with branch lengths was further reduced by pruning the most basal outgroup branch, as recommended in the manual for r8s (reducing the number of taxa to 26). Likewise, we followed other general recommendations in the manual, using the 'fixage' command applied to the basal *Mopalia* node for either 16 or 3 Mya, with other parameters constrained to the following: BLFORMAT ultrametric = no round = yes; COLLAPSE; DIVTIME method = pl algorithm = tn num_restarts = 10 perturb_factor = 0.05. The 'pl' refers to penalised likelihood

Table 1. Specimen and locality data for the 27 species analysed

Number of species, collection locations, the number (in parentheses) of individuals included in the 132-taxon phylogenetic analysis (Fig. 2), and the specimen voucher ID [in square brackets] of selected exemplars used for 27- or 26-taxon data subset analyses (see Methods, Fig. 3, and Table 2). Non-*Mopalia* species were used as outgroups in the phylogenetic analyses

Species	Localities (*n* individuals) [exemplar voucher ID]
Mopalia acuta (Carpenter, 1855)	Monterey, CA (5) [135815]
Mopalia ciliata (Sowerby, 1840)	Corona del Mar, CA (1); Monterey (2) [116048]
Mopalia cirrata Berry, 1919	Trinidad, CA (1); near Seward, AK (1) [141732]
Mopalia ferreirai Clark, 1991	Monterey, CA (4); San Juan Co., WA (1) [135749]
Mopalia hindsii (Sowerby MS, Reeve, 1847)	Monterey, CA (2); near Pismo Beach, CA (3); Santa Cruz, CA (1) [115810]
Mopalia imporcata Carpenter, 1864	Monterey, CA (5); San Juan Co., WA (1) [115843]
Mopalia kennerleyi Carpenter, 1864	Tacoma, WA (2); Anacortes, WA (1); San Juan Co., WA (3); near Juneau, AK (4) [135738]
Mopalia lignosa (Gould, 1846)	Cambria, CA (4); Monterey, CA (4) [115832]
Mopalia lionota Pilsbry, 1918	Monterey, CA (6), Santa Cruz, CA (1) [135809]
Mopalia lowei Pilsbry, 1918	Monterey, CA (6) [135742]
Mopalia muscosa (Gould, 1846)	Santa Barbara, CA (4) [115710]
Mopalia phorminx Berry, 1919	English Bay, British Columbia (1), Southern California (1) [156034]
Mopalia plumosa (Carpenter in Pilsbry, 1893)	Pta. San Carlos, Baja Calif. (2); Cabo San Quintín, Baja Calif. (3); Malibu, CA (2); Santa Cruz, CA (3) [144285]
Mopalia porifera Pilsbry, 1893	Cabo San Quintín, Baja Calif. (4) [136017]
Mopalia retifera Thiele, 1909	Vostok (East) Bay, Sea of Japan (3) [115888]
Mopalia sp. A	Off of Punta Asunción, Baja Calif. Sur (2) [DE133]
Mopalia seta Jakovleva, 1952	Vostok (East) Bay, Sea of Japan (3) [DE260]
Mopalia sinuata Carpenter, 1864	Monterey, CA (3); San Juan Co. (2); near Homer, AK (4) [135758]
Mopalia spectabilis Cowan & Cowan, 1977	Monterey, CA (2); San Juan Co., WA (1) [135685]
Mopalia swanii Carpenter, 1864	Anacortes, WA (2); San Juan Co., WA (8) [135733]
Mopalia vespertina (Gould, 1852)	Monterey, CA (2); San Juan Co., WA (3) [135710]
Dendrochiton flectens (Carpenter, 1864)	Monterey, CA (4) [115825]
Dendrochiton thamnoporus (Berry, 1911)	Monterey, CA (3) [115771]
Cryptochiton stelleri (von Middendorff, 1847)	Cambria, CA (1) [115886]
Tonicella lineata (Wood, 1815)	San Juan Co., WA (3) [141460]
Katharina tunicata (Wood, 1815)	near Trinidad, CA (1); near Juneau, AK (1); near Craig, AK (1) [144464]
Placiphorella velata Carpenter MS, Dall, 1879	Monterey, CA (5) [115790]

criterion (Sanderson 2002) and 'tn' refers to truncated Newton optimisation. We set 'smoothing = 1' based on the results of the cross validation routine implemented in r8s, but we also checked the results using higher values of smoothing, noting only slight differences in the results.

