

Short communication

Molecular phylogenetics and biogeography of the nacellid limpets of New Zealand (Mollusca: Patellogastropoda)

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1. Introduction

The origin and radiation of nacellid limpets (genera, *Cellana* and *Nacella*) are not well-resolved, but fossil records provide evidence of a historical range spanning throughout the Pacific, including North America (Lindberg and Hickman, 1986) north and south Australia, New Zealand, Java, Chile, and east and west Antarctica (Powell, 1973). The modern biogeographic range of *Cellana* is mostly tropical, extending north to Japan, East to Juan Fernandez and Hawaii, south to South Africa, Madagascar, south east Australia, New Zealand and the sub-Antarctic Islands, where it co-exists on Campbell Island with the sister taxon *Nacella* (Powell, 1973). *Nacella* is restricted to the sub-Antarctic and Megallenic region (Powell, 1973).

Molecular phylogenetic studies of Patellogastropods (Koufopanou et al., 1999; Nakano and Ozawa, 2004) have confirmed the monophyletic grouping of the Nacellidae limpets relative to the sister taxon Patellidae. The Nacellidae classification was first proposed in 1975 by Golikov and Starobogatov (1975), but was not widely accepted until Lindberg and Hickman (1986) confirmed the familial split through shell microstructure analysis.

New Zealand species of *Cellana* represent the southern most limits of the genus range (Powell, 1973) and are geographically placed between two potential sources of species immigrants: the tropics and the sub-Antarctics. Two species, *Cellana ornata* and *Cellana radians*, are distributed around the entire coastline of New Zealand. *Cellana flava* and *Cellana denticulata* are restricted to the north east coast while *Cellana strigilis redimiculum* is restricted to the south east. *Cellana stellifera* is the only sub-tidal species of this

genus and is distributed around the entire coast of New Zealand. The *Cellana strigilis* complex includes six sub-species: *C. s. redimiculum* on the main land; *C. s. chathamensis* from the Chatham Islands, and four *C. s. spp.* on the sub-Antarctic Islands.

The origin of marine biota in New Zealand is contentious. Although there is no evidence to support a southern or northern origin, Powell (1973) suggested that *Cellana* is of warm water origin with relict populations in the higher latitudes. Nakano and Ozawa (2004) presented evidence to suggest that the circum-polar current had influenced the range expansion of the *Cellana* genus, while Koufopanou et al. (1999) tentatively supported a southern origin for *Cellana* and *Nacella* with subsequent north and south radiation of the genera, respectively.

Indo-Pacific marine taxa have colonised New Zealand throughout the Cenozoic, although these taxa are almost exclusively species with teleplanic larvae capable of long distance dispersal and do not include limpet taxa (Fleming, 1979). Based on laboratory investigations of larval longevity of the sister taxon *Patella* (Branch, 1981), nacellids are thought to have restricted dispersal potential, not conducive to oceanic transport. A lecithotrophic period of 7–11 days follows the free spawning of gametes and a non-feeding trochophore stage of 1–2 days. This short period of larval dispersal and the homing behaviour of the adults suggest low levels of continental interchange and high endemism for the genus.

Eocene fossil deposits from Oregon, USA (Lindberg and Hickman, 1986) have been used for calibration of the minimum separation time between *Nacella* and *Cellana* (Koufopanou et al., 1999; Nakano and Ozawa, 2004). Powell (1973) suggested that the separation of *Patella* and *Cellana* was established before the Eocene. These proposed timings plus tentative fossil assignments suggest that the Nacellidae are an ancient taxon.

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In this paper, we used molecular phylogenetic analyses to examine two contrasting hypotheses about the biogeographic origins of the New Zealand *Cellana* limpets:

(1) *Cellana* is a warm water taxon, dispersing from northern waters of the Indo-Pacific.

(2) ancestral *Cellana* spp. arose via allopatric speciation through separation from Gondwana, Australia and Antarctica.

The mitochondrial 12S and 16S ribosomal RNA (rRNA) genes were used to examine our two biogeographic hypotheses. The use of these genes allowed the incorporation of published sequences from previous studies of Patellogastropods (Koufopanou et al., 1999; Nakano and Ozawa, 2004) and visualisation of ancient phylogenetic relationships, which fossil data suggest can be expected among limpet taxa.

