Received: 5 November 2018 | Revised: 10 August 2019 | Accepted: 20 August 2019

DOI: 10.1111/jzs.12342

SHORT COMMUNICATION

JOURNAL OF ZOOLOGICAL SYSTEMATICS AND EVOLUTIONARY RESEARCH

WILEY

Phylogenetic topology and timing of New Zealand olive shells are consistent with punctuated equilibrium

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Funding information
Ministry of Business, Innovation and Employment Te Tipu Pūtaiao Postdoctoral Fellowship, Grant/Award Number: CONT-22922-TTP-MAU; Royal Society of New Zealand Te Apārangi Marsden Fund, Grant/Award Number: 12-MAU-008; National Institute of Water and Atmospheric Research; Muséum National d'Histoire Naturelle

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Abstract

The olive shells of the genus *Amalda* comprises readily recognized species of marine neogastropod mollusks found around the world. The New Zealand *Amalda* fauna has particular notoriety as providing one of the best demonstrations of evolutionary morphological stasis, a prerequisite for punctuated equilibrium theory. An excellent fossil record includes representation of three extant endemic *Amalda* species used to explore patterns of form change. However, the phylogenetic relationship of the New Zealand *Amalda* species and the timing of their lineage splitting have not been studied, even though these would provide valuable evidence to test predictions of punctuated equilibrium. Here, we use entire mitogenome and long nuclear rRNA gene cassette data from 11 *Amalda* species, selected from New Zealand and around the world in light of high rates of endemicity among extant and fossil *Amalda*. Our inferred phylogenies do not refute the hypothesis that New Zealand *Amalda* are a natural monophyletic group and therefore an appropriate example of morphological stasis. Furthermore, estimates of the timing of cladogenesis from the molecular data for the New Zealand group are compatible with the fossil record for extant species and consistent with expectations of punctuated equilibrium.

KEYWORDS
Amalda, evolution, mitogenomics, New Zealand

1 | INTRODUCTION

The olive shells (*Amalda* H. & A. Adams, 1853, Ancillariidae) are marine gastropods largely confined to temperate regions (Olson, 1956) with almost 100 species throughout the Pacific and Atlantic oceans. The highest species diversity is in tropical waters with few species in the southern Pacific (Kantor, Fedosov, Puillandre, Bonillo, & Bouchet, 2017). *Amalda* generally live in sandy near-shore environments where they are predators, primarily feeding on bivalves (Powell, 1979). The benthic, soft-sediment habitat of *Amalda* lends itself to high probability of fossilization, and in New Zealand, *Amalda* has a fossil record dating back 45 million years to Eocene fossil beds (Beu & Maxwell, 1990). Of the seven currently recognized extant species in New Zealand waters, four have lineages that extend into the fossil record: *A. mucronata* Sowerby I, 1830, *A. australis* Sowerby I, 1830, *A. depressa* Sowerby II, 1859, and *A. novaezelandiae* Sowerby II, 1859 (Beu & Maxwell, 1990).

New Zealand *Amalda* have featured in debate about models of morphological evolution since they were presented as examples in support of punctuated equilibrium (Gould, 1991, 2009; Michaux, 1989). Fossil forms identified as "species" were inferred to be equivalent to reproductively isolated lineages identified with genetic markers and provided strong evidence of morphological stasis over millions of years (Michaux, 1987, 1989). However, accurate identification of lineages and morphological stasis are both important elements of the punctuated equilibrium theory and to date, no

molecular phylogenetic hypothesis of New Zealand *Amalda* has included species from outside of New Zealand. DNA sequences that are useful for phylogenetics also provide the opportunity to test the age of the most recent common ancestor of New Zealand *Amalda* that is suggested by available fossils (Beu & Maxwell, 1990; https://fred.org.nz/). Our molecular clock analysis avoids circularity of calibrating with the fossils species of interest in this analysis by using a rate estimate derived from a related taxon. Punctuated equilibrium predicts that cladogenesis and morphological change are linked, but paralogous sampling of separate evolutionary lineages could result in misinterpretation of this association in the fossil record. Geologically abrupt morphological evolution associated with lineage splitting needs to be distinguished from alternative ways a new species might appear at a location. Long-distance dispersal is an important component of biogeography (MacArthur & Wilson, 1967), and the abrupt appearance of a new species within a fauna by this means could be misconstrued as rapid in situ change in morphology. This is of special concern in the fossil record where there are no other lines of evidence (Van Bocxlaer, Damme, & Feibel, 2008). Colonization by long-distance dispersal is expected to result in a local fauna containing species diversity that is not monophyletic (Goldberg, Trewick, & Paterson, 2008; Waters & Craw, 2006). Thus, for interpretations of in situ speciation to be valid, the phylogenetic relationships of the set of regionally associated taxa under scrutiny should be examined. The inference of discrete but continuous lineages in a region can be supported if alternative explanations for abrupt change are excluded.

Previous analysis of New Zealand *Amalda* assumed that the species formed a natural group, on the basis of the widely held view that New Zealand's biota is a closed system, biologically isolated by physical isolation of New Zealand (e.g., Bellamy, Springett, Hayden, & Johnson, 1990). However, there is ample recent evidence showing that dispersal is a major contributor to New Zealand biodiversity (e.g., Trewick & Gibb, 2010; Wallis & Trewick, 2009), and molecular clock analysis of Trochidae topshells (Donald, Kennedy, & Spencer, 2005) and Buccinulidae whelks (Vaux, Hills, Marshall, Trewick, & Morgan-Richards, 2017) suggests colonization by dispersal of marine mollusks. In marine systems, the westerly circumpolar current is recognized as a dispersal vector to New Zealand (Beu, Griffin, & Maxwell, 1997; Fleming, 1979), and although the New Zealand *Amalda* species do not occur outside of New Zealand waters, many other olive shell species are recorded around the Pacific and Indian oceans (Kilburn & Bouchet, 1988; Pastorino, 2003). Thus, it is possible that all members of the genus *Amalda* are monophyletic without the New Zealand representatives being more closely related to one another than to New Caledonian or Australian species.

We examined this using two types of genetic marker, with different inheritance patterns; whole mitochondrial genomes and the nuclear rRNA gene cassette. This rich genomic dataset provides the opportunity to study the evolutionary relationships of the New Zealand *Amalda* with respect to selected representatives of worldwide *Amalda* species. We test whether the living New Zealand species that are represented in the fossil record form a monophyletic clade within our sampling and estimate timing of their putative in situ divergence.