Substitution rates were estimated using the ages estimated by r8s and branch lengths reported by PAUP* (criterion = likelihood) for a subset of our data comprised only of all the 3rd codon positions of COI, because it is easiest to compare rate estimates derived from such sites (presumed to be accumulating mostly silent substitutions) to comparable rates from other animal taxa. Rates were calculated as the sum of branch lengths, divided by the estimated age from r8s, divided by 2. Branch lengths were calculated as in Wares and Cunningham (2001; also C.W. Cunningham, personal communication) using PAUP*'s F84 maximum likelihood model with estimated base frequencies and equal rates between sites. Although this is a different model than we used in any of our analyses, this particular distance metric was chosen primarily so that our rate estimates were comparable to those in Wares and Cunningham (2001) because rate estimates are known to be sensitive to the model chosen. Rates were calculated only for the branches joined by the 15 most terminal nodes of our 26-taxa tree, selected because they were terminal or nearly so and thus expected to be less subject to the confounding influence of saturation of substitutions owing to multiple hits.

To further ensure that our molecular clock calculations were not seriously affected by multiple hits at third codon position sites, we constructed saturation graphs for these sites separately for transition and transversion substitutions. We followed the suggested approach of Sullivan and Joyce (2005), which tends to show more pronounced patterns of saturation than when distances that have not been corrected for multiple hits are used on the *x*-axis. Specifically, to calculate 'GTR+I+G' distances for 16S pairwise comparisons for our *x*-axis, we imported our best 132 taxon GARLI tree into PAUP*, executed to include only the 16S sites of our dataset and only those taxa in our reduced dataset used for molecular clock calculations and then additionally pruned the *Mopalia phorminx* exemplar because it lacked 16S data. With the optimality criterion set to likelihood, we specified 'previous' for the likelihood settings for 'pinvar' and 'shape' and these resulted in '0.43256931' and '0.65462669' values, respectively. These and additional settings different from the PAUP* 'factory' settings correspond to the PAUP* command: 'lset nst = 6 rmatrix = estimate basefreq = estimate rates = gamma shape = 0.43256931 pinvar = 0.65462669;'. We then calculated 16S pairwise distances using the 'Show pairwise distances' command in PAUP*. These were then graphed against the total number of transitition or transversion substitutions at third codon sites, calculated with MEGA4 (Tamura *et al*. 2007). For transition and transversion tallies, we excluded alignment sites that had missing data in any included taxon to ensure that tallies were comparable.

Results

Phylogenetic tree topology and support

Figure 2 describes the best GARLI-estimated maximum likelihood phylogenetic hypothesis we found for the members of *Mopalia*, with measures of node robustness from each type of analysis depicted at the corresponding nodes (See Fig. 2 caption).

Species were generally well supported as reciprocally monophyletic and occurred in well defined clusters with high levels of support. In contrast, the deepest internal nodes were found to be less robust. This may be due to relatively short and deep internal branch lengths between nodes. This pattern is consistent with, though not necessarily indicative of, a relatively rapid burst of speciation followed by more lengthy opportunities to accumulate apomorphies in each surviving lineage and its descendant species.

The placements of a few particular species or species groupings relative to each other are uncertain, especially *M. retifera*; *M. seta*; (*M. cirrata*, *M. sinuata*); and (*M. imporcata*, (*M. phorminx*, *M.* sp. A)), perhaps owing to attraction among their longer branches. An analysis of 16S+18S+28S without COI (that can be problematic for deeper nodes) also fails to achieve consensus on the placement of these clades. Interestingly, these species all share similar morphologies and all but one (*M. seta*) live in deeper water than other *Mopalia* species.

The resolution of *M. retifera* as basal within *Mopalia* was supported in the best trees found for parsimony, maximum likelihood and Baysian searches, but node support values at best only weakly supported this inference for our combined molecular dataset analysis (all algorithms). However, these low support values can be at least partly explained by different resolutions of the first outgroup. The alternative trees for especially parsimony and maximum likelihood results tended to have *Dendrochiton* spp. in a deeply nested position within the otherwise monophyletic *Mopalia* grouping (node highlighted with solid circle in Fig. 2). We think this instability in the basal resolution of *Dendrochiton* + *Mopalia* is probably related to the observation that our next most proximal outgroup, *Cryptochiton stelleri*, is considerably more distal, so there could be some tendency for spurious root attraction with various long branches within the *Mopalia* + *Dendrochiton* clade.

The fact that the Bayesian, likelihood (GARLI) and parsimony reconstructions of our ingroup agreed nearly exactly indicates that this topology is generally robust to the algorithm employed. Ignoring intraspecific topological details, the likelihood and Bayesian analyses were completely consistent with each other, despite the fact that we did not specify any *a priori* models for our different data subsets in our GARLI searches as we did in the MrBayes analyses. The maximum likelihood and parsimony bootstrap support values and parsimony Bremer support indices (not shown) also were in mutual agreement. The Bayesian posterior probability values were usually higher for those nodes only weakly supported by likelihood or parsimony. In contrast to the likelihood and Bayesian results, which strongly favoured *Tonicella lineata* and *Katharina tunicata* as sister taxa, the parsimony analysis weakly supported *T. lineata* as sister taxon to all Mopaliidae except for *K. tunicata* and *Placiphorella velata*. The basal relationships within Mopaliidae were not the focus of this study and this conflict is likely due to the lack of taxonomic sampling of the many other species of *Tonicella* or *Placiphorella* and the lack of any Mopaliidae outgroups.

