Molluscan Research 31(3): 141–151
http://www.mapress.com/mr/

ISSN 1323-5818
Magnolia Press

Molecular analysis of adults and egg masses reveals two independent lineages within the infaunal gastropod *Naticarius onca* (Röding, 1798) (Caenogastropoda: Naticidae)

THOMAS HUELSKEN[1*], HEIKE WÄGELE[2], BJÖRN PETERS[3], ANDREW MATHER[1] & MICHAEL HOLLMANN[3]

[1] School of Biological Sciences, The University of Queensland, Qld 4072 Brisbane, Australia
[2] Zoologisches Forschungsmuseum Alexander Koenig, Adenauerallee 160, 53113 Bonn
[3] Dept. Biochemistry I - Receptor Biochemistry, Ruhr University Bochum, Universitaetsstrasse 150, 44780 Bochum, Germany
*Corresponding author—Email: t.huelsken@gmail.com

Abstract

We analysed sympatrically occurring specimens from Lizard Island, Queensland, Australia identified on shell characters as *Naticarius onca* (Röding, 1798) and found them molecularly separated into two distinct clades. Additionally, we obtained sequences from nine morphologically similar, randomly collected naticid egg masses ('sand collars') from Lizard Island and two other Queensland localities. Eight out of the nine egg masses unambiguously grouped with one of the two *N. onca* clades. The two clades show no common haplotypes, resulting in a sequence divergence of 11.1% in the COI gene fragment, and 4.0% in the 16S gene fragment, while the intraspecific variability within the two taxa was 1.0–2.4% and 0.2–0.4% for COI and 16S, respectively. The adult shells of members of the two clades from Lizard Island are morphologically indistinguishable, showing overlapping intraspecific shell variability in colour pattern, shape, and protoconch morphology. Thus, *N. onca* contains at least two independent lineages that probably represent distinct species. One of the egg masses from Lizard Island, which is morphologically similar to *N. onca*, proved to be assigned to the rare *N. concinnus*, the first documented evidence of this species from the Great Barrier Reef. Our results illustrate the usefulness of egg masses in barcoding approaches and taxonomic assignments.

Key words: barcoding, protoconch, cryptic species, COI, mitochondrial DNA, Gastropoda, systematics

Introduction

The development of molecular techniques has substantially increased our capability to identify species and to discover sibling or cryptic species complexes (e.g., Knowlton 1993; Simison and Lindberg 1999; Collin 2000; Hebert *et al.* 2003; Lee and O´Foighil 2005). However, there has been little molecular work on burrowing, sand-associated gastropods because they are often difficult to collect. Consequently, these species are underrepresented in museum collections, with voucher specimens predominantly based on empty shells or fixed in formalin or weak ethanol and thus, such vouchers are inadequate for standard molecular analyses.

Here, we examine specimens assigned to the infaunal naticid species *Naticarius onca* (Röding, 1798) based on shell morphology. Röding´s definition of *Cochlis onca* was based on one of the perceived colour variants of "Pavimentum chinense" figured in Chemnitz (*non-binomial*, 1781, in Martini and Chemnitz 1769-1829; see Melville and Smith 1987). Röding restricted his *Cochlis onca* to only one of three specimens figured (plate 187, Figs 1887–1888; our Figs 1A, 1B), leaving aside the two differently coloured specimens figured on plate 187, Figs 1889–1891 (our Figs 1C, 1D). Based on the differences in their colour patterns, Röding believed the specimen of "Pavimentum chinense" on Chemnitz's plate 187, Figs 1889–1890, to represent a different species, which he named *Cochlis pavimentum* (Figs 1F, 1G). Röding did not assign a name to the third specimen on plate 187, fig. 1891 (our Fig. 1E), which he presumably also considered to be different as he did not include it in his definition of *Cochlis onca* (Röding 1798). Apparent similarity of the shell characters between the taxa, together with the observed variability in colouration of what was considered to be *N. onca*, hampered species separation and even led to subsequent proposals of synonymy of *N. pavimentum* with *N. onca* (e.g., Kabat 2000).

Röding's perception that the colour variants of "Pavimentum chinense" represent more than one species illustrates the taxonomic complexity of *N. onca*. Despite this, his assumption remained untested for more than 200 years. In this preliminary study, we used specimens and egg masses live-collected from three localities along the Great Barrier Reef, Australia, to test the monophyly of *N. onca.* Egg masses represent an alternative source of genetic data, and are particularly useful in difficult to collect and irregularly distributed infaunal naticids (Huelsken *et al.* 2008). While species identification of gastropod egg masses previously relied on serendipitous observations of individual specimens in aquaria or in the field (e.g., Puillandre *et al.* 2009), the advent of molecular barcoding (Hebert *et al.* 2003) now allows the assignment of egg masses to a species without direct observation of egg laying.

Materials and Methods

Material examined

Eleven naticid specimens and nine egg masses were collected at Lizard Island, Dingo Beach, and Pioneer Bay (Queensland, Australia). Additional specimens from other localities were provided by colleagues (Fig. 2; Table 1). The data are supplemented by sequence data from the only two Mediterranean *Naticarius* Duméril, 1806 species (*N. hebraeus* and *N. stercusmuscarum* from GenBank; Huelsken *et al.* 2008). We used sequences of *Cypraea annulus* (Linnaeus, 1758) [Cypraeidae; GenBank accession Nos.

COPYRIGHT © 2011 MALACOLOGICAL SOCIETY OF AUSTRALASIA & SOCIETY FOR THE STUDY OF MOLLUSCAN DIVERSITY

AY296832, DQ916532, AF033681] and *Bostrycapulus pritzkeri* (Collin, 2005) as outgroups [Calyptraeidae; GenBank accession Nos. AY061793, AY061767, DQ916531, DQ916451] (Colgan *et al.* 2007). Color photographs of all specimens used in the molecular analyses can be found via open access under the Morphobank (O'Leary and Kaufman 2007) projects 189 and 224 (http://morphobank.geongrid.org). From the 15 specimens analysed in this study 12 have been vouchered to the Muséum National d'Histoire Naturelle, Paris (MNHN), with two specimens vouchered in the Queensland Museum, Brisbane (QM) and one specimen vouchered in the Australian Museum, Sydney (AMS); for MNHN, QM, and AMS catalog numbers see Table 1. Sequences of all specimens analysed were uploaded to NCBI (accession numbers FJ384564-FJ384616, FJ469930-FJ469931). Protoconchs were measured as described by Solsona and Martinell (1999) and photographed using a digital binocular microscope. Height (h) and width (w) of shells were measured using callipers, and the ratio was calculated for each specimen (r = h/w) as described in Huelsken *et al.* (2006).

TABLE 1. Specimen identification numbers, collection sites and depths (in m), museum voucher numbers, Morphobank voucher numbers, and GenBank accession numbers of the *Naticarius* species analysed. Abbreviations: AMS—Australian Museum, Sydney; MNHN—Muséum National d'Histoire Naturelle, Paris; QM—Queensland Museum, Brisbane.