2. Methods and materials

2.1. Sampling

New Zealand limpets were collected from mainland and offshore islands (Table 1). Specimens were preserved in 70% ethanol or frozen in liquid nitrogen. Sequences for *Cellana*, *Scutellastra*, and *Nacella* of the Indo-West Pacific and Antarctica were obtained from GenBank Accession Nos.: AB106426–29, AB106473–80 (Nakano and Ozawa, 2004), AF058214–19, AF058178, AF058183, AF058231, and AF058263–68 (Koufopanou et al., 1999). The outgroup taxa, *Scutellastra flexuosa* was chosen from a recent Patellogastropod study (Koufopanou et al., 1999) because it exhibited the least difference in base composition with the nacellid limpets while maintaining sister taxa status.

Table 1
New Zealand *Cellana* species included in this study. Details of the collection location and GenBank accession numbers for 12S (upper) and 16S (lower) mitochondrial rRNA sequence of the reference specimen from each species are given

Species	Gen Bank Accession No.	Locality, Lat/Long for New Zealand locations
<i>Cellana denticulata</i> (Martyn, 1784)	AY621807	Kaikoura, 42.40045S, 173.68992E
	AY837759	
<i>Cellana flava</i> (Hutton, 1873)	AY621809	Kaikoura, 42.40045S, 173.68992E
	AY837761	
<i>Cellana ornata</i> (Dillwyn, 1817)	AY621812	Kaikoura, 42.40045S, 173.68992E
	AY837754	
<i>Cellana radians</i> (Gmelin, 1791)	AY621811	Kaikoura, 42.40045S, 173.68992E
	AY837762	
<i>Cellana stellifera</i> (Gmelin, 1791)	AY621810	Taupo Bay, 34.97401S, 173.69990E
	AY837760	
<i>Cellana strigilis redimiculum</i> (Reeve, 1854)	AY621808	Moeraki, 45.34575S, 170.84188E
	AY837755	
<i>Cellana strigilis chathamensis</i> (Pilsbry, 1891)	AY621806	Chatham Isl., 43.47894S, 176.41013W
	AY837758	
<i>Cellana strigilis strigilis</i> (Hombron and Jacquinot, 1841)	AY627627	Campbell Isl., 52.50S, 169.00E
	AY837756	
	AY627626	
	AY837757	
		Auckland Isl., 50.30S, 166.17E

2.2. DNA extraction

For each specimen a 3–5 mm² section of pedal tissue was cut from the centre of the foot. The tissue was rinsed with distilled water, and finely diced using flame-sterilized forceps and scissors. The diced tissue was digested and purified following a modified lithium chloride/chloroform protocol (Gemmell and Akiyama, 1996). DNA pellets were resuspended in 100 µl TE8 (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and stored at –20 °C.

2.3. Polymerase chain reaction amplification and sequencing

Partial fragments of the 12S and 16S mitochondrial genes were obtained using 12Sma, 12Smb and 16LRN13398, 16SRHTB primer pairs (Koufopanou et al., 1999). Polymerase chain reaction (PCR) amplifications were done in a 25 µl reaction volume, consisting of 2.5 µl of 10× buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.0), 1.5 mM MgCl₂, 200 µM dNTP's, 0.54 µM each primer, 1 U *Taq* (Invitrogen), 12.9 µl double-distilled, autoclaved water plus 2 µl of template DNA. Thermal cycling parameters included an initial denaturation at 94 °C for 2 min, followed by 36 cycles at 94 °C for 20 s, 47 °C (12S) and 51 °C (16S) for 20 s, and 72 °C for 30 s, before a final 7 min extension at 72 °C. PCR products were purified with Millipore Montage PCR₉₆ Multiscreen filter plates (Biolab, New Zealand).

PCR products were sequenced in both directions with the 12Sma, 12Smb, 16LRN13398, and 16SRHTB primers, using a Big Dye V3.1 sequencing kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions. Sequence products were purified using Sephadex-GS50 gel filtration (Amersham Bioscience, New Zealand). Capillary separation of samples was done by the Alan Wilson Centre

Genome Service on an ABI 3730 DNA analyser for 12S sequencing and by University of Canterbury Sequencing Service on an ABI 3100 DNA analyser for 16S sequencing. Sequences are deposited in the NCBI GenBank (Table 1).