Timing of Mopalia *diversification*

A chronogram (ultrametric tree assuming a molecular clock) as estimated in r8s is presented in Fig. 3, with two alternative time axes that correspond to whether the basal node of *Mopalia* is fixed at 16 Mya (during the known climatic optimum) or 3 Mya (the minimum age suggested by the alternative interpretation of the fossil record).

Rate substitution estimates could be underestimated if some COI third positions were saturated owing to the accumulation of multiple hits. Our plots of pairwise tallies of COI third codon position transitions (Fig. 4*A*) or transversions (Fig. 4*B*) versus pairwise maximum likelihood distances computed from the more slowly evolving 16S sites revealed that transversions were not saturated but that transitions were indeed somewhat satu-

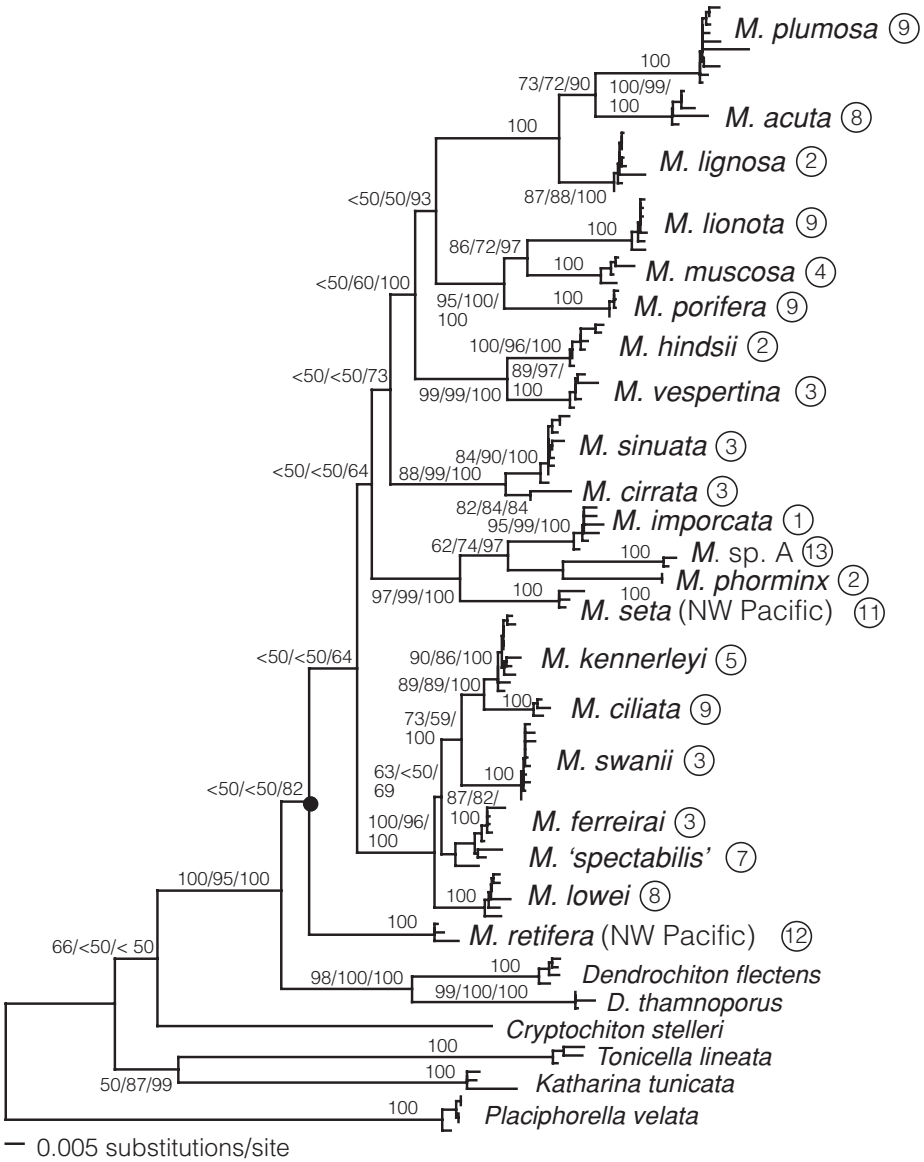

Fig. 2. A phylogenetic hypothesis for the genus *Mopalia*, based on simultaneous analysis of 2387 bp from COI, 16S, 18S and 28S loci. There was little overlap between intra- and interspecific levels of differentiation (see Genetic distance histogram, available as an Accessory Publication on the *Invertebrate Systematics* website). The tree shown was the best one computed by GARLI from five separate runs, as assessed by comparing final likelihood scores. Ignoring intraspecific topological differences, this tree generally matched the best Bayesian (MrBayes) and parsimony (PAUP*) trees found (see Results). The numbers at each node are listed in the following order, separated by slashes: (1) parsimony bootstrap values; (2) maximum likelihood (GARLI) bootstrap values; and (3) posterior probabilities from the Bayesian results using MrBayes. If all three had the same value then only one value is listed. Note that the inferred basal node of *Mopalia* (closed circle) is split between the northwestern Pacific species, *M. retifera*, and the remaining sampled *Mopalia* spp. The outgroups to *Mopalia* occur predominately in the north-eastern Pacific. Circled numbers after taxon names refer to range numbers in Fig. 1.