Species, author	Specimen ID, collection site	Morphobank Nos.	Voucher number
***Naticarius hebraeus* (Martyn, 1786)**	#27-1, #27-3 Campese Bay, Isola del Giglio, Italy, 6 m	M14567, M14583 - M14585	MNHN IM-2009-5588
***Naticarius stercusmuscarum* (Gmelin, 1791)**	#40-14 Campese Bay, Isola del Giglio, Italy, 6 m	M14574, M14586 - M14588	MNHN IM-2009-5589
***Naticarius concinnus* (Dunker, 1860)**	#82-1 Shark Bay, Western Australia, Australia, 2 m	M24876 - M24879, M24984	MNHN IM-2009-5590
***Naticarius onca* (Röding, 1798)**	#61-1, #61-2, #61-3, #61-4, #61-6, #61-7, #61-9 Casuarina Beach, Lizard Island, Queensland, Australia, 0.5-2 m	M24880 - M24891 M24900 - M24907 M24912 - M24915	MNHN IM-2009-5591 to -5593 MNHN IM-2009-5596 to -5597 MNHN IM-2009-5600
	#61-7, #61-8 Casuarina Beach, Lizard Island, Queensland, Australia, 0.5-2 m	M24896 - M24899 M24908 - M24911	QM MO80161, QM MO80162
	#61-5 Dingo Beach, Queensland, Australia, at low tide, 0 m	M24892 - M24895	MNHN IM-2009-5594
***Naticarius sertatus* (Menke, 1843)**	#79-1 Shark Bay, Western Australia, Australia, 5-8 m	M24918 - M24921, M24986	MNHN IM-2009-5599
	#79-2 Duke Group, Marble Island, Great Barrier Reef, Queensland, Australia	M24922 - M24926	AMS C.419737
***Naticarius zonalis* (Récluz, 1850)**	#60-1 Casuarina Beach, Lizard Island, Queensland, Australia, 2 m	M24927 - M24930, M24983	MNHN IM-2009-5600
Egg masses	L17, L24, L43, L54, L58 Casuarina Beach, Lizard Island, Australia, 0.5 m		
	TH190 Dingo Beach, Dingo Beach, Australia, at low tide, 0 m		
	TH191 Dingo Beach, Dingo Beach, Australia, at low tide, 0 m		
	TH192 Pioneer Bay, near Airlie Beach, Australia, at low tide, 0 m		
	TH205 Dingo Beach, Dingo Beach, Australia, at low tide, 0 m		

Nucleic acid isolation and sequence analysis

Total DNA was extracted from ethanol-preserved tissue using a modified protocol of the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as described in Huelsken *et al.* (2011) and stored in 0.1 mM Tris-EDTA pH 7.4. Amplification reactions using *iProof* polymerase (Bio-Rad Laboratories, Munich, Germany) were performed in MJ Research thermocyclers (Watertown, MA, USA). Primers used for partial amplification of CO1, 16S rRNA, 18S rRNA and H3 genes were taken from a previous study by Huelsken *et al.* (2008). The PCR products were purified using the JETSORB Gel Extraction Kit (Genomed, Löhne, Germany), and both strands were sequenced on an ABI 3130xl automated sequencer using the PCR primers and a BigDye Terminator v3.1 sequencing kit (both Applied Biosystems, Foster City, CA, USA). Amplification of the gene fragments resulted in alignments of the following lengths: COI (450 bp), 16S rRNA (489 bp), H3 (264 bp), 18S rRNA (407 bp). Egg mass

identification has been performed based on phylogenetic analyses of the COI gene fragments.

The best phylogenetic models for each gene fragment were calculated using *MrModeltest* 2.2 (Nylander 2004). Phylogenetic trees (Fig. 3) were calculated applying Bayesian inference, Distance analyses, and Maximum Parsimony by using the software packages *MrBayes* (Ronquist and Huelsenbeck 2003) and *PAUP*4.0b10* (Swofford 2003), respectively. NeighborNet analysis (Fig. 4) was performed using *SplitsTree v4.8* with the logdet model (Huson 1998; Huson and Bryant 2006). For the Bayesian analysis 5 x 10^6 generations were calculated, while Bootstrap analyses with 1000 replicates were performed for Maximum Parsimony and split-decomposition analysis. Data set analyses, uncorrected pairwise distance (p), and statistical tests were performed using *Paup*4.0b10* (Swofford 2003). To determine inter- and intraspecific variability of the genetic data, the fixation index (F_{ST}) and haplotype diversity (Hd) were calculated using the program DnaSP (Librado and Rozas 2009). The F_{ST} is a measure of population differentiation and genetic distance, based on genetic polymorphism data (COI haplotypes).

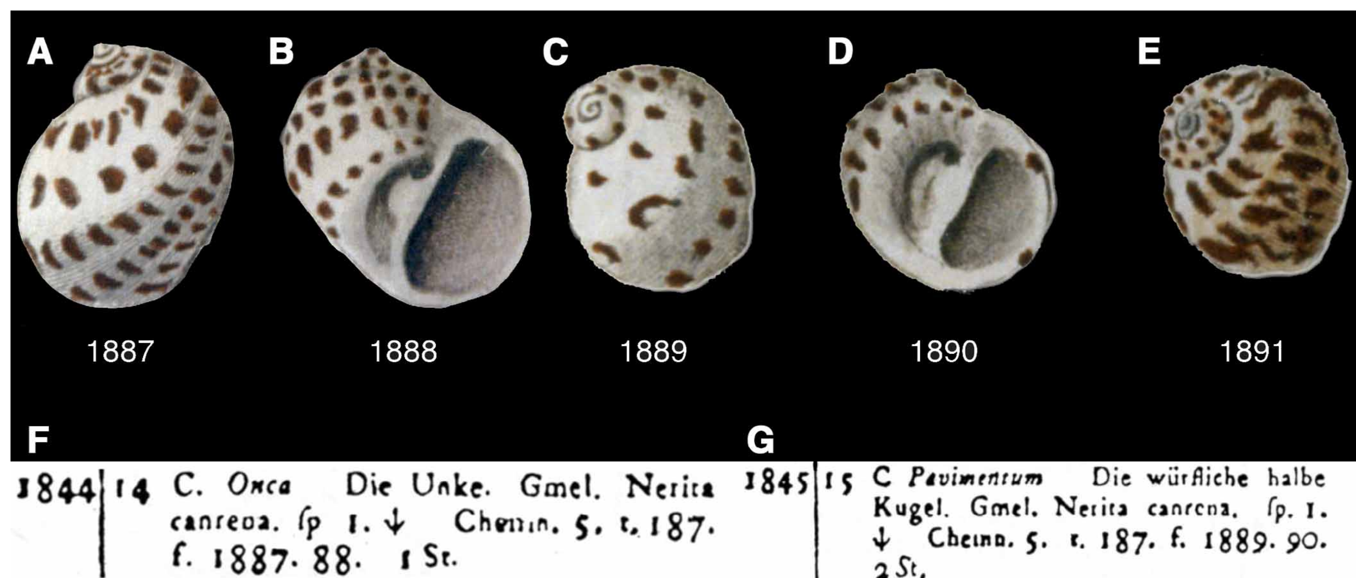

FIGURE 1. A–E. Type figures of "Pavimentum chinense" (*non-binomial*, Chemnitz 1781, in Martini and Chemnitz, 1769–1829). A, B, type figures referenced by Röding, 1798, in his original description of *Cochlis onca* (No. 1887, 1888). C, D, type figures referenced by Röding, 1798, in his original description of *Cochlis pavimentum* (No. 1889, 1890). E, Type figure not discussed by Röding, 1798 (No. 1891). **F–G.** Original descriptions of *Cochlis onca* (F) and *Cochlis pavimentum* (G) from Röding, 1798.

In this case, the supplementation of data obtained from EtOH-fixed specimens by data derived from egg masses was the only way to obtain adequate numbers of sequences for taxonomic analysis on this species group. As DNA extractions of naticid tissue often produced only limited amounts of amplifiable DNA (see Huelsken *et al.* 2011), we frequently had to use the entire tissue of a specimen for DNA extraction to be able to amplify sequences of high quality. Thus, anatomical work was not possible on this material.

Results

Phylogenetic Analyses

Irrespective of the genes used for phylogenetic and split analyses, our data demonstrate a distinct separation of the Indo-Pacific *Naticarius* species from their supposedly congeneric Mediterranean relatives, a conclusion supported by high bootstrap and Bayesian inference values (Figs 3, 4). Except for the 18S rRNA tree, *N. sertatus* (Röding, 1798) was basal within the Indo-Pacific *Naticarius* species. No further resolution concerning the relationships of *N. onca* and closely related species was obtained in the analyses of nuclear 18S rRNA and H3 sequences (Fig. 4). The phylogenetic reconstructions based on the mitochondrial COI and 16S rRNA gene fragments render *N. onca* paraphyletic.

