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Neverita delessertiana (Récluz *in* Chenu, 1843): a naticid species (Gastropoda: Caenogastropoda) distinct from *Neverita duplicata* (Say, 1822) based on molecular data, morphological characters, and geographical distribution

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Abstract

The members of the caenogastropod family Naticidae show highly conserved morphological characters, which in many cases complicate species separation. In such cases DNA sequence analysis may help to distinguish between species. In this work partial sequences from the small mitochondrial ribosomal RNA (16S rRNA) gene, the small nuclear ribosomal RNA (18S rRNA) gene, a short intron of the nuclear calmodulin (Cal) gene, and the mitochondrial cytochrome oxidase subunit I (COI) gene are shown to differ significantly between the genomes of what generally had been considered to be merely two morphological variants of the common Western Atlantic naticid *Neverita duplicata* (Say, 1822). Sequence differences between the two forms of *Neverita duplicata* are similar to differences between either of these two forms and the Eastern Pacific *Neverita reclusiana* (Deshayes, 1839), the Indopacific *Neverita didyma* (Röding, 1798), and the Mediterranean *Neverita josephinia* (Risso, 1826). The COI sequences divergence between the two forms of *Neverita duplicata* is in the range of the average COI sequences divergence reported for congeneric species of Mollusca (Hebert 2003). We conclude that in addition to *Neverita duplicata* a second shallow water species of *Neverita* exists along the US Atlantic and Gulf coasts for which the name *Neverita delessertiana* (Récluz, 1843) is available.

Key words: *Neverita duplicata*, *Neverita delessertiana*, 18S rRNA, 16S rRNA, COI, calmodulin intron, molecular phylogeny, Naticidae, Polinicinae

Introduction

In current compilations of Western Atlantic gastropod species (Abbott 1974; Camp 1998; Rosenberg 2005) a single shallow water representative of the naticid genus *Neverita* is

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recognized, *Neverita duplicata* (Say, 1822), vernacularly known as the „Shark´s Eye Snail“, „Atlantic Moon Snail“ or „Double Moon Snail“ (Eisenberg 1981). However, in the 19th century within a span of six years three eminent malacologists independently described an additional species, similar to but separable from *Neverita duplicata*, under three different names. Récluz in 1843 figured and named *Natica delessertiana*, albeit without a description (Chenu 1843; *Natica* pl. 4, figs. 5, 5a; 6, 6a), and distinguished it from *Natica duplicata*, which he figured on the same plate (Chenu 1843; pl. Natica 4, Figs. 1, 1a; 3, 3a). He figured two specimens as *N. delessertiana* of which only one, however, his figs. 6, 6a (see our Figs. 1A-D), appears to be distinct from *N. duplicata* as it has a distinctive, deeply excavated umbilical channel. In 1847 Gould described a similar form of *N. duplicata* from Florida, USA, as *Natica fossata* (Gould 1847). The specimen of *Natica delessertiana* figured by Récluz on his *Natica* plate 4, Figs. 6, 6a, shows a deeply excavated, longitudinally striated umbilical channel, a feature also described by Gould as characteristic for his *Natica fossata*. Therefore, we believe that *Natica fossata* is merely a junior synonym of *N. delessertiana*. In 1849, Philippi recognized the existence of a variant, deeply excavated form of *Natica duplicata* from Galveston, Texas, USA, and named it *Natica texasiana* (Philippi 1849a). His figure of the type (shown in Fig. 1M; reproduced from Philippi 1849–53: pl. 5, fig. 3) shows an extreme form of the striated umbilical excavation, and Philippi commented on the distinct features of this species in several publications (Philippi 1849a; 1849b; 1849–53). We conclude from these descriptions that *Natica texasiana* is merely a junior synonym of *N. delessertiana.* Tryon (1886) included *N. delessertiana, N. fossata* and *N. texasiana* in the synonymy of *N. duplicata*, and he mentioned that *N. delessertiana* combines the typical form of *N. duplicata* with features described by Gould for *N. fossata.*

Kabat *et al.* (1997: 19) examined the type material of *N. delessertiana* preserved in the Geneva Museum of Natural History and reported the hitherto unpublished type locality to be „Lousiana, at the banks of the Mississippi“. Thus, all three taxa describing a form of *N. duplicata* with an excavated umbilical channel were reported from the US Gulf Coast and Florida. These three taxa in the past decades consistently have been treated as synonyms of *N. duplicata* (Kabat *et al.* 1997) and were not considered distinct at the species level, with the exception of one report, recently published on the internet (Poland 1998).