rated for the deepest nodes that we used for estimation (i.e. those joined at Node 15). Because transversions were relatively common and our branch length estimates were based on both transition and transversion substitutions, corrected for multiple hits with a tree-based (not pairwise) maximum likelihood approach, we concluded that our estimations were either little affected for the case of our shallowest nodes or only slightly underestimated for the deepest nodes. Still, using these estimates to calculate the age of an even deeper node might have led to an underestimation of the age of the basal node of *Mopalia*.

In Table 2, we report estimated substitution rates of all COI third position sites for only those branches of the 26 taxon tree that connect two or more closely-related species. Specifically, the estimates are for the branches that descend from the 15 relatively shallow nodes that are highlighted in Fig. 3. With the exception of the pairwise comparison between *M.* '*spectabilis*' and *M. ferreirai*, the substitution rate estimates showed little variation across branches in the tree but, as expected, the rates assuming a most recent common ancestor (MRCA) of sampled *Mopalia* species at 16 Mya were 5.3 times slower than the 3 Mya MRCA estimates, averaging 3.5×10^{-8} or 1.8×10^{-7} substitutions per site per million years, respectively (Table 2). Although no fossil calibration is yet available for any other chitons, the former estimate is consistent with mutation rates for a diverse variety of other marine taxa (McCartney *et al*. 2000; Wares and Cunningham 2001; Hellberg 2006). For example, Wares and Cunningham (2001) found that most estimates were ~4×10^{-8}, with a range from ~2×10^{-8} to 9×10^{-8}. Our 3 Mya MRCA estimates would imply rates faster than any of these, ~4 times typical mitochondrial rates of surveyed marine animals. This evidence thus strongly suggests that the ancestor of *Mopalia* species included here arose no later than the Miocene and that the most recent speciation events leading to extant *Mopalia* species average ~5 Mya.