The specimens of *N. onca* formed two highly supported and independent clades. In the COI dendrogram, *N. concinnus* and *N. zonalis* (Röding, 1798) are more closely related to *N. onca* clade 1 (Fig. 3) while both species were arranged in a terminal taxon together with *N. onca* clade 2 in the 16S rRNA tree (Fig. 4). However, the similar lengths of branches in the COI NeigborNet analyses (Fig. 5) support distinctness of *N. onca* clade 1, *N. onca* clade 2, *N. zonalis, N. sertatus, N. concinnus, N. stercusmuscarum,* and *N. hebraeus*. Of the nine egg masses, the one from Dingo Beach unambiguously grouped with *N. onca* clade 1, while seven grouped with *N. onca* clade 2 and one with *N. concinnus* (Figs 3, 5).

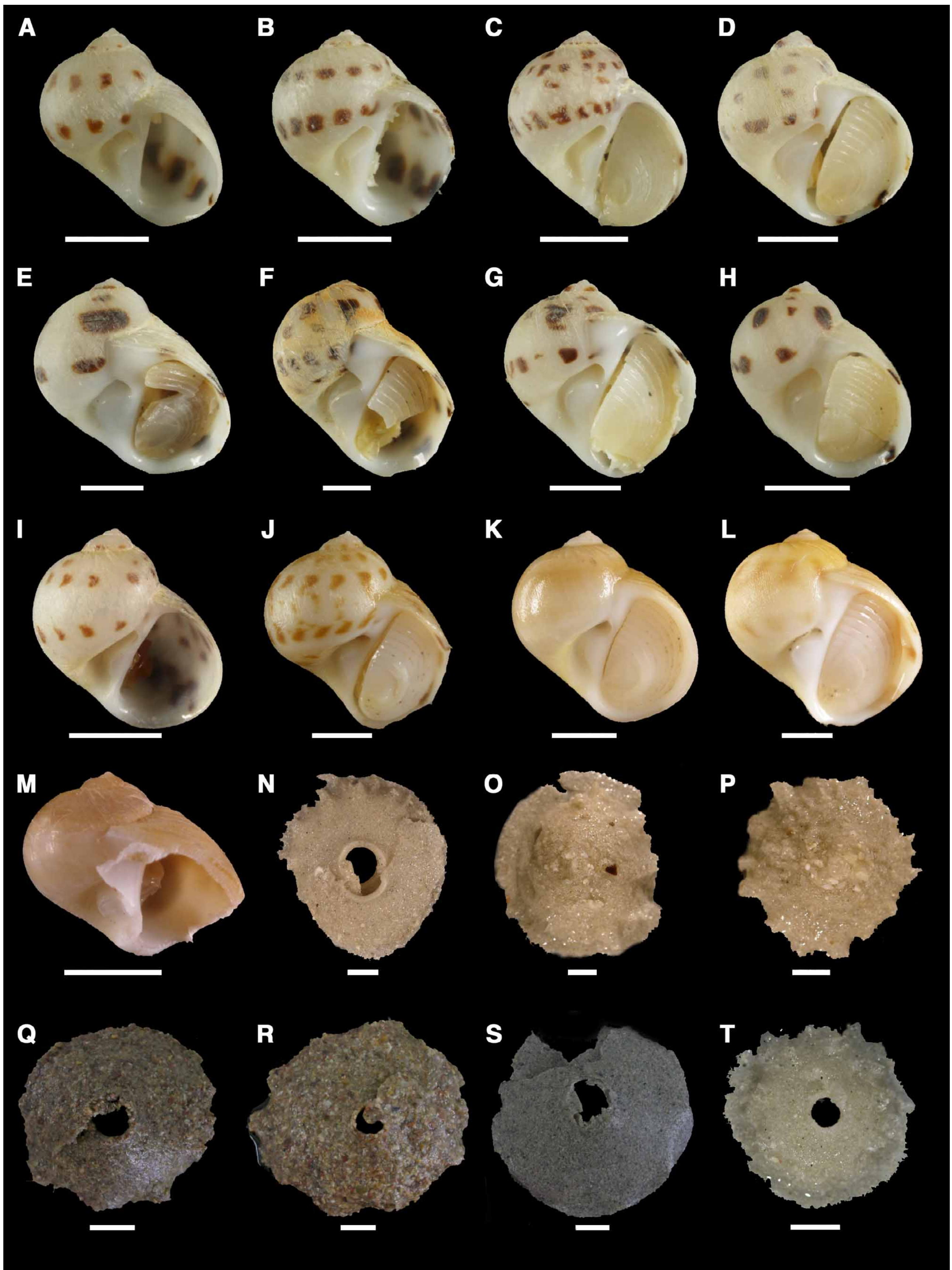

FIGURE 2. A–E. *Naticarius onca* clade 1. A, #61-1, B, #61-2, C, #61-4, D, #61-7, Lizard Island, Queensland; E, #61-5, Dingo Beach, Queensland. **F–I**. *Naticarius onca* clade 2. F, #61-3, G, #61-8, H, #61-9, I, #61-6 - Lizard Island, Queensland. **J**, *Naticarius concinnus* (Dunker, 1860), Shark Bay, Western Australia (#82-1). **K**, *Naticarius zonalis* (Récluz, 1850), Lizard Island, Queensland (#60-1). **L**, *Naticarius sertatus* (Menke, 1843), Lizard Island, Queensland (#79-1). **M**, *Naticarius sertatus* (Menke, 1843), Shark Bay, Western Australia (#79-2). **N–R**. Egg masses of *N. onca* clade 2; N, #L24, O, #L54, P, #L58, Lizard Island, Queensland; Q, #TH190, R, #TH191, Dingo Beach, Queensland. **S**, TH205, egg mass of *N. onca* clade 1, Dingo Beach, Queensland. **T**, Egg mass of *N. concinnus*, Lizard Island, Queensland. # numbers refer to collection number used in analysis. Scale bars = 0.5 cm.