2.4. Sequence analysis

Multiple alignment of all sequences for both genes was performed using default parameters in ClustalX (Thompson et al., 1997): *Cellana* and *Nacella* sequences were aligned and then a profile-to-profile alignment was done with the outgroup taxa, maintaining secondary structures and conserved regions (Hickson et al., 1996; Lydeard et al., 2000).

2.5. Phylogenetic analysis

Analysis of the 12S and 16S data sets was done separately and congruence between them was confirmed with a partition homogeneity test (Farris et al., 1995) for a combined analysis in PAUP*4.0b10 (Swofford, 1998). Nucleotides identified as stem sequence, based on the 12S and 16S secondary structure (Hickson et al., 1996) were initially down-weighted by 20% (Dixon and Hillis, 1993). However, analyses run without weighting of stems did not change or add resolution to the results so weighting was eliminated from further analysis of these data. Maximum parsimony and LogDet distance (Steel et al., 2000) analyses were conducted on the combined data set with 10,000 bootstrap replicates, using PAUP*4.0b10 (Swofford, 1998). Maximum likelihood analysis was performed with 100 bootstrap replicates using PAUP*4.0b10 (Swofford, 1998). The GTR+I+G (I, 0.29; α , 0.37) model of evolution was used in maximum likelihood analysis as determined by modeltest v. 3.06 (Posada and Crandall, 1998). Phylogenetic signal was confirmed with the g_1 statistic (Hillis and Huelsenbeck, 1992).

2.6. Tree topology tests

The Kishino–Hasegawa test of tree topology (Kishino and Hasegawa, 1989), using maximum likelihood scores was applied to three constrained trees and run using PAUP*4.0b10 (Swofford, 1998). Trees were representative of the contrasting a priori hypotheses, although Tree 3 (T3) takes into account the polyphyletic nature of the New Zealand species. Tree 1 = ((NZ + Aus)(Asia + Pacific Islands)); T2 = ((NZ + Asia + PI)(Aus)); T3 = ((*C. ornata* + Aus) (NZ + Asia + PI)).

2.7. Molecular clock assignment

Tests of base composition and nucleotide change as well as evolutionary rate estimation were conducted on the New Zealand sequence data in MEGA version 2.1 (Kumar et al., 2001) for each gene separately. Tajima's Relative Rates test (Tajima, 1993) was performed using *S. flexuosa* as the outgroup taxon. Pairwise transition and transversion differ-

ences were calculated separately and plotted against pairwise LogDet distances to test for substitution saturation within the data. Pattern homogeneity (Disparity Index) with Monte-Carlo testing (Kumar and Gadagkar, 2001) was conducted in MEGA version 2.1 to confirm base composition consistency among taxa. A Neighbour-Joining tree with 10,000 bootstrap replicates based on pairwise LogDet distance was transformed to a linearized tree to estimate divergence rates between clades (Takezaki et al., 1995). The Eocene fossil record from Oregon (Lindberg and Squires, 1990) was used to calibrate the rate of divergence between *Cellana* and *Nacella*. Two fossils from the Late Eocene (38 mya) in New Zealand show similarities to present day *C. strigilis* and *C. denticulata* (Beu and Maxwell, 1990). The shell microstructure of these fossils has not been examined and so allocation to the genus *Cellana* is tentative. However, alternative rates were obtained by initially setting 38 mya as the minimum divergence time of the New Zealand clade. A third rate was obtained by setting the minimum appearance of *C. ornata* as 38 mya.

3. Results

We resolved 360 and 490 base pairs of mitochondrial 12S and 16S rRNA sequence respectively, for each of the New Zealand species (Table 1). There was no intraspecific variation in either gene. When combined, with sequences from GenBank the total aligned sequence lengths were 260 and 419 bp, respectively.

3.1. Phylogenetic analysis

Partition homogeneity tests showed congruence ($P > 0.1$) between the 12S and 16S data, so phylogenetic analysis was done on the combined data set. Analysis for the combined data was based on 675 bp, comprising 284 variable sites, 171 of which were parsimony informative. The g_1 statistic showed that significant phylogenetic signal was obtained ($P < 0.01$).