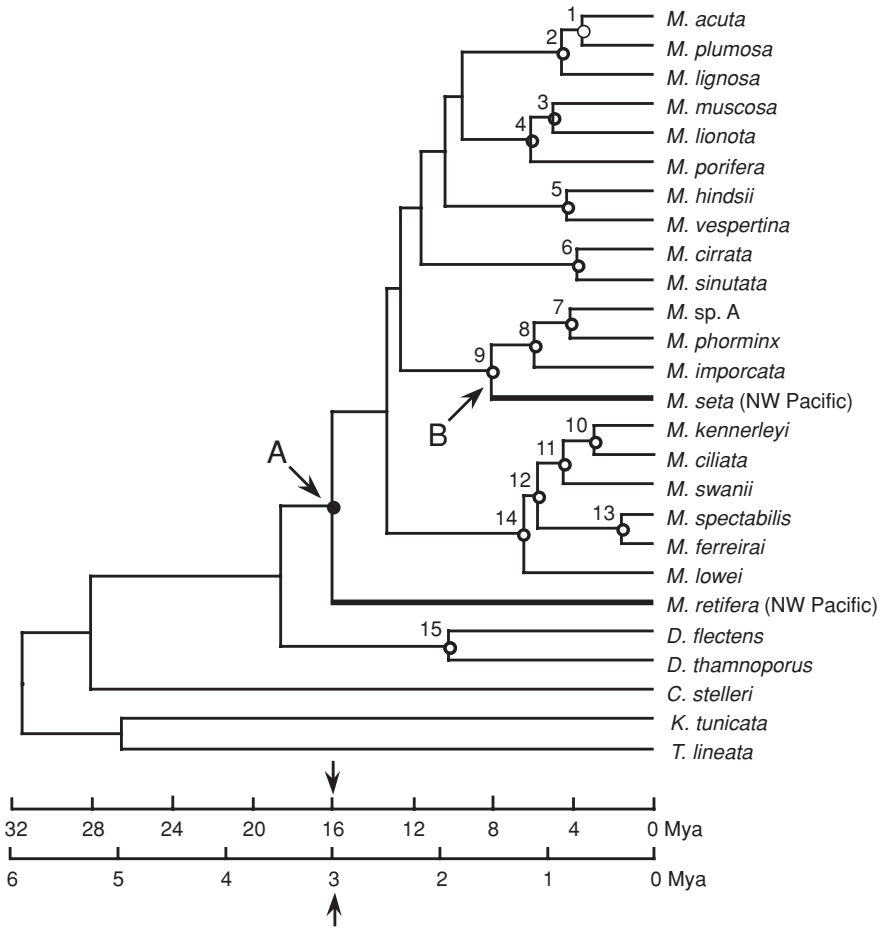

Fig. 3. Chronogram computed with r8s for a 26-taxon data subset as listed in Table 1. The two scale bars reflect two alternative fixed date calibrations at the level of the basal node for *Mopalia* (closed circle and arrows by the scale bars), each reflecting a different scenario (16 or 3 Mya) for the earliest trans-Pacific spread of *Mopalia*. The 15 selected nodes with numbers and open circle symbols were used for the substitution rate calculations presented in Table 2. Two trans-Pacific invasion events are inferred, at nodes labelled A and B, with the north-western Pacific branch highlighted in bold. Genus abbreviations are as follows: *M*., *Mopalia*; *D*., *Dendrochiton*; *C*., *Cryptochiton*; *K*., *Katharina*; *T*., *Tonicella*.

Discussion

Phylogeny and historical biogeography

The tentative placement of *M. retifera* as sister to the other 20 sampled *Mopalia* species suggests an early divergence between lineages on either side of the northern Pacific Ocean. *Mopalia retifera* occurs in the north-western Pacific, whereas most (20 of 24 total) species of *Mopalia* and all five species of *Dendrochiton* (the most proximal outgroup of *Mopalia*, based on D. J. Eernisse unpubl. data and this study) are found exclusively along western North America between Alaska and the Gulf of California. Besides *M. retifera*, the only other north-western Pacific *Mopalia* species included here, *M. seta*, is nested well within this clade, so its range is probably best