2 | MATERIALS AND METHODS

2.1 | Taxon sampling

The global diversity of living *Amalda* exceeds 90 recognized species (plus 30–60 fossil taxa) making a full analysis of all taxa prohibitive as few specimens are preserved in a manner to yield quality DNA. While sampling gaps will always limit phylogenetic inference (Crisp, Trewick, & Cook, 2011), our sampling effort includes a range of species and localities focusing on local coverage of the *Amalda* genus, with representatives of five of the seven nominal subgenera (Table 1; Kantor et al., 2017). The use of subgenera classification within *Amalda* has not been fully tested with molecular data but preliminary analysis suggests genetic distances within the genus are relatively shallow (Kantor et al., 2017) and species descriptions often avoid use of subgenera classification (e.g., Kilburn & Bouchet, 1988). Our analysis emphasized character-rich DNA data to maximize resolution of phylogenetic relationships, and selective taxon sampling directed at maximizing power to falsify our prediction that the New Zealand species from a natural group, and to estimate timing of lineage branching.

Seven extant species are recognized from New Zealand in two subgenera: *Baryspira* Fischer, 1883, (*A. australis* Sowerby I, 1830; *A. bathamae* Dell, 1956; *A. depressa* Sowerby II, 1859; *A. mucronata* Sowerby I, 1830) and *Gracilispira* Olsen, 1956, (*A. benthicola* Dell, 1956; *A. northlandica* Hart, 1995; *A. novaezelandiae* Sowerby II, 1859). Type species of the subgenera are *A. australis* and *A. novaezelandiae*, respectively (Olson, 1956). An eighth species *A. raoulensis* Powell, 1967, is recognized from subtropical Raoul Island which is in New Zealand territorial waters but is >1,000 km northeast of New Zealand. *Amalda* specimens were collected from coastal marine habitat around New Zealand using dredging or hand sampling (Figure 1; Table 1). Specimens collected from Spirits Bay in Northland and near the Three Kings Islands were included as they were considered to be putative new species. Representatives of species not collected in our sampling came from Te Papa Tongawera National Museum of New Zealand (Te Papa; Table 1). In total, specimens used from the New Zealand fauna included 24 *A. australis*, two *A. benthicola*, 23 *A. depressa*, 13 *A. mucronata*, one *A. northlandica*, and two *A. novaezelandiae*. Single specimens of the available New Zealand species, except *A. benthicola*, were used for high-throughput sequencing. No tissue samples of the deep water New Zealand species *A. bathamae* or the Raoul Island species were available for DNA sequencing, and neither is represented in the fossil record.

We note that *Amalda* species show high levels of regional endemicity consistent with the idea of insular faunas, and the living and fossil *Amalda* from New Zealand waters are endemic species (Beu & Maxwell, 1990; Horton et al., 2019). Indeed, New Zealand marine mollusk fauna generally displays high endemicity rates and many endemic cenozoic fossil species (Crampton et al., 2006; Spencer, Marshall, & Crampton, 2009). Thus, the analysis has a Pacific focus,

TABLE 1 Olive shell (*Amalda*) and neogastropod specimens used in molecular phylogenetic analyses of these benthic marine gastropods. Collection locations are indicated and water depth at collection location provided where information is available. Sequences obtained from GenBank do not have location information. Representatives of nominal subgenera within *Amalda*: *Alocospira* (*A. bellonrium*, *A. marginata*); *Pinguispira* (*A. opima*); *Austrancilla* (*A. edithae*); *Baryspira* (*A. australis*, *A. muronata*, *A. depressa*); *and Gracilispira* (*A. novaezelandiae*). Specimens with no voucher codes used in *COI* analysis only were not museum voucher specimens and are currently stored at Massey University. For sequences obtained from GenBank, accession numbers are indicated. Sequence types are mt g, mitochondrial genome; rRNA, rRNA gene cassette; *COI*, mitochondrial cytochrome c oxidase subunit I gene. The alignments containing sequences for all specimens are available via treebase submission 23,923

Species	Location	Number of specimens	Voucher/GenBank	Sequence type	Coordinates	Depth (m)
Alcithoe arabica		1	gi:341605632	mt g		
Alcithoe benthicola		1	gi:341605590	mt g		
Alcithoe flemingi		1	gi:341605597	mt g		
Amalda aureomarginata	Ile des Pins, New Caledonia	1	MNHN-IM-2007-43655	*COI*	22°53′8.412″S; 167°13′4.8″E	420–450
Amalda australis	Doubtless Bay, NZ	10		*COI*	34°54′12.37″S; 173°24′52.18″E	2–25
Amalda australis	Kaipara, NZ	1	M.325908/MN400052	mt g, rRNA	36°32′32.84″S; 174°22′55.17″E	12
Amalda australis	Golden Bay, NZ	1		*COI*	40°44′49.65″S; 172°55′11.22″E	5–40
Amalda australis	Mahia Peninsular, NZ	3		*COI*	39°4′58.65″S; 177°55'52.67″E	<10
Amalda australis	Nelson, NZ	3		*COI*	41°15′18.60″S; 173°17′15.84″E	<6
Amalda australis	Cloudy Bay, NZ	5		*COI*	41°27′16.16″S; 174°4'2.46″E	5
Amalda bellonorium	N Bella, New Caledonia	1	MNHN-IM-2007-33273/MN400056	mt g, rRNA	20°23′39.00″S; 158°45′19.28″E	324–330
Amalda benthicola	Chatham Rise, NZ	2		*COI*	43°28′35.42″S; 179°57′46.14″E	500
Amalda depressa	Tauranga, NZ	1	M.325909/MN400051	mt g, rRNA	37°39′11.80″S; 176°8′56.79″E	<10
Amalda depressa	Mahia Peninsular, NZ	10		*COI*	39°4′58.65″S; 177°55′52.67″E	<10
Amalda depressa	Doubtless Bay, NZ	6		*COI*	34°54′12.37″S; 173°24′52.18″E	2–25
Amalda depressa	Nelson, NZ	6		*COI*	41°15′18.60″S; 173°17′15.84″E	<6
Amalda edithae	Melbourne, Australia	1	M.325910	mt g, rRNA	38°18′49.15″S; 144°43′3.16″E	10
Amalda fuscolingua	Ile des Pins, New Caledonia	1	MNHN-IM-2007-43649/MN400054	mt g, rRNA	22°47′26.41″S; 167°12′20.41″E	390–410
Amalda hilgendorfi	Grand Passage, New Caledonia	1	MNHN-IM-2009-11966	mt g, rRNA	18°1′58.80″S; 163°1′30.58″E	430–438
Amalda hilgendorfi	Ile des Pins, New Caledonia	1	MNHN-IM-2009-11968	*COI*	18°16′16.20″S; 162°54′8.99″E	–
Amalda marginata	Melbourne, Australia	1	M.325911	mt g, rRNA	38°16′23.14″S; 144°39′45.55″E	6
Amalda mucronata	Wellington, NZ	1	M.325914/MN385249	mt g, rRNA	41°21′31.06″S; 174°46′36.80″E	<40
Amalda mucronata	Golden Bay, NZ	12		*COI*	40°44′49.65″S; 172°55′11.22″E	5–40
Amalda northlandica	Spirits Bay, NZ	1	M.289187 gi:27048647	mt g	34°26′43.89″S; 172°49′38.40″E	<5
Amalda novaezelandiae	Tauranga, NZ	1	M.325912/MN400053	mt g, rRNA	37°39′11.80″S; 176°8′56.79″E	<10
Amalda novaezelandiae	Golden Bay, NZ	1		*COI*	40°44′49.65″S; 172°55′11.22″E	5–40