The two forms of *N. duplicata* are found sympatrically in waters along the Gulf coast of Florida. However, the typical form of *N. duplicata* is found in shallow as well as in deeper water while the excavated form is predominantly found in shallow water, particularly in bays (Poland 1998; data of specimens in the collection of Michael Hollmann [MHC]). Dall (1892) reported morphological differences, in particular overall size and relative spire height as a secondary sexual dimorphism, a statement which was reiterated by Jacobson (1973). In his interpretation, specimens of *N. fossata* (and thus similarly *N. delessertiana* and *N. texasiana*) are considered male specimens while the „typical“ *N. duplicata* are regarded as females. However, none of these authors detailed how the sex of the snails was determined; thus, it is not clear whether the sex was verified

anatomically, or merely deduced from the shell form:

> „ ... the usual differences exist between sexes, the male beeing smaller, more conical, generally with a proportionately higher spire and darker colored ... The small, elevated dark males were described by Dr. Gould as *N. fossata*....“ (Dall 1892: 368-369)
>
> „ ... according to Dall (1892) this difference is a secondary sexual characteristic, the heavier shell being the male, the lower, tighter one the female ... “ (Jacobson 1973: 27)

These reports show that the morphological differences in the two forms of *N. duplicata* seem to be present in many populations at different locations along the eastern coast of the USA and the US Gulf coast.

In this study we set out to analyze potential genetic differences between the two forms based on partial sequences of four different genes, the nuclear 18S rRNA gene, a small intron of the nuclear calmodulin gene, the mitochondrial 16S rRNA gene, and the mitochondrial cytochrome oxidase subunit I gene. Additionally, the localities of 278 specimens of both forms were mapped and their shell ratios were measured. We conclude that the two forms of *N. duplicata* investigated belong to two distinct species, *N. duplicata* (Say, 1822) with a narrow umbilicus showing evenly rounded walls of the body whorl, and *N. delessertiana* (Récluz, 1843) with a widely excavated, longitudinally striated umbilicus, showing a strong ridge or keel (see Figs. 1A-B, E-F, H) on the walls of the body whorl.

FIGURE 1: Photos of type specimens, reproductions of original figures and figured specimens of *Natica delessertiana* Récluz *in* Chenu, 1843, *N. duplicata* Say, 1822, and taxa synonymous with these two species. A: Figure of one syntype of *N. delessertiana* from Chenu 1843: Fig. 6a; B-D: Syntype 1 of *N. delessertiana*, MHNG 1300/48/1, herein selected as the lectotype of *N. delessertiana*, type locality: Lousiana, close to the Mississippi, 46.5 x 46.8 mm; E: Syntype 2 of *N. delessertiana*, MHNG 1300/47/1; F: Syntype 3 of *N. delessertiana*, MHNG 1300/47/2; G: Figure of another syntype of *N. delessertiana* from Chenu 1843: Fig. 5; H: Syntype 4 of *N. delessertiana*, MHNG 1300/48/2. The figured syntype of *N. delessertiana* in A was probably drawn from the specimen shown in B-D. It is unclear on which specimen the other figured syntype (G, Chenu 1843) was based. None of the four syntypes of *N. delessertiana* preserved in the Récluz collection at the MHNG has the operculum that is shown in Chenu's figure 5; I: *N. duplicata* Say, 1822, *sensu* Récluz (Chenu 1843: *Natica* pl. 4, fig. 3a); J: *N. duplicata* Say, 1822, ex coll. Récluz, MHNG 1300/36, 44.5 x 50 mm, presumably the specimen figured in I; K: *N. campeachiensis* Reeve, 1855 *ex* Récluz MS (Reeve 1855: pl. 1, fig. 1b); L: *N. campechiensis* Récluz *in* Chenu, 1843 (Chenu 1843: *Natica* pl. 4, fig. 2); M: Original figure of *N. texasiana* Philippi, 1849 (Philippi 1849-53; pl. 5 fig. 3); N: Original figure of *N. texasiana var.* Philippi, 1849 (Philippi 1849-53; pl. 12 fig. 10); O: Original figure of *N. listeri* Philippi, 1850 (Philippi 1849-53; pl. 12 fig. 11); P: *N. duplicata* Say, 1822, *sensu* Philippi (Philippi 1849-53; pl. 5 fig. 1). The most distinctive morphological character of *N. delessertiana*, the deep umbilical channel, is identifiable in all four syntype specimens of *N. delessertiana* (A-B, E-F, H), and is also evident in the original figures of *N. texasiana* (M) and *N. texasiana var.* (N).