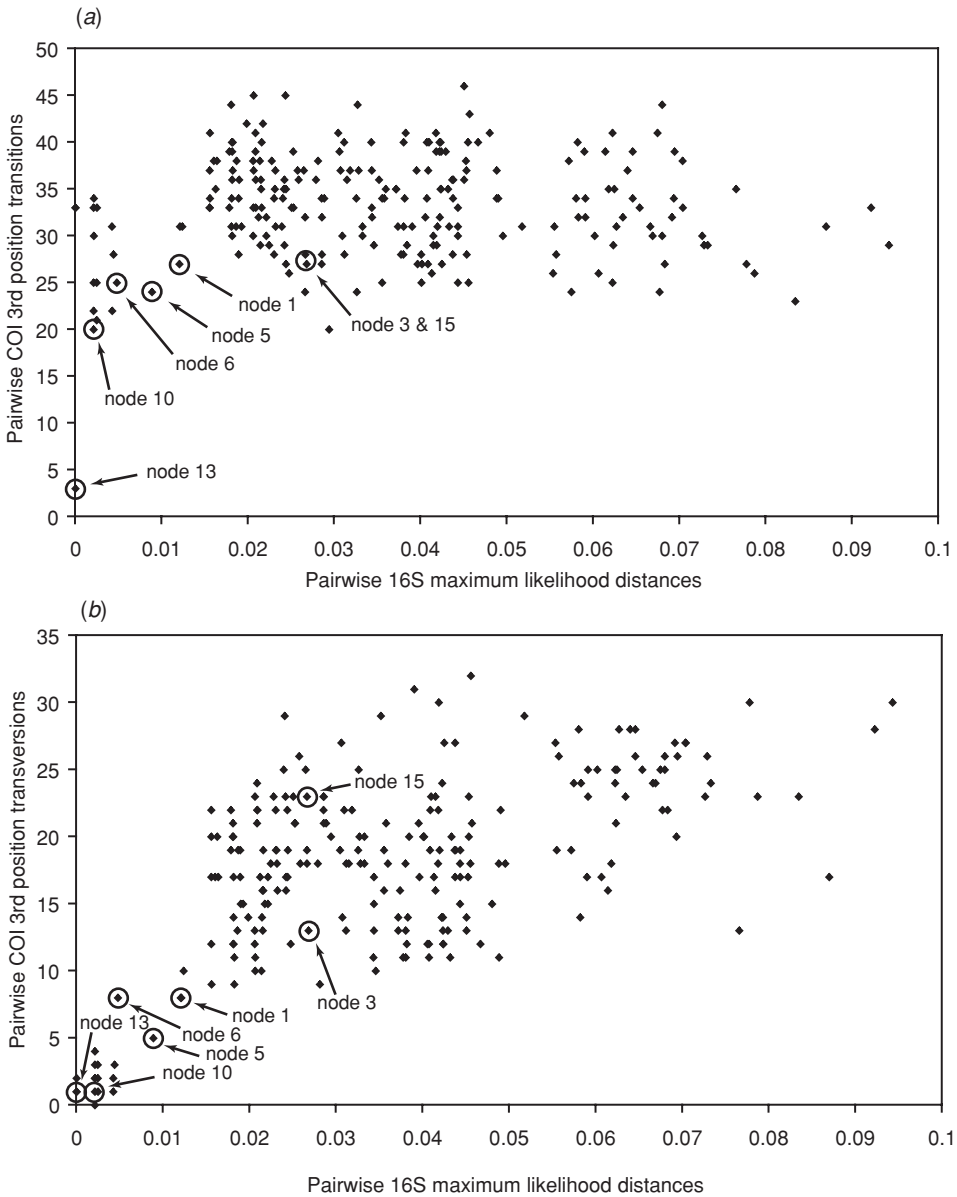

Fig. 4. Pairwise comparisons of the total number of transitions (A) and transversions (B) at COI 3rd codon position versus maximum likelihood pairwise distances (see Methods) for the 16S data subset only, which generally evolves at a slower rate. Each point corresponds to specific pairwise comparison between two terminals in Fig. 3 except that there was no 16S data for *M. phorminx* so it was not included, and all pairwise comparisons whose 16S distance was greater than 0.1 (*x*-axis) are not included in this graph. Note the expected saturation of transition substitutions (A) at 3rd codon sites with increasing pairwise maximum likelihood distance. The open circles (with arrows pointing to them) highlight all comparisons between sister species of those 15 nodes used for substitution rate calculations (Fig. 3 and Table 2). The pairwise comparisons for Node 13 corresponds to the shallowest node and Nodes 3 and 15 correspond to the deepest nodes in Fig. 3, where branch length is based on maximum likelihood based calculations using both transition and transversion substitutions.

explained as a secondary trans-Pacific invasion event. Some only slightly sub-optimal topologies have both *M. retifera* and *M. seta* as basal lineages in separate *Mopalia* subclades, whereas we never found trees with *M. retifera* and *M. seta* as sister taxa. For our best-supported trees for all algorithms, the basal split between *M. retifera* and other *Mopalia* implies either that the shared common ancestor was spread across the northern Pacific, then separated by a later vicariant event, or else a dispersal event across the northern Pacific gave rise to the present day north-eastern *Mopalia* species. Based on morphology (Saito and Okutani 1991), the other two north-western Pacific species are most similar to either *M. seta* or the north-eastern Pacific *M. ciliata*, respectively, so only the latter case implies one additional secondary trans-Pacific invasion event. The directionality of these events is uncertain except that both the preponderance of species and the trends observed for other taxa (see below) would seem to favour the north-western Pacific *Mopalia* fauna as younger.

The north Pacific in the Miocene and the timing of Mopalia *diversification*

Our results indicate that speciation events leading to extant north-eastern Pacific *Mopalia* species mostly occurred in the late Miocene to the early Pliocene. Worldwide, this timing coincides approximately with one of the few relatively warm periods (*c*. 5.3–3.5 Mya) of the last 49 Mya, which has otherwise generally been characterised by cooling trends (Vermeij 2001). However, it also coincides with the earliest opening of the Bering Strait (Durham and MacNeil 1967) at least *c*. 5.5 to 5.4 Mya as confirmed by the invasion of the Arctic bivalve, *Astarte*, into the northern Pacific (Gladenkov *et al*. 2002). At first, cold low salinity flowed southward (Marincovich Jr. and Gladenkov 1999), eventually reversing and leading to the well known trans-Arctic invasion of north Pacific fauna and flora to the northern Atlantic, especially around 3.5 Mya. By 2.7 Mya, the earliest ice sheets were accumulating in the Arctic, partly as a consequence of the closing of the Panamic Isthmus and the diversion of the Gulf Stream towards the north Atlantic, bringing moisture-laden weather patterns towards the Arctic. Meanwhile, the north Pacific became increasingly dominated by kelps, which provided a wealth of nutrition for molluscan grazers such as abalones and chitons (Estes and Steinberg 1988; Estes *et al*. 2005).