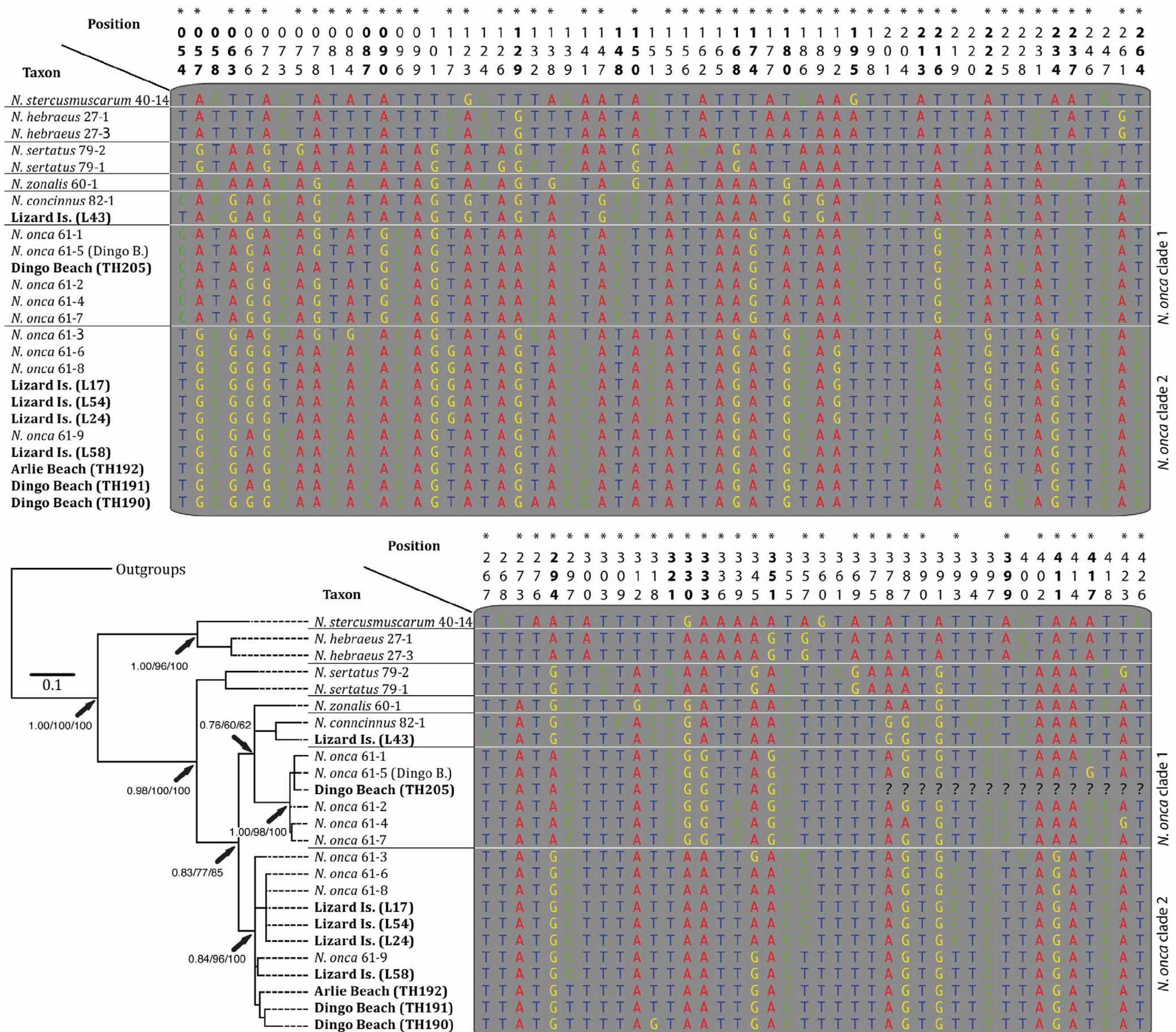

FIGURE 3. Phylogenetic tree (COI) and differing positions in COI haplotypes of the analyzed *Naticarius* species (GTR+I+G, CI: 0.63). The three statistical values shown at the nodes indicate the percentage of trees calculated using three different phylogenetic methods (Bayesian inference/Parsimony bootstrap/Distance bootstrap). Positions with fixed differences between *N. onca* clades are marked bold. Third codon positions are marked with asterisks (*)

Haplotype Analysis

The phylogenetic arrangement of the *Naticarius* lineages was consistent with the uncorrected pairwise distance (p) within the COI gene fragment between the *Naticarius* species from different geographic areas (p = 0.169–0.190). These values were much greater than the inter-specific genetic distance among the Mediterranean species (p = 0.074) or Indo-Pacific species (p = 0.063–0.124) (Table 2). Within the *N. onca* clades, p is 0.011 ± 0.006 for *N. onca* clade 1 and 0.028 ± 0.014 for *N. onca* clade 2 while p is 0.111 ± 0.009 between the two clades. The F_{ST} value between *N. onca* clade 1 and *N. onca* clade 2 (F_{ST} = 0.85) is similar to values observed in pairwise comparisons between the taxonomically well-separated *Naticarius* species *N. concinnus, N. sertatus, N. stercusmuscarum,* and *N. hebraeus* (F_{ST} = 0.72–0.96) (Table 2). The haplotype analysis demonstrated significant levels of heterozygosity within each *N. onca* clade (Fig. 3, Table 3). *N. onca* clade 1 showed a haplotype diversity (Hd) of 0.73 with an average number of 2.2 differences, while *N. onca* clade 2 showed a Hd of 0.93 with an average number of 8.3 differences (Table 3).

Most positions differing between the COI haplotypes were the third codon positions (marked with asterisks in Fig. 3). In most cases only two alternatives were observed at alignment positions showing nucleotide variation, i.e. haplotypes contained either one or the other nucleotide at a certain position. The positions were mostly identical within species/clades (e.g., position 123*, nucleotide change A to G in Fig. 3). In some cases, however, the positions showed overlapping nucleotides between species/clades. A total of 98 positions were found to differ between haplotypes, 71 differed between clades, while 37 showed genetic variability

within clades. Between the two *N. onca* clades 18 positions showed overlapping sites of nucleotides, 27 positions have fixed differences between the clades, while 38 positions were constant (marked bold in Fig. 3).

TABLE 2. Average uncorrected pairwise distances (p) and F_{ST} values calculated for *Naticarius* taxa based on their COI sequences (n/a = not applicable).

p\F_{ST}	*N. hebraeus*	*N. stercusm.*	*N. onca* cl. 1	*N. onca* cl. 2	*N. sertatus*	*N. concinnus*	*N. zonalis*
N. hebraeus	0.000 ± n/a	*n/a*	*0.98*	*0.92*	*0.90*	*0.96*	*n/a*
N. stercusmuscarum	0.074 ± 0.000	n/a	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*
***N. onca* clade 1**	*0.169 ± 0.005*	0.190 ± n/a	0.011 ± 0.006	***0.85***	*0.86*	*0.89*	*n/a*
***N. onca* clade 2**	*0.185 ± 0.012*	0.186 ± 0.017	**0.111 ± 0.009**	0.028 ± 0.014	*0.72*	*0.77*	*n/a*
N. sertatus	*0.166 ± 0.003*	0.178 ± 0.000	0.124 ± 0.009	0.103 ± 0.012	0.030 ± n/a	*0.82*	*n/a*
N. concinnus	*0.187 ± 0.003*	0.190 ± 0.008	0.092 ± 0.006	0.091 ± 0.010	0.122 ± 0.005	0.014 ± n/a	*n/a*
N. zonalis	*0.172 ± 0.001*	0.175 ± n/a	0.085 ± 0.008	0.080 ± 0.011	0.108 ± 0.001	0.063 ± 0.002	n/a