Material and Methods

a) Material examined

Our sequence data are based on seven specimens of the form with an excavated, longitudinally striated umbilical channel (= "excavated from") and four specimens of the "typical" form of *N. duplicata* from the Florida coastline, one specimen of *N. reclusiana*, which was collected on the Pacific coast of Mexico, and one specimen of *N. josephinia* from Italy. An additional partial COI sequence of the Indopacific *N. didyma* collected off Taiwan was obtained from GenBank (AF550509; 647 base-pairs (bp); Collin 2003; Table 1; voucher specimen UF282591 at the University of Florida, Gainesville, 41 x 53 mm). Sample specimens of the species included in the molecular, morphological, and geographical analysis are shown in Figure 2. The specimens except for *N. didyma* (University of Florida, Gainesville) are stored in the collections of the Department of Biochemistry, Faculty of Chemistry, Ruhr University Bochum, Germany, under the unique identifier numbers listed in Table 1. The molecular data set is used for phylogenetic reconstruction as shown in Figure 3. The specimens of the two forms were classified by their morphological characters, in particular the umbilical area, as illustrated in Figure 4.

The ratios of height and width of shells were measured to compare these to the comments on differences in shell morphology by Dall (1892) and Jacobsen (1973). For this, additional specimens in the MHC collection were classified based on their umbilical morphology. In total, the height and width of 278 specimens of both forms of *N. duplicata* (Table 2) were measured and their ratios r = height (mm) / width (mm) calculated. The data set also includes the shells that were analyzed by molecular methods. The significance of difference between the two groups was verified by a two-paired non-parametric t-test (Mann-Whitney) using the Program PRISM v3.0 (Fig. 5).

FIGURE 2: Specimens of the species used for molecular analysis in this study; A-D, *Neverita josephinia* #2 (Isola del Giglio, Italy, 16.5 x 23.1 mm); E-H, "typical" form of *Neverita duplicata* #4 (Jacksonville, Florida, USA; 22.7 x 30.1 mm); I-L, "excavated" form of *N. duplicata* #14 (Cedar Key, Florida, USA, 21.6 x 27.3 mm); M-O, *Neverita didyma*, (Taiwan, 41 x 53 mm; voucher specimen UF282591 of the University of Florida, Gainesville); P-S, *Neverita reclusiana* #1 (Cholla Bay, Puerto Penasco, Mexico, 19.0 x 21.4 mm).