Maximum parsimony and maximum likelihood analyses revealed a polyphyletic topology for the New Zealand species (Fig. 1). A monophyletic clade (A) composed solely of New Zealand limpets is supported in 99% of bootstrap replicates. However, this clade does not include *C. ornata* which stands unresolved with weak bootstrap support (35%) placing it in a basal position within this genus. Although the New Zealand clade is clustered with Australian species, the low resolution of the data does not confirm that these species are phylogenetically closer than the Asian or Pacific Island species. This was further shown by the Kishino–Hasagawa tests which were non-significant ($P > 0.5$) for all three tree topologies.

Four other clades are well supported by both analyses. An Asian Clade (B) is well supported at 95 and 89%, and is composed of two species restricted to Japan and Hong Kong. Clade C includes *C. radiata capensis* and *C. radiata orientalis* occurring together 95 and 85% of the time. Taxonomic classification suggests that *C. r. capensis* and *C. r. orientalis*

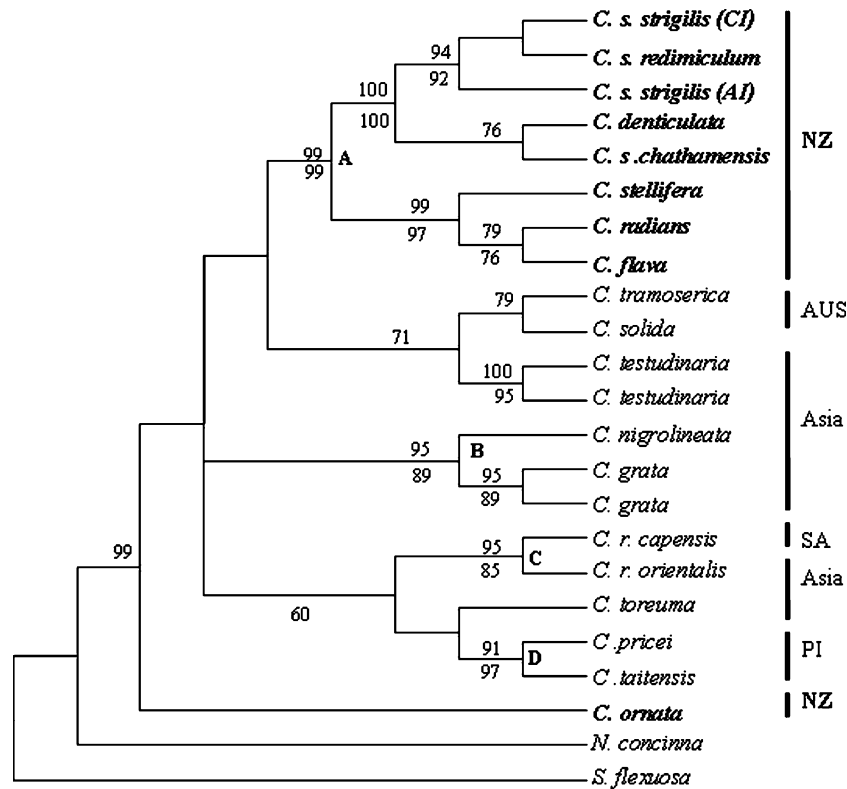


Fig. 1. Maximum parsimony tree of combined 12S and 16S rRNA data. Bootstrap support values for 10,000 replicates are shown above the branches. Maximum likelihood bootstrap support values for 100 replicates are shown below the branches; values of less than 50% are not shown. Generic abbreviations: C, *Cellana*; C. s., *Cellana strigilis*; C. r., *Cellana radiata*; N, *Nacella*; S, *Scutellastra*. Geographic abbreviations: AI, Auckland Islands; CI, Campbell Islands; NZ, New Zealand; Aus, Australia; SA, South Africa; PI, Pacific Islands; Asia includes Japan, Hong Kong and Vietnam. New Zealand taxa are shown in bold print.

are sub-species of *C. radiata* (Powell, 1973), and while our data are insufficient to explore this, they lend support to a close taxonomic relationship between these species. A Pacific Island lineage, D, includes Tahiti and Western Samoa, and has a bootstrap support of 91 and 97%.