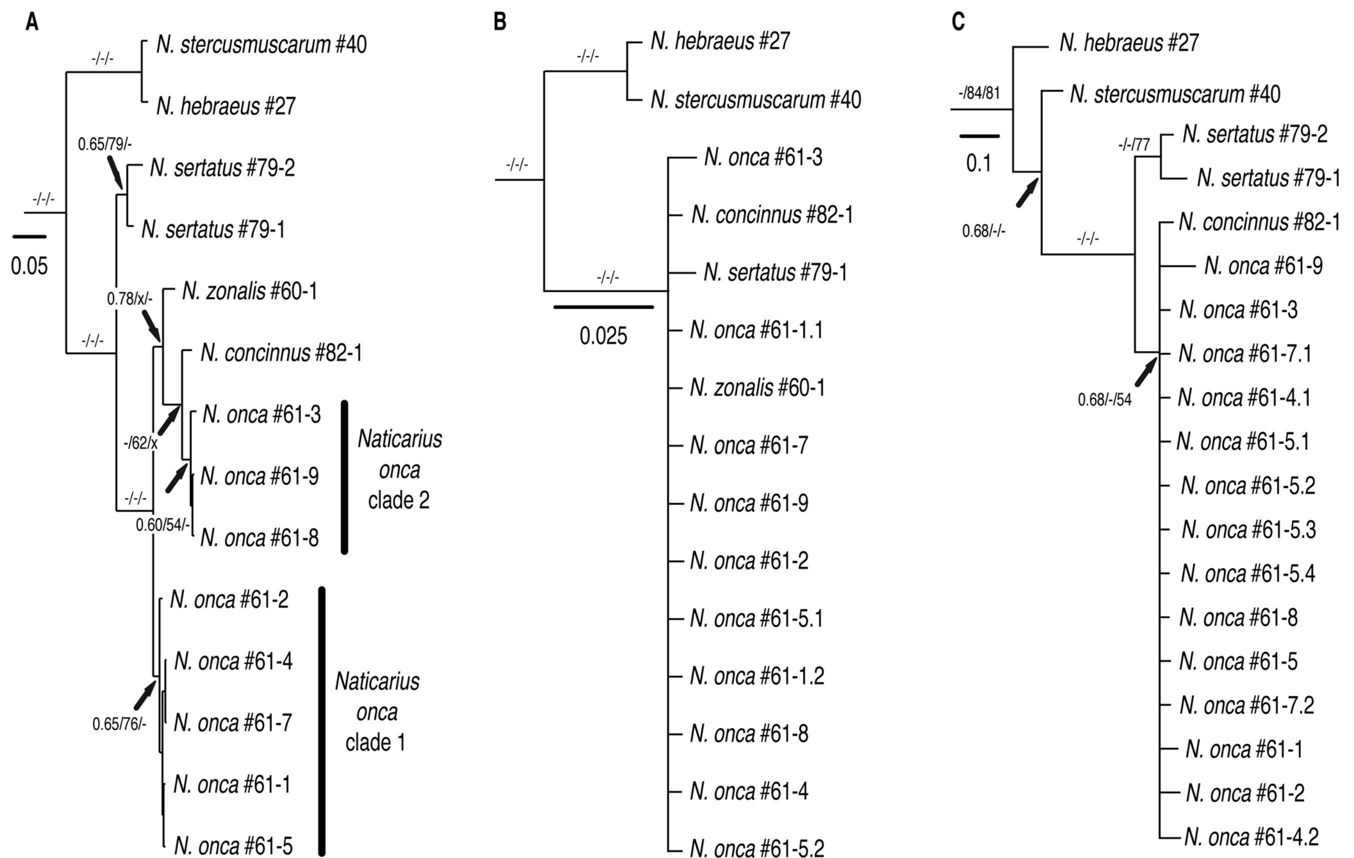

FIGURE 4. Phylogenetic trees of members of the genus *Naticarius* calculated for three separate gene fragments (only ingroup taxa are shown). **A**, Phylogenetic tree based on the 16S gene fragment (GTR+G, CI: 0.78). **B**, Phylogenetic tree based on the 18S gene fragment (K80+I, CI: 0.91). **C**, Phylogenetic tree based on the histone H3 gene fragment (GTR+G, CI: 0.75). The three statistical values shown at the bifurcating branch points indicate the percentage of trees calculated by three different phylogenetic models (Bayesian inference/Parsimony bootstrap/Distance bootstrap). Values larger than 90% are marked as dashes.

Shell characters

All Indo-Pacific *Naticarius* species analyzed here showed similar shell characters (Figs 1, 2; Table 4). The average ratios of width to height (character SR) of all shells were virtually identical (SR = 1.1 ± 0.1) and ranged from 1.0 to 1.2 (Table 4), while the average aperture height as a percentage of total shell height (character AR, see Table 4) ranged from 74.9% ± 7.0 in *N. onca* clade 2 to 85.0% ± 6.2 in *N. sertatus.* AR varied strongly in each taxon for which more than one specimen was analyzed (*N. onca* clade 1: 70.0–

87.5%; *N. onca* clade 2: 66.7–83.3%; *N. sertata*: 80.6–89.3%). Furthermore, all specimens displayed a broad funicle (character F) that almost fills the entire open umbilicus (character U), and a multi-sulcate operculum (character O; see Table 3).

TABLE 3. Haplotype analysis for each *Naticarius* clade with at least two taxa. Abbreviations: **No. hp**—total number of haplotypes; **Hd**—haplotype diversity; **No. diff**.—total number of differing positions within clades.

Clade	No. seqs.	No. hp	Hd	No. diff
***N. onca* clade 1**	11	6	0.80	8.1
***N. onca* clade 2**	6	3	0.73	2.2
N. concinnus	2	2	1.0	4.0
N. sertatus	2	2	1.0	10.0
N. hebraeus	2	1	0.0	0.0

Protoconch morphology did not differ significantly between the two clades of *N. onca* (Table 4). Members of both clades displayed paucispiral protoconchs with 1.25 whorls that have diameters of 610 ± 50 µm (range: 580–680 µm) and 600 ± 40 µm (range: 560–660 µm), respectively. The specimens of *N. sertatus* and *N. concinnus* had 1.5 protoconch whorls each, with maximum diameters of 1020 ± 20 µm (range: 1000–1040 µm) and 760 µm, respectively (Table 4). The smallest protoconch was found in *N. zonalis* with 1.5 whorls and a maximal diameter of 200 µm. Following the assumption of the planktotrophic/lecithotrophic dichotomy (Jablonski and Lutz 1980; Jablonski and Lutz 1983; Bouchet 1989; Pastorino *et al.* 2009), *N. sertatus* (> 1000 µm) may have the shortest PLD with a lecithotrophic or possibly benthic development. *N. onca* clade 1, *N. onca* clade 2 and *N. concinnus* have virtually identical protoconchs (560–760 µm), the size of which may indicate lecithotrophic development, while *N. zonalis* (200 µm) has the longest PLD and most likely develops planktotrophically.

Typically, the Indo-Pacific *Naticarius* shells analysed were marked by rows of regularly or irregularly arranged, spiral- or hook-shaped, light-brown to dark-brown dots and blotches of variable size, while only *N. zonalis* displayed plainly coloured bands that encircle the entire shell (Figs 1, 2; Table 4). The *N. onca* specimens had three to five spiral rows of dark-brownish spots that varied from the prototypical "*onca* arrangement" of five rows (see type referred to by Röding, figured by Chemnitz in: Martini and Chemnitz 1769–1829, pl. 187, figs 1887–1888, our Fig. 1) with evenly spaced, squarish, uniformly shaped dots to an arrangement of three rows of irregularly distributed, variable dots of different sizes and shapes. The dots were either squarish to oval, formed axial lines, or were hook-shaped. In some cases, the topmost and/or the middle rows consisted of only a few irregularly distributed dots of variable size, or were reduced entirely (e.g., #61-1, #61-2). These colour variations were found independently in both clades of *N. onca* analyzed here (Fig. 2). The single specimen of *N. concinnus* analysed molecularly also had three bands of densely and regularly distributed, brownish, hook-shaped dots, of which a few faded vertically into the uncoloured zones (Fig. 2). Thus, *N. concinnus* showed a highly similar shape of dots and an overlapping colour pattern compared to both of the two *N. onca* clades. Finally, the two specimens of *N. sertatus* displayed two bands of fuzzy, regularly distributed, hook-shaped brownish stripes (Fig. 2).

Egg mass morphology

The egg masses of Indo-Pacific *Naticarius* species (*N. onca* clade 1, *N. onca* clade 2, *N. concinnus*) were circularly arranged bands, pyramidal in shape reaching diameters of 2–3 cm and consisted of up to three coils of sand grain-embedded mucus bands with a small central opening which may also even be closed (Figs 2, 4). The egg masses identified as *N. concinnus* and *N. onca* clade 2 showed wavy margins (e.g., L43, L54, L58, TH190, TH191) while the margins of the single *N. onca* clade 1 egg mass had a smooth margin (TH205).

Discussion

Our molecular data demonstrates that the widely distributed Indo-Pacific *N. onca* consists of two independent clades with apparently identical shell morphology. Both *N. onca* clades occur sympatrically within the Great Barrier Reef and are distinct from the well-defined congeneric taxa *N. concinnus, N. sertatus* and *N. zonalis*. Thus, the observed molecular diversity of specimens assigned to *N. onca* by genetic data could either indicate that *N. onca* is a non-monophyletic taxon or represents two species showing identical shell morphology, but probably differing in egg mass morphology.