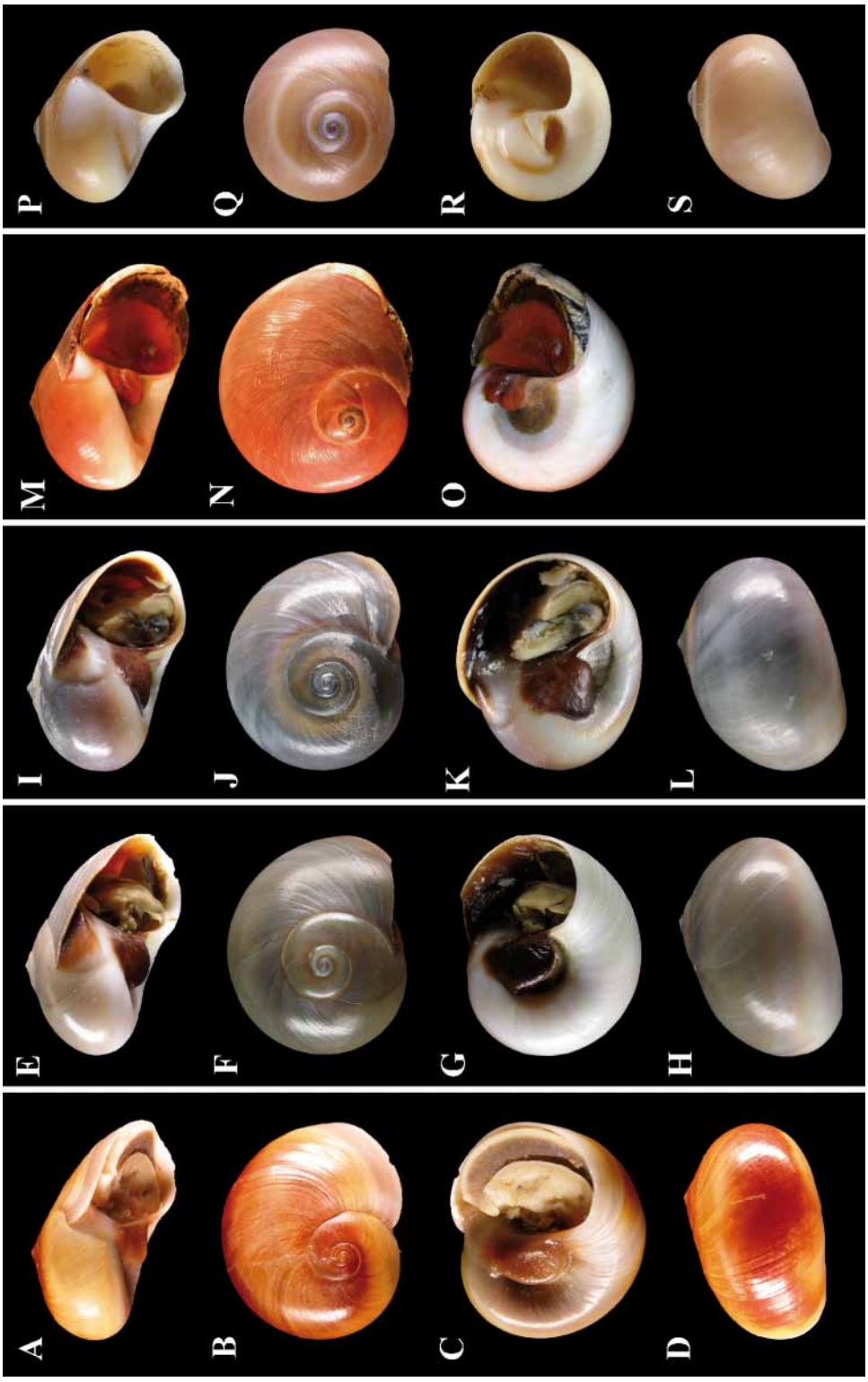


TABLE 1: Collecting sites of the specimens used for molecular analysis in this study, and sequences analyzed of the species investigated. For 16S and COI several sequences from different specimens of both forms of *N. duplicata* were analyzed. "Collection No." refers to a unique identifier in the collections of the Department of Biochemistry I, Faculty of Chemistry, Ruhr University Bochum (RUB-BC), except for *N. didyma*, which is stored at the University of Florida (UF). "Spec. No." refers to the specimen numbers used throughout this paper.