(Continues)

TABLE 1 (Continued)

Species	Location	Number of specimens	Voucher/GenBank	Sequence type	Coordinates	Depth (m)
Amalda optima	Maputo, Mozambique	1	MNHN-IM-2007-38287/MN400055	mt g, rRNA	25°52′41.99″S; 33°6′56.41″E	112–127
Amalda sp	Ile des Pins, New Caledonia	1	MNHN-IM-2009-11963	*COI*	22°49′48″S; 167°16′5.4228″E	–
Amalda sp	Ile des Pins, New Caledonia	1	MNHN-IM-2007-43661	*COI*	22°51′18″S; 167°15′24.012″E	–
Amalda sp	Ile des Pins, New Caledonia	1	MNHN-IM-2007-43662	*COI*	22°51′18″S; 167°15′24.012″E	–
Amalda sp	Ile des Pins, New Caledonia	1	MNHN-IM-2007-43611	*COI*	23°0′11.412″S; 167°16′3E + 1″E	400–420
Amalda sp (Spirits Bay)	Spirits Bay, NZ	1	M.325913/MN400057	mt g, rRNA	34°26′43.89″S; 172°49'38.40″E	<5
Amalda sp (Spirits Bay)	Spirits Bay, NZ	2		*COI*	34°27′6.36″S; 172°47′51.72″E	>5
Amalda sp (Three kings)	Three Kings, NZ	1		*COI*	34°9′50.70″S; 172°7′25.38″E	–
Ancilla cf sum	Bohol Sea, Philippines	1	MNHN-IM-2007-31956	*COI*	9°31′24.00″N; 124°0′36.01″E	602–738
Babylonia areolata		1	gi:311788616	mt g		
Babylonia lani		1	gi:316992410	mt g		
Bolinus brandaris		1	gi:192757907	mt g		
Buccinulum pallidum		1		mt g		
Buccinum pemphigus		1	gi:977902732	mt g		
Buccinum undatum		1		mt g		
Cancellaria cancellata		1	gi:1025815325	mt g		
Cominella adspersa		1	GI:1464312081	mt g		
Concholepas concholepas		1	gi:378925699	mt g		
Conus borgesi		1	gi:192757844	mt g		
Conus consors		1	gi:575525360	mt g		
Conus gloriamaris		1	gi:1025815325	mt g		
Conus textile		1	gi:110809860	mt g		
Conus tribblei		1	gi:924859541	mt g		
Conus tulipa		1	gi:1025726014	mt g		
Cymatium parthenopeum		1	gi:192757872	mt g		
Cymbium olla		1	gi:192757858	mt g		
Fusiturris similis		1	gi:192757830	mt g		
Glaphyrina caudata		1		mt g		
Ilyanassa obsoleta		1	gi:78057821	mt g		
Littorina saxatilis		1	gi:1043378047	mt g		

(Continues)

TABLE 1 (Continued)

Species	Location	Number of specimens	Voucher/GenBank	Sequence type	Coordinates	Depth (m)
Lophiotoma cerithiformis		1	gi:82395857	mt g		
Nassarius reticulatus		1	gi:192757886	mt g		
Olive sp	Kaoka Bay, Solomon Islands	2	MNHN-IM-2007-36204	*COI*	9°40′6.00″S; 160°44′54.013″E	650–725
Oncomelania hupensis hupensis		1		mt g		
Oncomelania hupensis robertsoni		1		mt g		
Penion sulcatus		1	gi:1347479676	mt g		
Potamopyrgus antipodarum		1		mt g		
Potamopyrgus estuarinus		1	gi:517501622	mt g		
Rapana venosa		1	gi:157696072	mt g		
Reishia clavigera		1	gi:161561766	mt g		
Strombus gigas		1		mt g		
Taron dubius		1		mt g		
Terebra dimidiata		1	gi:213390631	mt g		
Varicinassa variciferus		1	gi:702073820	mt g		
Volutharpa perryi		1	gi:937500896	mt g		
Volutopsius norwegicus		1		mt g		

including five species from New Caledonia (where seven species are recognized) and two species from Australia (where 36 species are recognized, 10 of which are present in southern Australia), in addition to the New Zealand fauna.

Species of *Amalda* from outside of New Zealand were obtained from Museum collections. To maximize the chances of sampling species that are part of the New Zealand *Amalda* species clade, we compared preliminary DNA sequences from New Zealand species mitochondrial cytochrome c oxidase subunit I gene (*COI*) with published data (Kantor et al., 2017). This preliminary comparison indicated taxa from New Caledonia, Philippines, Solomon Islands, and Africa as relatively similar to the New Zealand species (Figure S1). Whole specimens of five *Amalda* species, (*A. bellonorium* Kilburn & Bouchet, 1988, *A. fuscolingua* Kilburn & Bouchet, 1988, *A. hilgendorfi* Martens, 1897, *A. optima* Sowerby III, , *A. aureomarginata* Kilburn & Bouchet, 1988), and two out-group species (*Ancilla cf sum* and an olive species) were provided by the Muséum National d'Histoire Naturelle (MNHN), France. Despite 2,000 km wide Tasman Sea preventing ongoing gene flow, some coastal New Zealand gastropods are sister to taxa in southern Australia, (Waters, McCulloch, & Eason, 2007) and we therefore included *Amalda* species from south Australia from which we could obtain suitable tissue samples; *A. marginata* Lamarck, 1811, and *A. edithae* Pritchard & Gatliff, 1899 (provided by Geoff Macaulay; Malacological Society of Australasia). The full mtDNA of *A. northlandica* (see McComish, Hills, Biggs, & Penny, 2010) and mitochondrial genomes of neogastropod mollusks were accessed via GenBank, and specimens that were used for mitochondrial genome construction were accessioned to Te Papa, National Museum of New Zealand (Table 1).