Based on molecular barcoding of egg masses we provide evidence that the rare species *N. concinnus*, which has hitherto been known from Japan (Dunker 1861; Kabat and Kilias 1991), South Korea (Noseworthy *et al.* 2007), and the Indo-West Pacific (Poppe 2008), also occurs in the northern Great Barrier Reef. Our findings support earlier studies (Huelsken *et al.* 2008) demonstrating the usefulness of molecular barcoding of egg masses and phylogenetic analyses in the identification of naticid species.

The phylogenetic arrangement and genetic distance observed for the two *N. onca* clades correspond well with results of an earlier analysis (Huelsken *et al.* 2006), where similar results were shown to separate the sister taxa *Neverita delessertiana* (Récluz in Chenu, 1943) and *Neverita duplicata* (Say, 1822) as two distinct, valid species. Certainly, high inter-clade genetic distances of mitochondrial sequences have occasionally been found for marine gastropods (e.g., Davison 2000; Funk and Omland 2003; Meyer and Paulay 2005; Wägele *et al.* 2010) but are very uncommon at the magnitude observed for the two *N. onca* clades (COI: 11.1%; 16S: 4.0%). The molecular results therefore support inter-specific differences rather than intra-specific clade divergence.

This is supported by the phylogenetic arrangement of taxa collected at two independent and not directly connected

localities within the Great Barrier Reef (750 km apart). The egg masses assigned to *N. onca* clade 1 collected from Pioneer Point (near Airlie Beach) and Dingo Beach (TH190-TH192; ca. 50 km apart) are sister taxa and more closely related to each other than to the Lizard Island specimens/egg masses of *N. onca* clade 1, thus showing evidence for intra-specific population structure usually expected for populations of the same species (Lizard vs. Dingo Beach/ Pioneer Bay, $F_{ST} = 0.17$).

TABLE 4. Shell characters of the molecularly analyzed specimens. Abbreviations: **NW**—Number of embryonal whorls. **MD**—Maximal diameter of first embryonal whorl. **DN**—Diameter of the nucleus (first half-whorl). **SR**—Size ratio of shell height and shell width (shell height/shell width). **AR—**Aperture ratio: aperture height as percentage of total height. **F**—Funicle (+, present). **U** - Umbilical structure (o, open). **O**—Operculum (ms, multi-sulcate). **C**—Colouration (A, two bands of regularly distributed dots; B, two bands of regularly distributed dots plus two additional bands of irregularly distributed dots of variable size; C, four bands of regularly distributed dots; D, five bands of regularly distributed dots; E, three bands of dense and regularly distributed brownish dots that sometimes fade into the uncoloured zones; F, two bands of regularly distributed, hook-shaped brownish stripes; G, plain brownish, evenly coloured zones that surround the entire shell.)

Species	NW	MD [µm]	DN [µm]	SR	AR [%]	F	U	O	C
N. onca clade 1 (#61-1)	1.25	580	220	1.1	87.5	+	o	ms	A
N. onca clade 1 (#61-2)	1.25	580	240	1.1	70.0	+	o	ms	A
N. onca clade 1 (#61-4)	1.25	560	220	1.2	71.4	+	o	ms	B
N. onca clade 1 (#61-5)	1.25	680	240	1.1	76.5	+	o	ms	D
N. onca clade 1 (#61-7)	1.25	640	200	1.1	80.0	+	o	ms	C
***N. onca* clade 1 (average)**	**1.25**	**610 ± 40**	**220 ± 20**	**1.1 ± 0.04**	**77.1 ± 7.1**	**+**	**o**	**ms**	**A–D**
N. onca clade 2 (#61-3)	1.25	560	220	1.2	77.0	+	o	ms	D
N. onca clade 2 (#61-6)	1.25	580	180	1.0	66.7	+	o	ms	B
N. onca clade 2 (#61-8)	1.25	600	220	1.2	72.7	+	o	ms	A
N. onca clade 2 (#61-9)	1.25	660	220	1.0	83.3	+	o	ms	C
***N. onca* clade 2 (average)**	**1.25**	**600 ± 40**	**210 ± 20**	**1.1 ± 0.1**	**74.9 ± 7.0**	**+**	**o**	**ms**	**A–D**
N. concinnus	**1.5**	**760**	**240**	**1.2**	**71.4**	**+**	**o**	**ms**	**E**
N. sertatus (#79-1)	1.5	1,040	300	1.1	80.6	+	o	ms	F
N. sertatus (#79-2)	1.5	1,000	300	1.0	89.3	+	o	ms	F
***N. sertatus* (average)**	**1.5**	**1.02 ± 30**	**300 ± 0**	**1.1 ± 0.1**	**85.0 ± 6.2**	**+**	**o**	**ms**	**F**
N. zonalis	**1.5**	**200**	**70**	**1.1**	**82.1**	**+**	**o**	**ms**	**G**

The protoconch is often considered to be an important character in caenogastropod taxonomy (e.g., Solsona and Martinell 1999; Reid 2002). However, the obvious overlapping variability in embryonal whorl proportions within the *N. onca* clades and among taxonomically separable species (*N. concinnus* - *N. onca*) supports earlier observations that protoconch features are often not sufficiently differentiated and therefore difficult to interpret (Bouchet and Warén 1993; Bandel 1997). Protoconch features therefore cannot help to decide whether the observed clades of *N. onca*, which have identical protoconch structure, represent different species or not.

Although generally distinguishable from each other, *N. concinnus* shows striking similarities in colour pattern when compared to *N. onca*. Thus, the observed overlapping colouration pattern within the Indo-Pacific *Naticarius* species could be based on the repeated combinations of genetically-stable multistate traits (see Hoagland 1977). Shell colouration in the *N. onca* clades therefore may not necessarily indicate that the two clades belong to a non-monophyletic species, but this distinctive colour pattern could have evolved at least twice in the Indo-Pacific *Naticarius*.

Little or nothing is known about the developmental biology of most naticid species (e.g., Amio 1955; Giglioli 1955; Bandel 1976; Knudsen 1992; Pastorino et al. 2009). Earlier studies attempted to find a correlation between morphological features of the egg mass (larval size, egg capsule morphology, egg mass shape, and rigidity of the sand collar walls) and particular naticid species (Giglioli 1955; Pastorino *et al*. 2009) with no success (Giglioli 1955; Bandel 1976; Aronowsky 2003).

In the present study we show that egg mass morphology is geographically variable within the traditional genus *Naticarius*. Both *N. onca* clades as well as *N. concinnus* have similar egg mass morphology, but this morphology differs

markedly from that of the Mediterranean *N. hebraeus* (Figs 2, 5; see Huelsken *et al.* 2008). In fact, the pyramidal shape and tight coiling of egg masses of Indo-Pacific *Naticarius* species is different from any previously published type of naticid egg masses (Figs 2, 5). The egg collars of *N. hebraeus*, however, are structurally similar to egg masses known from *Neverita heros* (Say, 1822), *Neverita lewisii* (Gould, 1847), *Euspira nitida* (Donovan, 1804), *Euspira montagui* (Forbes, 1838), and *Natica traillii* (Reeve, 1855) (see Giglioli 1955; Huelsken *et al.* 2008). Thus, egg masses may be homogeneous in morphology across closely related taxa occurring in the same geographical area, but may differ from congeneric species in other geographic areas.