This scenario for the timing of *Mopalia* diversification closely matches that of other groups: several of the taxa that today dominate the cool temperate rocky shores of the west coast (Oregonian Province) appear to have origins in the eastern Pacific, probably from warmer-water North American ancestors (Vermeij 2001). Some extant genera have fossil records extending back to the Eocene-Oligocene Transition when there was a general deterioration of the climate in the northern Pacific leading to a dramatic turnover of species and much cooler conditions (Hickman 2003; Oleinik and Marincovich Jr. 2003; Squires 2003). However, most of the molluscan genera that first appeared in the Oligocene and are still extant live on deep muddy bottoms, not rocky shores (Vermeij 2001). Fossil evidence suggests that various rocky shore genera generally arose later, by the Early Miocene (*c*. 23 to 16 Mya). Familiar gastropod examples include teguline gastropods (Hellberg 1998), a clade of large-bodied abalones (Estes *et al*., 2005), *Littorina* (Reid 1996), the several carnivorous genera *Nucella* (Collins *et al*. 1996), *Ceratostoma* (Amano and Vermeij 1998) and *Lirabuccinum* (Amano and Vermeij 2003). Other examples are consistent with an Early Miocene origin as well, including other gastropods as well as bivalves, barnacles and probably also kelp (Estes and Steinberg 1988; but see Vermeij 2001, who discusses the earlier formation of the northern Pacific fauna).

As inferred from those genera that do have a fossil record, there was a general trans-Pacific spread ~17 to 14.5 Mya, mostly from east to west (to the north-western Pacific), which closely coincides with a ‘climatic optimum’ interval that should

Table 2. Alternative substitution rates for node to tip branches calculated from a 26-taxon data subset for 15 nodes, each with the corresponding number and an open circle symbol in Fig. 2

Each node joins two branches, each leading to a terminal species exemplar or a nested clade, as listed in the ‘Terminal A’ and ‘Terminal B’ columns. All species names refer to *Mopalia* except for Node 15, which refers to *Dendrochiton*. Branch lengths are maximum likelihood estimates (see Methods) for a data subset of all COI 3rd codon positions. Node age (in millions of years) and substitution rate estimates (substitutions per site per million years) are provided based on fixing the basal node for *Mopalia* (close circle in Fig. 2) to either 16 or 3 Mya

Node	Terminal A	Tip Dist A	Terminal B	Tip Dist B	Comb Dist	Age 16 (Mya)	Rate 16	Age 3 (Mya)	Rate 3
1	*acuta*	0.147	*plumosa*	0.205	0.352	3.5	5.0E-08	0.81	2.2E-07
2	*lignosa*	0.149	node 1	0.264	0.413	4.6	4.5E-08	1.04	2.0E-07
3	*muscosa*	0.197	*lionota*	0.264	0.461	4.99	4.6E-08	1.10	2.1E-07
4	*porifera*	0.234	node 3	0.230	0.465	6.12	3.8E-08	1.33	1.7E-07
5	*hindsii*	0.153	*vespertina*	0.127	0.280	4.27	3.3E-08	0.87	1.6E-07
6	*cirrata*	0.176	*sinuata*	0.048	0.224	3.77	3.0E-08	0.74	1.5E-07
7	sp. A	0.183	*phorminx*	0.163	0.346	4.14	4.2E-08	0.92	1.9E-07
8	*imporcata*	0.186	node 7	0.242	0.429	5.99	3.6E-08	1.29	1.7E-07
9	*seta*	0.239	node 8	0.354	0.593	8.05	3.7E-08	1.67	1.8E-07
10	*kennerleyi*	0.028	*ciliata*	0.137	0.165	2.89	2.8E-08	0.48	1.7E-07
11	*swanii*	0.142	node 10	0.101	0.243	4.4	2.8E-08	0.72	1.7E-07
12	node 11	0.291	node 13	0.124	0.415	5.66	3.7E-08	0.93	2.2E-07
13	*spectabilis*	0.010	*ferreirai*	0.034	0.044	1.47	1.5E-08	0.22	9.9E-08
14	*lowei*	0.105	node 12	0.238	0.342	6.3	2.7E-08	1.04	1.6E-07
15	*flectens*	0.210	*thamnoporus*	0.531	0.741	10.21	3.6E-08	1.88	2.0E-07
Average		0.163		0.204	0.367	5.09	3.5E-08	1.00	1.8E-07

have made the northern Pacific habitable and is supported by ample evidence of northern shifts in the range of the Miocene marine fauna (e.g. Itoigawa 1993). This area was at this time still bounded on the north by an impenetrable Bering land bridge (Vermeij 2005; Lyle *et al*. in press). Later, the north-western and north-eastern Pacific faunas became separated again as the northern Pacific cooled considerably, at least in part owing to the opening of the Bering Strait. This occurred possibly as early as 9 Mya but at least by the latest Miocene or earliest Pliocene (*c*. 5.3 Mya) and likely resulted in dramatic cooling and localised lowered salinities at the shallow depths inhabited by *Mopalia* spp. Trans-Pacific invasions since then have been more sporadic and probably more west to east than east to west (Vermeij 2001). The best-studied snail, bivalve and barnacle examples seem similar in many respects and match our *Mopalia* results but it should be emphasised that they represent only a small fraction of an extremely diverse north Pacific fauna. For example, other chiton genera besides *Mopalia* as well as the diverse limpet genus, *Lottia*, are like *Mopalia* in having at best only scant pre-Pleistocene fossil records. Applying the present approach to these and other north Pacific taxa could help test the generality of a Miocene to early Pliocene radiation of the north Pacific fauna.