2.2 | DNA isolation

All New Zealand snails were frozen after collection and transferred to the laboratory. Specimens were thawed, snails removed from their shells, and stored in ample 95% ethanol until required. DNA was isolated from ethanol preserved snail foot tissue using either the Geneaid™ column extraction kit or a modified CTAB extraction protocol involving clean up with Ampure™ microbeads as necessary (Doyle & Doyle, 1990). In most cases, the CTAB method produced higher yields of genomic DNA. DNA quantity and quality were assessed using agarose gel electrophoresis and with Qubit™ broad-range DNA assay.

2.3 | Confirmation of species identification

An initial survey of New Zealand *Amalda* specimens was undertaken using analysis of a short fragment of the *COI* to confirm lineage

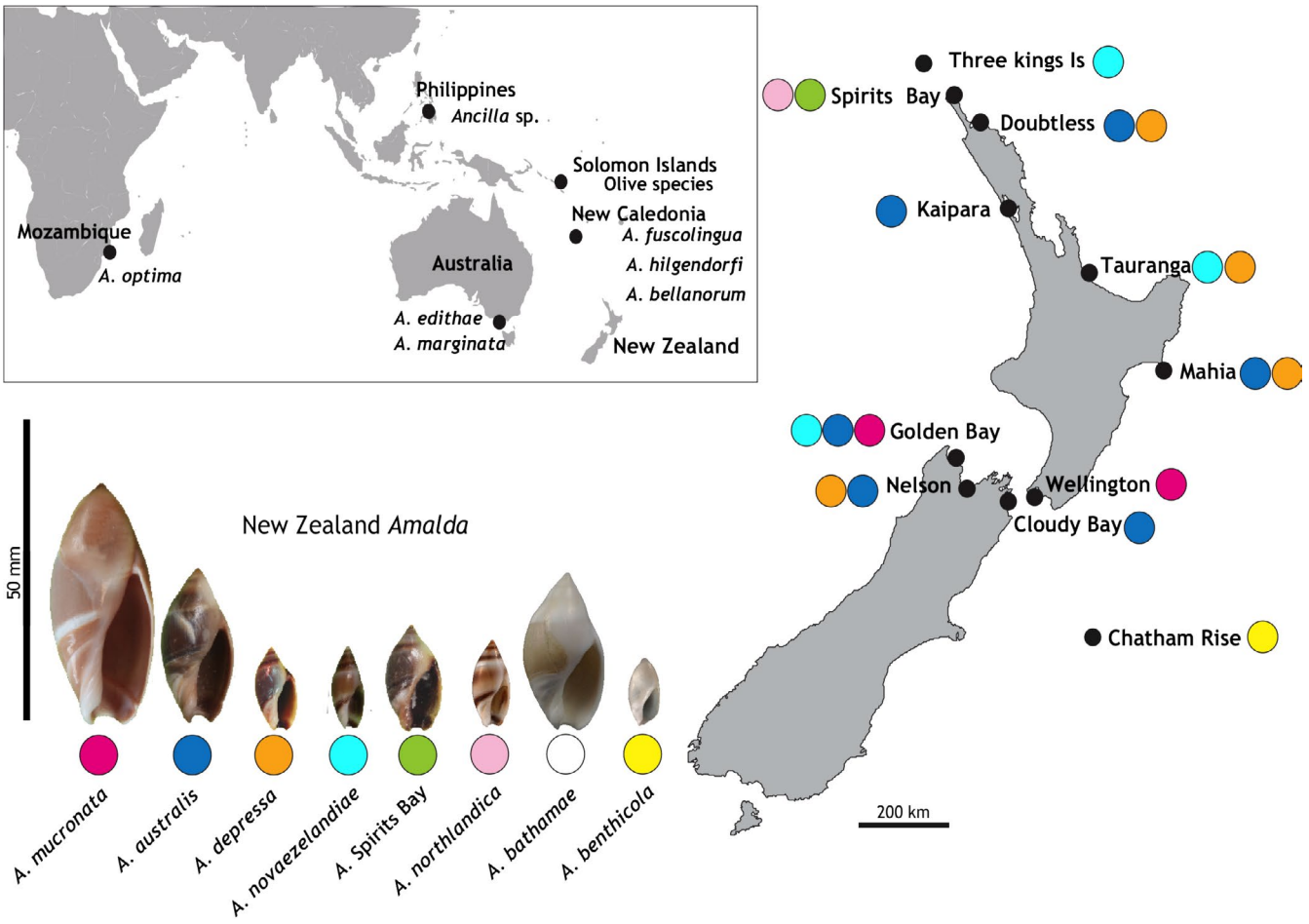

FIGURE 1 Collection locations of marine gastropods in the genus *Amalda* and Olividae out-groups in the western Pacific and Indian oceans. The shells are the New Zealand taxa. Colored spots indicate New Zealand collection locations by species. Locations with multiple colored species spots show that these species are sympatric. In the case of *A. australis* and *A. depressa*, species are syntopic

assignment of multiple individuals. New Zealand species are sympatric (Figure 1), and correct identification of juvenile specimens based on shell shape can be difficult (Michaux, 1987). For this, a 709 bp region of *COI* was amplified by PCR using the primers LCO1490 (GGTCAACAAATCATAAAGATATTGG) and HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA; Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994), sequenced from the HCO2198 end, and trimmed to 585 bp to ensure sequences of equal length. Phylogenetic analysis was inferred from the alignment of *COI* using MrBayes (chain length 1,000,000 burn-in 10,000). Results assisted the selection of samples for high-throughput (next-generation) DNA sequencing and provided preliminary evidence of evolutionary relationships of species that were not suitable for high-throughput sequencing.