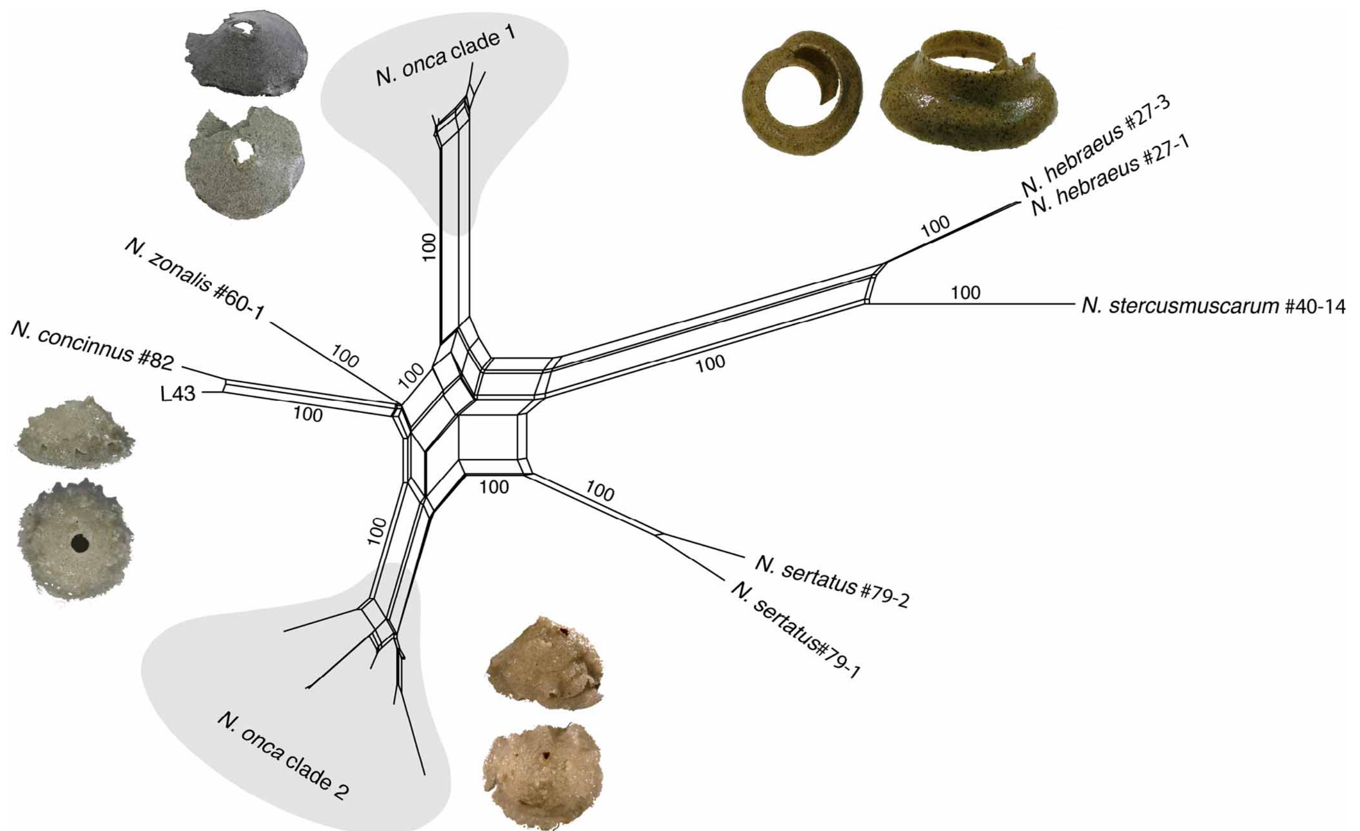

FIGURE 5. Neighbour net analysis of the analyzed Indo-Pacific *Naticarius* species based on the COI data set. The calculations of 1000 bootstrap replicates were performed by applying the LogDet model.

The morphology of egg mass margins (smooth or wavy) was predicted to differ significantly between species (Giglioli 1955). The observed differences in margin morphology between the molecularly separated *N. onca* clade 1 and *N. onca* clade 2 thus might represent a valid differentiating character. However, this variability may be environmentally induced as egg masses by necessity are assembled from different sand types. Thus, further research is needed to prove whether this observed morphological difference constitutes merely anecdotal evidence or can be used as a diagnostic character between the clades.

Conclusion and taxonomic implications

Taken together, it remains unresolved if the two *N. onca* clades represent a non-monophyletic species or two distinct species with identical shells. However, the data presented suggest that the most parsimonious explanation is a separation at the species level. If the two lineages indeed represent two different, congeneric species, it would have important implications for the taxonomy of this broadly distributed taxon, given Röding's (1798) selection of two morphologically different variants of Chemnitz's "Pavimentum chinense" as separate species (*Cochlis onca*, *Cochlis pavimentum*) based on obviously different colour patterns (Fig. 1). The data presented in our study strongly supports Röding's implicit assumption that the non-binomial "Pavimentum chinense" Chemnitz, 1781 represents more than one taxon. However, as the two clades are not separable based on their shell colouration, they are not unequivocally assignable to either of the two taxa erected by Röding. At this point, we therefore cannot resolve Röding's definition of *Cochlis onca* and *Cochlis pavimentum* as both species could be assigned to either of the two taxa identified. Thus, further analyses of egg mass and soft body morphology, as well as anatomical and biogeographical studies are needed to corroborate distinction at the species level.

Acknowledgements

We are grateful to Dr. Philippe Bouchet of the *Muséum National d'Histoire Naturelle* (MNHN) and Richard E. Petit of North Myrtle Beach, South Carolina, for their comments and help with taxonomic questions. We thank Ian Loch of the *Australian Museum* in Sydney and Hugh Morrison of Kingsley, Western Australia, for providing ethanol-preserved specimens for sequence analyses. We also thank Dr. Ralph Tollrian and Eileen Wilmsen (Ruhr-Universität Bochum) for the opportunity to use their digital binocular microscope for taking pictures of protoconchs. We additionally would like to thank Dr. Lyle Vail and Dr. Anne Hogget of the Lizard Island Research Station, Queensland, Australia, for their kind help during collecting, and Annette Tolle for expert sequencing. Finally, we would like to thank Dr. Daniel Tapken (University of Copenhagen), Dr. Cynthia Riginos, Libby Liggins, Carrie Sims and Jude Keyse (The University of Queensland), two anonymous reviewers and the editors of MR for their useful comments on the manuscript. Field and laboratory work were conducted in accordance with the Great Barrier Reef Marine Park Authority (GBRMPA) permit G05/16526.1 to Thomas Huelsken and Michael Hollmann. This study was financed by the German Academic Exchange Service (DAAD) and the Deutsche Forschungsgemeinschaft (Hu 1806/1-1) to TH.

References

Amio, M. (1955) On the egg masses and larvae of seven species of Naticidae (Gastropoda) from the coastal region of Japan. *The Journal of the Shimonoseki College of Fisheries* 5, 137–158.

Aronowsky, A. (2003) *Evolutionary biology of naticid gastropods*. UMI Dissertation Services, Ann Arbour, Michigan.

Bandel, K. (1976) Die Gelege karibischer Vertreter aus den Überfamilien Strombacea, Naticacea und Tonnacea (Mesogastropoda) sowie Beobachtungen im Meer und Aquarium. *Mitteilungen aus dem Institut Colombo Alemán de Investigaciones Cientificas* 8, 105–139.

Bandel, K. (1997) Planctonic Gastropod Larvae from the Red Sea: A Synopsis. *Ophelia* 47, 151–202.

Bouchet, P. (1989) A review of poecilogony in gastropods. *Journal of Molluscan Studies* 55, 67–78.

Bouchet, P. & Warén, A. (1993) Revision of the Northeast Atlantic bathyal and abyssal Mesogastropoda. *Bollettino Malacologico* Supplement 3, 577–840.

Colgan, D.J., Ponder, W.F., Beacham, E. & Macaranas, J. (2007) Molecular phylogenetics of Caenogastropoda (Gastropoda : Mollusca). *Molecular Phylogenetics and Evolution* 42, 717–737.

Collin, R. (2000) Phylogeny of the *Crepidula plana* (Gastropoda: Calyptraeidae) cryptic species complex in North America. *Canadian Journal of Zoology-Revue Canadienne De Zoologie* 78, 1500–1514.