Species (form)	**Collection No.**	**Spec. No.**	**Collecting site**	**COI**	**16S**	**18S**	**Cal**
Neverita duplicata („typical form“)	RUB-BC 21-1	#1	Clearwater, Florida, USA	X	X	X	X
	RUB-BC 21-2	#2	Jacksonville, Florida, USA	X	X		
	RUB-BC 21-3	#3	Jacksonville, Florida, USA	X	X		
	RUB-BC 21-4	#4	Jacksonville, Florida, USA	X	X		
Neverita duplicata („excavated form“)	RUB-BC 19-1	#1	Clearwater, Florida, USA	X	X		
	RUB-BC 19-3	#3	Clearwater, Florida, USA		X	X	X
	RUB-BC 19-5	#5	Clearwater, Florida, USA	X	X		
	RUB-BC 19-7	#7	Tampa, Florida, USA		X		
	RUB-BC 19-8	#8	Tampa, Florida, USA		X		
	RUB-BC 19-9	#9	Tampa, Florida, USA	X			
	RUB-BC 19-14	#14	Cedar Key, Florida, USA	X			
Neverita didyma	UF282591	----	Taiwan [GenBank, AF550509]	X	----	----	----
Neverita reclusiana	RUB-BC 33-1	#1	Cholla Bay, Puerto Penasco, Mexico	X	X	X	----
Neverita josephinia	RUB-BC 46-2	#2	Isola del Giglio, Toscana, Italy	X	----	----	----

TABLE 2: Numbers and collecting sites of the specimens used for morphological analysis.

Collecting site	No. of **“typical”** shells	No. of **“excavated”** shells
Collecting sites at the US Atlantic coast (North to South)		
Lynn Harbor, **Massachusetts**, USA	9	---
Cape Cod, **Massachusetts**, USA	7	---
Lyme, **Massachusetts**, USA	3	---
Bristol Harbor, **Rhode Island**, USA	2	---
Westbrook, **Connecticut**, USA	1	---
Hempstead, **New York**, USA	1	---
New Jersey, USA	2	---
Le Jeune, **North Carolina**, USA	3	---

.....continued on the next page

TABLE 2 *(continued)*

Collecting site	No. of **"typical"** shells	No. of **"excavated"** shells
Charleston, **North Carolina**, USA	10	---
Isle of Palms, **South Carolina**, USA	11	---
St. Simon´s Island, **Georgia**, USA	3	---
Jacksonville, **Florida**, USA	3	---
Daytona, **Florida**, USA	2	---
Cocoa, **Florida**, USA	3	3
Cape Canaveral, **Florida**, USA	11	---
Boca Raton, **Florida**, USA	1	---
Collecting sites at the Gulf coast (East to West)		
Key West, **Florida**, USA	2	2
Marco Island, **Florida**, USA	2	6
Pine Island & Fort Myers, **Florida**, USA	5	8
Sanibel Island, **Florida**, USA	41	42
Sarasota, **Florida**, USA	13	2
Joe Island & Tampa, **Florida**, USA	2	11
Clearwater, **Florida**, USA	4	7
Cedar Key, **Florida**, USA	---	1
Mullet Key, **Florida**, USA	1	9
Jefferson County, **Florida**, USA	2	---
Indian Pass, Gulf County, **Florida**, USA	2	---
Panama City, **Florida**, USA	2	---
Carrabelle, **Florida**, USA	2	---
Alabama, USA	---	1
El Cuyo, **Yucatan**, Mexico	6	---
Freeport, **Texas**, USA	2	---
Sabine Pass, **Texas**, USA	2	---
Aransas, **Texas**, USA	---	1
Mustang Island, **Texas**, USA	12	---
Other collecting sites		
Caribbean, **Honduras**	3	---
Florida, USA	6	4
	181	97

Additionally, the collecting sites of all specimens were plotted on a map comprising the east coast of the USA, the Gulf of Mexico, and the Caribbean Sea (Fig. 6) to depict the pattern of distribution of the two forms.