2.4 | High-throughput sequencing

Eleven samples representing five endemic New Zealand species and six species from elsewhere (Australia, New Caledonia, and Mozambique) were selected for sequencing using the ThruPLEX DNA-seq Kit (Rubicon Genomics). Library preparation for high-throughput sequencing was done following the protocol outlined in Vaux et al. (2017). Whole mitochondrial genomes were assembled by mapping reads to the published annotated *Amalda northlandica* mitochondrial genome (GenBank accession no. GU196685.1). The nuclear ribosomal gene cassettes (*18S*, *ITS1*, *5.8S*, *ITS2*, *28S*) were constructed by mapping paired reads to a reference annotated molluskan ribosomal sequence, following the method of Vaux et al. (2017).

2.5 | Phylogenetic reconstruction

Mitochondrial genomes and the rRNA gene cassette sequences were aligned using Geneious 9.1.3 using the default settings on the Geneious alignment tool. Alignments were checked by eye for anomalies and translated to identify start and stop codons and ensure genes were in agreement with the correct reading frame. DNA sequence alignments can be found on the open access repository of phylogenetic data TreeBASE (https://www.treebase.org/treebase-web; submission 23,923).

We aligned our new mtDNA genomes with those from other marine snails (eight Littorinimorpha and 43 Neogastropoda), encompassing 12 families and five neogastropod superfamilies (Table 1).

We inferred a phylogeny using protein-coding genes from these mitochondrial genomes to provide a broader context of neogastropod diversity and the positioning of *Amalda* as representatives of Olividae. Phylogenetic reconstruction was carried out using Bayesian inference with MrBayes (Ronquist et al., 2012; Geneious 9 plugin), 10,000,000 MCMC iterations and a burn-in length of 2,000,000.

Complete mitochondrial genome and rRNA gene cassette sequence alignments were used to separately infer phylogenetic relationships for the members of the *Amalda* clade. Molecular phylogenies were estimated using Bayesian MCMC inference in MrBayes 3.2 (Ronquist et al., 2012) with 10,000,000 iterations and a burn-in length of 2,000,000. Maximum-likelihood inferences were performed using RAxML, with the same gene partitions. A rapid hill-climbing algorithm was implemented with 500 bootstrap replicates. Phylogenies were rooted with *Amalda bellonarum* based on preliminary findings from the richer taxon datasets. A Bayesian phylogeny of the mitochondrial genomes rooted with *Babylonia areolata* was also constructed. We consider a clade well supported if posterior probabilities are >0.97 and bootstrap resampling score >85% (Cummings et al., 2003).

To infer approximate time of cladogenesis, we implemented a molecular clock analysis in BEAST 2.5.0 (Bouckaert et al., 2014), using a concatenated mtDNA sequence alignment from nine *Amalda* species. An uncorrelated lognormal relaxed clock prior was calibrated with an mean rate of 0.0075 substitutions per site per million years, inferred for related New Zealand marine snails (Volutes, *Alcithoe*; Hills, Crampton, Trewick, & Morgan-Richards, 2012). We did not incorporate *Amalda* fossil calibrations into our molecular clock analysis so that we could test whether current fossil material provides reliable estimates of the age of origin of New Zealand *Amalda* species. The DNA substitution rate used to calibrate this molecular clock analysis was inferred using fossil calibrations of both extant species and a common ancestor in a study of a single marine snail genus (*Alcithoe*) using sequences of two-thirds of the mtDNA genome (Hills et al., 2012). Thus, the DNA substitution rate we used is based on an analysis of related gastropods with a similar depth of divergence as this study. The DNA substitution rate used here is within the range commonly cited for interspecific invertebrate mitochondrial evolution including marine gastropods (Knowlton & Weigt, 1998; Lynch & Jarrell, 1993; Ozawa & Okamoto, 1993; Papadopoulou, Anastasiou, & Vogler, 2010; Reid, Rumbak, & Thomas, 1996). A GTR+I+G nucleotide substitution model was used, and the birth–death model was applied for tree prior. Unless stated otherwise, default parameters were used. An MCMC chain length of 10,000,000 was used, sampling every 1,000.

3 | RESULTS

3.1 | Sample selection and *Amalda* taxonomy

The 585 bp region of *COI* aligned from 61 individuals of the New Zealand *Amalda* had 79.7% identical sites and showed no evidence of ambiguities, indels, or stop codons that are indicative of nuclear copies. Preliminary phylogenetic relationships were inferred using partial *COI* sequences from 77 specimens of *Amalda* and related species (Figure S1). The level of sequence divergence and the clustering of *COI* haplotypes support current species-level taxonomy; however, the single haplotype from *A. northlandica* and the unidentified specimens from Spirits Bay, New Zealand, grouped within the *A. depressa* haplotype cluster. Although the tissue we had from the New Zealand species *A. benthicola* was not suitable for high-throughput sequencing, we did successfully amplify the *COI* fragment from one specimen and this short sequence suggests it is part of the New Zealand *Amalda* clade, sister to *A. mucronata* (Figure S1).

TABLE 2 Summary of olive shell (*Amalda*) DNA sequence statistics for new mitochondrial genomes and rRNA gene cassette (*45S*) sequenced and assemble for this study

		mt genome				*45S*			
	Reads	Reads mapped	Mean coverage	% missing	Length	Reads mapped	Mean coverage	% missing	*45S* length
A. australis	33,306,422	7,657	47.5	0	15,403	13,489	187.0	0	5,984
A. bellonorium	4,399,343	1,491	11.2	0	15,249	2,954	45.9	0	5,820
A. depressa	77,366,938	5,864	18.6	0.4	15,347	18,079	113.9	0	5,809
A. edithae	16,977,846	2,664	17.1	0	15,332	16,676	31.0	0	5,998
A. fuscolingua	3,065,376	511	4.1	0.7	15,357	13,361	4.1	0	5,813
A. hilgendorfi	4,135,126	343	3.7	23.7	14,354	3,195	41.1	0	5,950
A. marginata	15,583,022	2,765	18.5	0	15,072	2,214	134.8	0	6,083
A. mucronata	28,217,402	4,698	30.5	0	15,393	34,009	279.2	0	5,963
A. novaezelandiae	79,893,938	5,099	15.1	0	15,331	55,096	425.4	0	5,839
A. optima	9,166,880	1,158	9.4	0	15,339	4,562	67.4	0	5,974
A. sp (Spirits Bay)	20,558,288	1538	9.9	11	15,358	7,479	124.7	0	6,010