Davison, A. (2000) The inheritance of divergent mitochondria in the land snail, *Cepaea nemoralis*. *Journal of Molluscan Studies* 66, 143–147.

Dunker, W.B.R.H. (1861) *Mollusca japonica descripta et tabulis tribus iconum illustrata a Guilielmo Dunker*. Typis et sumtibus E. Schweizerbart, Stuttgart.

Funk, D.J. & Omland, K.E. (2003) Species-level paraphyly and polyphyly: Frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Annual Review of Ecology Evolution and Systematics* 34, 397–423.

Giglioli, M.E.C. (1955) The egg masses of the Naticidae (Gastropoda). *Journal of the Fisheries Research Board of Canada* 12, 287–327.

Gmelin, J.F. (1791) *Tomus 1, pars VI, Vermes testacea. Lipsiae. In: Editio decima tertia, a., reformata, cura J.F. Gmelin (Ed.), Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonomis, locis*. G. E. Beer.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003) Biological identifications through barcodes. *Proceedings of the Royal Society of London, Series B* 270, 313–321.

Hoagland, K.E. (1977) Gastropod color polymorphism - one adaptive strategy of phenotypic variation. *Biological Bulletin* 152, 360–372.

Huelsken, T., Clemmensen, M. & Hollmann, M. (2006) *Neverita delessertiana* (Recluz in Chenu, 1843): a naticid species (Gastropoda: Caenogastropoda) distinct from *Neverita duplicata* (Say, 1822) based on molecular data, morphological characters, and geographical distribution. *Zootaxa* 1257, 1–25.

Huelsken, T., Marek, C., Schreiber, S., Schmidt, I. & Hollmann, M. (2008) The Naticidae (Mollusca : Gastropoda) of Giglio Island (Tuscany, Italy): Shell characters, live animals, and a molecular analysis of egg masses. *Zootaxa* 1770, 1–40.

Huelsken, T., Schreiber, S. & Hollmann, M. (2011) COI amplification success from mucus-rich marine gastropods (Gastropoda: Naticidae) depends on DNA extraction method and preserving agent. *Mitteilungen der Deutschen Malakozoologischen Gesellschaft* 85, 17–26.

Huson, D.H. (1998) Splitstree: analyzing and visualizing evolutionary data. *Bioinformatics* 14, 68–73.

Huson, D.H. & Bryant, D. (2006) Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* 23, 245–267.

Jablonski, D. & Lutz, R. (1980) Molluscan larval shell morphology, ecological and paleontological applications. *In:* L. R. Rhoads DC (Ed), *Skeletal growth of aquatic organisms*. Plenum Press, New York, pp. 323–377

Jablonski, D. & Lutz, R.A. (1983) Larval Ecology of Marine Benthic Invertebrates - Paleobiological Implications. *Biological Reviews of the Cambridge Philosophical Society* 58, 21–89.

Kabat, A.R. (2000) Results of the Rumphius Biohistorical Expedition to Ambon. Part 10: Mollusca, Gastropoda, Naticidae. *Zoologische Mededelingen* 1, 345–380.

Kabat, A.R. & Kilias, R. (1991) Catalogue of the types of Naticidae (Gastropoda, Streptoneura) of the mollusk collection of the Zoological Museum, Berlin. *Mitteilungen des Zoologischen Museums Berlin* 67, 319–324.

Knowlton, N. (1993) Sibling Species in the Sea. *Annual Review of Ecology and Systematics* 24, 189–216.

Knudsen, J. (1992) Observations on egg capsules and reproduction of some marine prosobranch molluscs from Hong Kong waters. *Proceedings of the 4th International Marine Biological Workshop*, 723–744.

Lee, T. & O´Foighil, D. (2005) Placing the floridian marine genetic disjunction into a regional evolutionary context using the scorched mussel, *Brachidontes exustus*, species complex. *Evolution* 59, 2139–2158.

Librado, P. & Rozas, J. (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–1452.

Martini, F.H.W. & Chemnitz, J.H. (1769–1829) *Neues systematisches Conchylien-Cabinet.* Nürnberg, Vol. 5 [of 12], xxii + 324, pls. 160–198.

Martyn, T. (1786) *The universal conchologist, exhibiting the figure of every known shell, accurately drawn, and painted afternature with a new systematic arrangement by the author Thomas*

Martyn, London, 4, Vol. 3.

Melville, R.V. & Smith, J.D.D. (1987) *Official lists and indexes of names and works in zoology.* The International Trust for Zoological Nomenclature, London.

Menke, K.T. (1843) *Molluscorum Novae Hollandiae specimen, quod ad celebrandum diem jubilaeum vicesimum sextum mensis Februarii anni MDCCCXLIII, quo per vingtii quinque Annos Professoris muneri in illustre Gymnasio academico Hamburgensi praefuit vir doctissimus, clarissimus J.G C. Lehman*., Hannoverae, In libraria aulica Hahniana, 46 pp.

Meyer, C.P. & Paulay, G. (2005) DNA barcoding: error rates based on comprehensive sampling. *PLoS Biology* 3, 422.

Noseworthy, R.G., Lim, N.-R. & Choi, K.-S. (2007) A catalogue of the Mollusks of Jeju Island, South Korea. *Korean Journal of Malacology* 23, 65–104.

Nylander, J.A.A. (2004) MrModeltest v2. Program distributed by the author. Available from: Evolutionary Biology Centre, Uppsala University.

O'Leary, M.A. & Kaufman, S.G. (2007) MorphoBank 2.5: Web application for morphological phylogenetics and taxonomy. Available from: http://www.morphobank.org.

Pastorino, G., Averbuj, A. & Penchaszadeh, P.E. (2009) On the egg masses, eggs and embryos of *Notocochlis isabelleana* (D'orbigny, 1840) (Gastropoda: Naticidae) from Northern Patagonia. *Malacologia* 51, 395–402.

Poppe, G.T. (2008) *Philippine marine mollusks (Gastropoda - Part 1).* ConchBooks, Hackenheim.

Puillandre, N., Strong, E.E., Bouchet, P., Boissellier, M.C., Couloux, A. & Samadi, S. (2009) Identifying gastropod spawn from DNA barcodes: possible but not yet practicable *Molecular Ecology Resources* 9, 1311–1321.

Récluz, C.-A. (1850) Description de Natices nouvelles. *Journal de Conchyliologie* 1, 379–402.

Reid, D.G. (2002) Morphological review and phylogenetic analysis of *Nodilittorina* (Gastropoda : Littorinidae). *Journal of Molluscan Studies* 68, 259–281.

Röding, P.F. (1798) *Museum Boltenianum, sive catalogus cimeliorum e tribus regnis naturae quae olim collegerat Joa. Fried. Bolten, M.D.p.d. Pars secunda, continens conchylia sive testacea univalvia, bivalvia & multivalvia* (Vol. 7). Joachim Friedrich Bolten, Hamburg [Reprinted, 1906, Watford (England)].

Ronquist, F. & Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19, 1572–1574.

Simison, W.B. & Lindberg, D.R. (1999) Morphological and molecular resolution of a putative cryptic species complex: a case study of *Notacmea fascicularis* (Menke, 1851). *Journal of Molluscan Studies* 65, 99–109.

Solsona, M. & Martinell, J. (1999) Protoconch as a taxonomic tool in Gastropoda systematics: Application in the Pliocene Mediterranean Naticidae. *Geobios* 32, 409–419

Swofford, D.L. (2003) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.* Sinauer Associates, Sunderland, Massachusetts.

Wägele, H., Stemmer, K., Burghardt, I. & Händeler, K. (2010) Two new sacoglossan sea slug species (Opisthobranchia, Gastropoda): *Ercolania annelyleorum* sp. nov. (Limapontioidea) and *Elysia asbecki* sp. nov. (Plakobranchoidea), with notes on anatomy, histology and biology. *Zootaxa* 2676, 1–28.