3.2. Divergence rate estimates

Pattern homogeneity tests for several taxa pairs showed significant disparity in nucleotide composition, for both genes. LogDet distances were calculated as this method is robust to compositional heterogeneity (Steel et al., 2000). Substitution saturation was not evident and the transition/

transversion ratio was 1.6 (12S) and 1.7 (16S). Tajima's Relative Rates test (Tajima, 1993) shows no significant difference between ingroup taxa for either gene.

The three estimated divergence rates (Table 2) show that placing datum at the *Nacella-Cellana* split increases the rate of divergence and is less compatible with published rates (Koufopanou et al., 1999) than the rates obtained from the New Zealand fossil data.

4. Discussion

We set out to test two contrasting hypotheses of nacellid entry into New Zealand: entry by dispersal from the Indo-West Pacific versus allopatric fragmentation from Gondwana stock. We hypothesised that the addition of New Zealand species, the most southern limpets of the family living within the cold-temperate and sub-Antarctic waters, should help to resolve these opposing theories.

The polyphyletic nature of the New Zealand species was unexpected. All the species, aside from the sub-tidal *C. stellifera*, occur in the same habitat with up to five species co-occurring on the same platform. The polyphyletic topology suggests that *C. ornata* is basal to New Zealand and possibly the entire genus and that a second colonisation event may have brought a second ancestor into New Zealand, from which rapid diversification occurred. Alternatively, sympatric speciation occurred within New

Table 2

Contrasting divergence rates expressed as nucleotide changes per site per million years. The 'Point of Calibration' represents the node used to calibrate the divergence time of 38 million years: the rate for Patellids was estimated by Koufopanou et al. (1999) using the *Nacella-Cellana* split for the 38 million year calibration point

Point of Calibration	12S	16S	Combined
<i>Cellana/Nacella</i> —38 mya	0.0031	0.0023	0.0026
NZ clade divergence—38 mya	0.0016	0.00088	0.0012
<i>C. ornata</i> appearance—38 mya	0.0021	0.0014	0.0015
Patellids Koufopanou et al. (1999)	0.0016	0.0014	
<i>Littorina</i> Koufopanou et al. (1999)	0.0011	0.00084	

Note. *Littorina* divergence rates are added for comparison (Koufopanou et al., 1999).

Zealand millions of years after the entry of the *C. ornata* like ancestor. The basal nature of the sub-tidal species, *C. stellifera*, nested within the New Zealand clade suggests that sea level changes may have been a driving factor in the diversification within this lineage.

Comprehensive phylogenetic studies of the Littorininae (Reid et al., 1996; Williams et al., 2003) show many consistencies with our data. Here too molecular data were unable to conclusively resolve among competing biogeographic hypotheses. Williams et al. (2003) suggest that the lack of resolution for the main Littorininae genera may be due to rapid differentiation. Like *Cellana*, fossils pre-dating the Eocene have not been found for littorinines, but molecular data have the group as late Cretaceous age. However, unlike *Cellana* where the radiations occur within regions, the phylogenetic reconstruction for *Littorina* found a mixture of tropical and temperate clades, which may be a result of diversification into new habitats.

Koufopanou et al. (1999) have shown, through molecular analysis, that *Patella s. s.* occurred much earlier than the first confirmed fossil of the Pliocene. The rates obtained in this study are more similar to the evolutionary rates observed for the patellids and *Littorina* (Koufopanou et al., 1999) when the New Zealand fossil data was used. In contrast, assuming that the Oregon fossil represents the first appearance of *Cellana* over inflates the rate of divergence. The altered dates give a *Nacella*, *Cellana* split time of approximately 100mya, pushing the split of this family back into the Cretaceous. This date matches tentative fossil records of Northern Australia (Powell, 1973) and coincides with a time of continental movement splitting Antarctica, Africa and India (Hay et al., 1999).

The lack of resolution obtained from this data even though important biogeographic species were included shows that the focus of future studies should turn to the confirmation of available fossil records and the inclusion of more species from the *Nacella* genus. This would enhance the ability of molecular studies to further resolve the phylogenetics and biogeography of the nacellid limpets, and in turn further clarify Patellogastropod evolution.

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