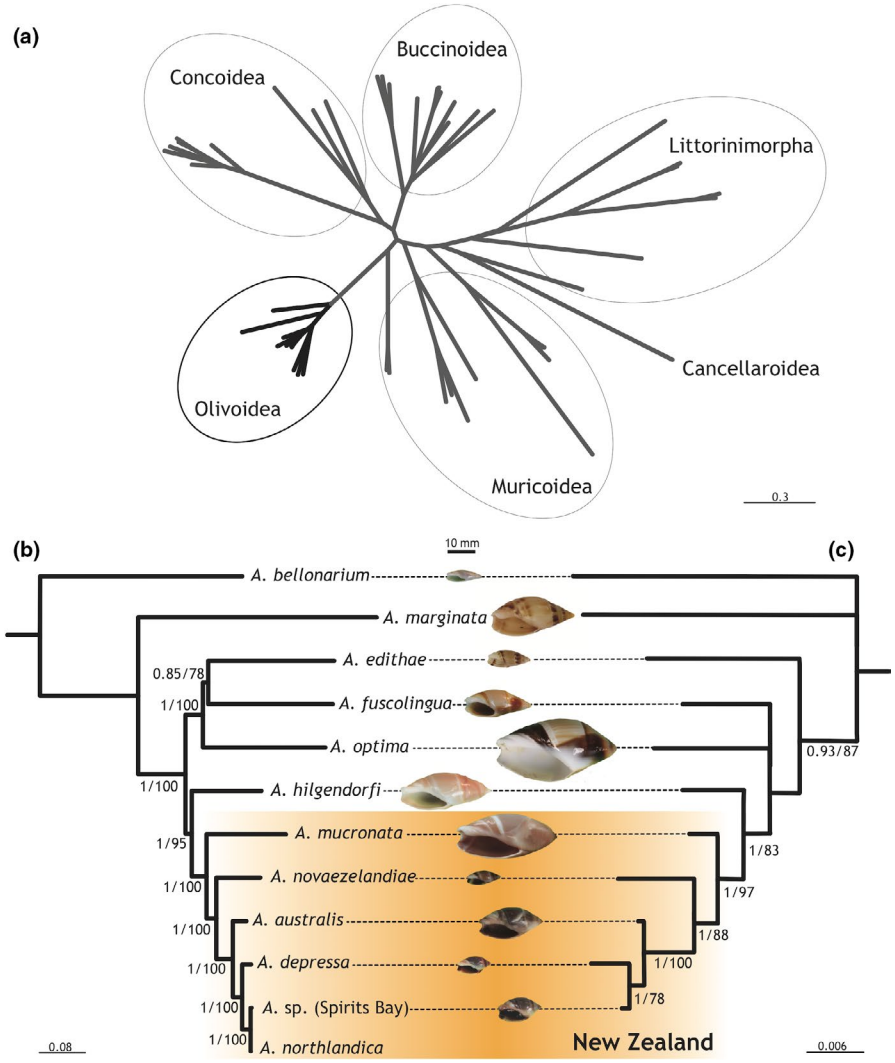

FIGURE 2 (a) Evolutionary relationships of marine snail superfamilies inferred from mitochondrial DNA sequences (unrooted Bayesian tree of 48 available Neogastropods and six Littorinomorphs using 10,215 bp of concatenated protein-coding genes). All posterior probabilities were 1 except for a few nodes within family or genus groups. Representatives of the superfamily Muricoidea appear in three clusters. Two *Babylonia* species might be sister to the Olivoidea represented here (depending on where the root is). (b+c) Monophyly of New Zealand olive shell species inferred from mitochondrial (b) and nuclear DNA (c) sequences. Phylogenies of *Amalda* based on analysis of whole mitochondrial genome sequences (15,300 bp; left) and nuclear rRNA gene cassette sequences (~6,000 bp; right). Likelihood scores from RAxML and Bayesian posterior probabilities from MrBayes are shown. Representative shells of each species are shown to the same scale. The clade of New Zealand *Amalda* is highlighted in orange

3.2 | Mitochondrial genomes and rDNA sequences

New mitochondrial genomes were assembled from 11 *Amalda* samples using high-throughput sequencing. Mitochondrial DNA sequence length ranged from 15,249 to 15,404 bp (Table 2). Average coverage for the samples ranged from 67.4 reads per site in *Amalda optima* to 3.9 in *A. hilgendorfi*. For four other *Amalda* species, short regions of low coverage resulted in incomplete genomes (Table 2). The mitochondrial genomes of these 11 *Amalda* species contain many (36.8%) variable sites, providing information for inferring evolutionary relationships. Phylogenetic analysis of 10,282 bp of concatenated mitochondrial protein-coding genes indicates that four of the superfamilies represented form monophyletic clades while Muricoidea is paraphyletic. The Olivoidea are here represented solely by specimens from the *Amalda* genus and are most closely related to the Muricoidea (Figure 2).

The full DNA sequences of nuclear rRNA gene cassette were assembled from each of the 11 specimens (mean length 5,971 bp; Table 2). *ITS1* ranged in size from 510 to 556 bp, and *ITS2* ranged from 124 to 338 bp. The rRNA gene cassette sequences were much more conserved than the mitochondrial genome, varying among species by no more than 4.1%.

3.3 | New Zealand *Amalda* relationships

Phylogenetic analysis of mtDNA genomes and nuclear rRNA gene cassettes produced similar topologies; both resolved monophyly of the six New Zealand taxa with respect to the other *Amalda* species sampled (Figure 2). There was some discordance between the mitochondrial genome tree and the nuclear ribosomal DNA tree in the placement of species outside the New Zealand clade. The placement of *A. optima*, *A. edithae*, and *A. fuscolingua* varied; either as a separate clade (mtDNA) or *A. optima* and *A. fuscolingua* sisters to the clade containing *A. hilgendorfi* and the New Zealand *Amalda*. However, from both mtDNA and the nuclear markers, maximum support possible was observed for the clade containing all New Zealand species sampled including the species represented in the fossil record (posterior probability = 1, bootstrap = 100). However, within the New Zealand clade, the species that are recognized in the fossil record were not monophyletic. In both these phylogenies, the New Caledonian species *A. hilgendorfi* was sister to the monophyletic New Zealand clade. The longer and more variable mitochondrial DNA sequences provided better resolution of phylogenetic relationships than the shorter more conservative nuclear marker (i.e., one