The combination of warm tropics and cold northern seas must have intensified the latitudinal gradients along western North America and such gradients probably contributed to the 'packing' of species into increasingly distinct provinces along latitudinal gradients, leading to an increase of diversity (Jablonski *et al*. 2003; Briggs 2006). Opportunities for vicariance associated with province boundaries must have existed, along with the potential for sympatric 'ecological' speciation driven by natural selection based on local habitat partitioning or divergence in food sources. We have had more success in clarifying the tempo, rather than the mode, of speciation in *Mopalia* because our inferred speciation events are older that the intervening glaciation events with their tremendous potential to obscure historical signal.

Speciation mode

No consensus has yet emerged about the dominant mode of speciation in the sea. Hellberg (1998) and Collin (2003) both report many pairs of marine molluscan sister species with broadly overlapping geographic distributions, implying a minimal role for classical allopatric speciation in those taxa. Instead, Hellberg suggested that 'transient' allopatry could account for the majority of speciation events in teguline gastropods. Briggs (2006) has advocated at least some role for sympatric speciation as a common mode of speciation within highly diverse areas such as the north Pacific and has further argued that such speciation can be much (perhaps 20 times) faster than allopatric speciation, with natural selection involved in the earliest stages of reproductive isolation. Examples of such speciation include Hellberg and Vacquier (1999), Hendry *et al*. (2000), Dawson *et al*. (2002) and Jones *et al*. (2003). However Meyer's (2003) comprehensive work on cowries suggests that vicariant speciation in allopatry is the most common mechanism for divergence among Indo-West Pacific cypraeid gastropods. Similarly, *Nucella* appears to be subject to vicariance owing to climatic cycling (Collins *et al*. 1996).

Sister taxa with adjacent or narrowly overlapping ranges (<10%) have been seen as evidence for vicariant speciation (Marko 1998; Knowlton 2000; Meyer 2003). According to this criterion, only one of seven *Mopalia* sister-species pairs shows evidence of vicariance: *M. ciliata* is found predominantly in central California and to the south, whereas its sister taxon, *M. kennerleyi*, is predominantly more northern. The two species overlap in northern California, with *M. kennerleyi* being apparently absent or rare south of Pt Reyes. Other species pairs do not show such geographic separation.

Thus, distributional data indicate vicariance in only one of seven *Mopalia* species pairs. However it is worth noting that the apparently vicariant pair, *M. ciliata*/*M. kennerleyi*, has the most recent divergence date among sister species pairs (Table 2; not including *M. 'spectabilis'* and *M. ferreirai*, which are not reciprocally monophyletic). This could be an indication that only the most recent speciation events retain a geographic signal of vicariance; the other species ranges have had sufficient time to shift and erase any such signal (see Losos and Glor 2003). Based on extensive observations of *Mopalia* spp. in the vicinity of Monterey, California (Anthony Draeger, personal communication), it appears that most species of *Mopalia* are sympatric even at fine scales, such that microhabitat differences among species are insufficient to account for the observed range overlap. However, most speciation events appear to be sufficiently ancient that the overlapping distributions need not be interpreted as the result of massive sympatric speciation.

Conclusion

This work represents the first genus-wide phylogeny for any chiton and also serves to substantiate morphological species distinctions, supporting the validity of a new species and the revival of two previously synonymised species. In addition, the molecular substitution rate calculations for *Mopalia* species strongly suggest that the group had already spread across the north Pacific by the middle Miocene, rather than much later in the Pliocene. This is a case in which the molecular data have clarified an ambiguous fossil record and in the process revealed a complex biogeographic history including at least two trans-Pacific interchange events.

The mechanisms of speciation remain cloudy. The majority of species have broadly overlapping ranges but an average of ~5 Mya has elapsed since the cladogenic events that led to them, and this is sufficient opportunity to erase traces of allopatric distribution that could have been involved in speciation, either through vicariance or dispersal. A single pair of *Mopalia* sister species are presently allopatric, which provides at least one case where sympatric speciation is unlikely to have been involved. *Mopalia* species typically range over ~20 to even 40 degrees of latitude with little restriction to gene flow and no phylogeographic structure, despite high levels of genetic variation (R. Kelly and D. Eernisse, unpubl. data). It remains to be seen how such species became fragmented into divergent lineages and whether these same processes are ongoing.

Mopalia is an example of a group with substantial genetic divergences but little morphological change, which seems to have radiated relatively rapidly within the north Pacific by an uncertain mechanism. Such taxa challenge traditional ideas of

marine speciation and biogeography, hinting at an intricate evolutionary history.

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