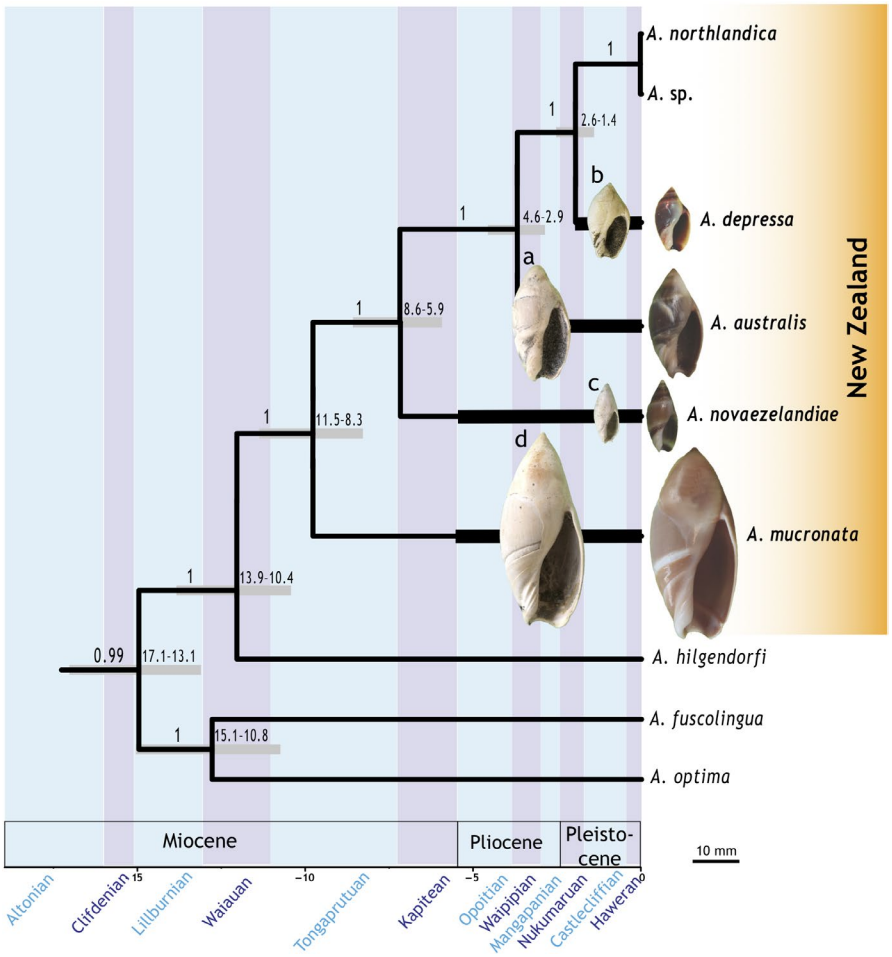

FIGURE 3 Estimated divergence times of New Zealand *Amalda* lineages. Bayesian calibrated mtDNA phylogeny of New Zealand olive shells based on an alignment of nine concatenated mitochondrial genomes (10,282 bp of protein-coding genes) calibrated with marine gastropod DNA substitution rate. 95% highest posterior density intervals are shown for each lineage divergence. Fossil evidence for four living species is illustrated by thickened branches and images of shells representing both fossil and recent specimens. Approximate durations of New Zealand geological stages indicate age of photographed fossil specimens. The tree was rooted with *Amalda bellonarum* based on our analyses with richer taxon datasets

node had low posterior probability (<0.97) in the mitogenomic tree cf. four nodes with low support in the rRNA gene cassette tree). When the phylogeny was rooted with *Babylonia areolata*, topology and support within *Amalda* remained the same.

We used a DNA substitution rate inferred for a genus of related marine snails (*Alcithoe*; Hills et al., 2012) to estimate timing of cladogenesis of the extant New Zealand *Amalda* species. The most recent common ancestor of the New Zealand clade is estimated to have lived 11.5–8 Mya, significantly before the oldest recorded *A. mucronata* fossil (5.3 Mya; Beu & Maxwell, 1990), and consistent with this lineage including the extant New Zealand species *A. benthicola*. The most recent common ancestor of *A. novaezelandiae*, *A. australis*, *A. depressa*, and *A. northlandica* we estimated to be 8.6–5.9 Mya, slightly pre-dating the currently recognized earliest fossil of *A. novaezelandiae* at 5.3 Mya (Beu & Maxwell, 1990). The earliest fossil currently recognized as *A. australis* (2.4 Mya; Beu & Maxwell, 1990) also is slightly younger than our estimate of the most recent common ancestor of *A. australis*, *A. depressa*, and *A. northlandica* (4.6–2.8 Mya). Thus, most of our estimates of the most recent common ancestor occur just before species appear within the New Zealand fossil record (see electronic database; https://fred.org.nz/) and before morphological stasis is documented (Michaux, 1989). The first appearance of *A. depressa* in the fossil record (2.4 Mya; Beu & Maxwell, 1990; Beu & Raine, 2009) is also compatible with the most recent common ancestor of *A. depressa* and *A. northlandica* 2.6–1.44 Mya. Thus, our molecular clock estimates of lineage age are all compatible with the available fossil record of these extant species (Figure 3).

4 | DISCUSSION

Four species of New Zealand *Amalda* (*A. australis*, *A. depressa*, *A. novaezelandiae*, and *A. mucronata*) have previously been examined for concordance of taxonomy (based on shell size, shape, and color) and genetic variation (Michaux, 1987). Combinations of these marine snail species occur in sympatry. For example, in Doubtless Bay, *A. australis and A. depressa* were collected from the sea floor in 2–25 m of water, and in Golden Bay, *A. australis*, *A. novaezelandiae*, and *A. mucronata* were all collected in 5–40 m of water (Table 1), and there are no known differences among the species in their microhabitats or diet (Michaux, 1987). With sympatric species, fixed differences at nuclear loci provide strong evidence of reproductive isolation and support for four taxa (Michaux, 1989). Phylogenies constructed from genomic data and haplotype data are consistent with the current species taxonomy. Three of these New Zealand *Amalda* species are abundant in the fossil record and provide evidence of morphological stasis (Michaux, 1989). Our molecular phylogenies of *Amalda* did not resolve the two New Zealand subgenera of *Gracilispira* and

Baryspira as currently recognized within this genus. The type species of *Gracilispira* has a smaller and narrower shell than *Baryspira*, which although larger has a relatively short spire and wide aperture (Beu & Raine, 2009). *Amalda novaezelandiae* (*Gracilispira*) is nested within the New Zealand group as sister to the clade of *A. australis* and *A. depressa* (both *Baryspira*). The short *COI* sequence suggests *A. benthicola* (*Gracilispira*) is sister to *A. mucronata* (*Baryspira*), and together both are sister to other New Zealand *Amalda* species. Given that olive shells have few diagnostic traits in general (Kantor et al., 2017), it is perhaps not surprising that we found no support for the current subgenera classification. Classification of *Amalda* species into subgenera (subclades) is not stable (Kantor et al., 2017; World Register of Marine species (WoRMS) Horton et al., 2019). Our sampling included representatives of five of the seven putative subgenera although there is disagreement as to whether *Amalda optima* is within *Pinguispira* (Kantor et al., 2017) or *Baryspira* (WoRMS). Paraphyly of subgenera *Alocospira*, *Baryspira*, and *Gracilispira* in our molecular phylogenies, despite few taxa, suggest these subgeneric classifications do not represent evolutionary relationships within *Amalda*, and therefore, subgeneric designations may have little value within *Amalda*.

Fewer than 50 complete mitochondrial genomes of the Neogastropoda have been published to date. Only six of the seven extant superfamilies have full mitogenomic sequences available for any of their members. The Neogastropoda, long considered a natural group based on morphological characters, was not supported by phylogenetic analysis which suggests the inclusion of littorinomorph species within the clade (Cunha, Grande, & Zardoya, 2009; Kantor et al., 2017; Osca, Templado, & Zardoya, 2015). Our mitogenomic tree is consistent with this due to the placement of the Littorinimorpha specimen *Monoplex parthenopeum* (formerly *Cymatium parthenopeus*) within the Neogastropoda. Recent studies have included data from *Amalda* (Fedosov, Puillandre, Kantor, & Bouchet, 2015; Kantor et al., 2017) and indicate that the genus is a monophyletic clade within the Ancillariidae. Olivoidea are represented only by the genus *Amalda* in our mitogenomic phylogeny and here are sister to the *Babylonia* (Muricoidea).

In the context of our sampling, DNA sequence from nuclear and mitochondrial genes from *Amalda* species provides support for the hypothesis that the New Zealand *Amalda* lineage evolved in isolation. We were able to include only a fraction of the species diversity of the genus but found close relatives in New Caledonia. Of the more than 34 million tree topologies possible with the current taxon sampling, only 0.03% would resolve New Zealand monophyly by chance. Further sampling of *Amalda* taxa might identify species that have dispersed from New Zealand, but the shallow diversity within the New Zealand fauna and monophyly inferred with this subset of species is consistent with the regional endemicity and evidence of a single extant evolutionary lineage (Daugherty, Gibbs, & Hitchmough, 1993; Spencer et al., 2009). The closest relative of the New Zealand species in our dataset is *A. hilgendorfi* from New Caledonia, a species that in terms of size, color and shape is more similar to New Zealand taxa than any of the other species sampled. Currently available information on the New Zealand *Amalda* species is consistent with the hypothesis of diversification of a single shallow local lineage and does not indicate multiple dispersal events.

We cannot yet refute that the extant *Amalda* species that occur as fossils are part of a monophyletic New Zealand clade. It is possible that morphological changes detected in the New Zealand fossil record are the result of evolutionary events in situ rather than the result of the arrival of a new species from elsewhere in the Pacific. Our estimates of the age of extant New Zealand *Amalda* taxa did not incorporate New Zealand fossils to calibrate the molecular clock tree, thus allowing us to determine that our molecular phylogeny with a relaxed rate of DNA substitution is compatible with the earliest known dates for the origin of these species. The most recent common ancestor of the extant New Zealand diversity was estimated to be no more than about 12 million years old, suggesting a Miocene arrive in New Zealand waters of the current lineage. New phenotypes may well result from evolution within this local lineage. Our inferred age of each New Zealand *Amalda* lineage does not invalidate inferences of stasis for the three taxa *A. australis*, *A. depressa*, *and A. mucronata* (Michaux, 1989). Our molecular clock estimates of the age of the most recent common ancestors within the New Zealand clade are almost all slightly before the first fossil record of the associated extant species, suggesting morphological stasis is independent of cladogenesis, and thus, we can continue to regard these species as some of the best examples in the literature that support punctuated equilibrium (Gould, 1991).

ACKNOWLEDGEMENTS

Snail specimens were provided by New Zealand's National Institute of Water and Atmospheric Research, Cloudy Bay Clams, Te Papa Tongawera National Museum of New Zealand, the Muséum National d'Histoire Naturelle (MNHN), France, Geoff Macaulay (Malacological Society of Australasia). Assistance with dredging was provided by Leigh (Wildcat Charters) in Golden Bay, Phil Green and Wilma Datema in Northland, and Felix Vaux. Alan Beu and Bruce Marshall provided expert species identification and discussions. Gillian Gibb helped with phylogenetic software. Members of the Phoenix Laboratory (evolves.massey.ac.nz) provided helpful discussions. The manuscript benefited from very constructive comments and suggestions from Julia Sigwart and two anonymous reviewers. This work was supported by the Royal Society of New Zealand Te Apārangi Marsden Fund grant (12-MAU-008) and a Ministry of Business, Innovation and Employment Te Tipu Pūtaiao Postdoctoral Fellowship (CONT-22922-TTP-MAU).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Preliminary phylogeny of Olive shell species (*Amalda*) inferred from an alignment of 585 bp of the mitochondrial *cox1* from 83 specimens. *indicates samples selected for whole mitochondrial genome and nuclear 45S analysis. Outgroups are a Sumatran *Ancilla* species and an unidentified olive shell species. Where more than one specimen from a species is included in analysis species clusters have been coloured for clarity. Bayesian posterior probability indicated where less than 1. (MrBayes Chain length 1,000,000 burn-in 10,000 within species posterior probability omitted). The positioning of the *A. northlandica* specimen within the *A. depressa* cluster requires further study as it may represent a misidentified colourmorph of *A. depressa*. Likewise the unidentified specimen from Spirits Bay New Zealand is likely to be a colourmorph of *A. depressa*. The specimen from Three Kings clusters with other *A. novaezelandiae* specimens.

How to cite this article: Gemmell MR, Trewick SA, Hills SFK, Morgan-Richards M. Phylogenetic topology and timing of New Zealand olive shells are consistent with punctuated equilibrium. *J Zool Syst Evol Res*. 2019;00:1–12. https://doi.org/10.1111/jzs.12342