b) Nucleic acid isolation, subcloning and sequence analysis

Total DNA was extracted from ethanol-preserved tissue by a modified CTAB extraction (Doyle & Doyle 1987) or using the DNeasy Extraktion Kit (Qiagen, Hilden, Germany) and stored in Tris-EDTA pH 7.4. A 345 bp fragment of the 16S rRNA gene, 355 bp of the COI gene, 241 bp of a small intron of the Cal gene, and 394 bp of the 18S rRNA gene were sequenced from the same individuals of each species (sequence length without primers). Amplification reactions using Phusion (Finnzymes, Espoo, Finnland) or Taq Polymerase (Invitrogen, Karlsruhe, Germany) were done in MJ Research thermocyclers (Watertown, MA, USA). Amplification primers used were P256 and P259 for 16S rRNA, P388 and P390 for COI, P225 and P226 for the intron of the calmodulin gene, and P398 and P399 for the partial 18S rRNA sequence (Table 3). The PCR products were purified using gel extraction kits „Jetstar“ (Genomed, Löhne, Germany) and were subcloned into an EcoRV-cut vector, pBluescript SK- (Stratagene, La Jolla, USA) or pGEMt (Invitrogen, Karlsruhe, Germany). At least two independent colonies were sequenced for most subcloned fragments and were found to be either identical or differing in only a single base, which likely reflects the polymerase error rate. Both strands were cycle-sequenced on an ALF automated sequencer (Pharmacia, Freiburg, Germany) using T7/Rev primers (pBSK-) or T7/SP6 primers (pGEMt) and an ALF sequencing kit (Amersham, Freiburg, Germany). Sequences obtained are summarized in Table 1.

TABLE 3: Primers used and length of fragments obtained in PCR reactions to amplify partial sequence of the four genes 16S rRNA, 18S rRNA, cytochrome oxidase subunit I, and the small intron of the calmodulin gene.

Primer	Sequence (5´→3´)	Fragment size (bp)
P388 [COI sense]	gct ttt gtt ata att tty tt	455 bp
P390 [COI antisense]	cga tca gtt aaa art atw gta at	
P256 [16S sense]	ccg tgc aaa ggt agc ata at	373 bp
P259 [16S antisense]	aac atc gag gtc aca amc	
P225 [Cal sense]	gag gtg gat gcc gat ggt at	279 bp
P226 [Cal antisense]	cgt cag gaa ctc ggg gaa gt	
P398 [18S sense]	gtg gtt gat yct gcc agt	378 bp
P399 [18S antisense]	tct cag gct ccy tct ccg	

c) Tree calculations

The sequences were aligned with the MegAlign program (DNAStar), and sequence divergence was calculated using PAUP* 4.0b (Swofford 2003). In a first step the heterogeneity of base composition was determined, using the chi-square test. Next, the permutation tail probability test (PTP) was performed. Both tests are implemented in PAUP*. The base heterogeneity did not differ significantly in both data sets among taxa (16S: chi-square = 1.22 [dF = 21], P = 1.0; COI: chi-square = 4.51 [dF = 30], P = 0.99). The permutation test assesses the randomness of the data structure. 100 permutation test replicates resulted in $P < 0.01$ for both data sets, demonstrating absence of randomness.

Absolute differences were transformed into distances. Distances were corrected using LogDet, the mathematical model also used for dendrogram calculations. Unrooted equal-weighted phenetic (neighbor joining = NJ) as well as phylogenetic analyses (maximum likelihood = ML, and maximum parsimony = MP) were performed on each of the two data sets using a heuristic search with tree bisection-reconnection (TBR) branch swapping. Sequence additon by As-is method, 1 tree was held from each step. Steepest descent option and multress option were used, the maxtrees was set to 100. During the analyses this value of maxtrees was never reached for any of the data sets.

In MP analyses, 48 positions in the COI data set were parsimony-informative, and 46 were parsimony-uninformative, while 261 were constant. The 16S alignment contained nine positions that were parsimony-informative, 15 were parsimony-uninformative, while 322 were constant. Gaps were treated as "missing" in all analyses. Statistical bootstrap analysis was based on 10,000 replicates.

Results

1) Molecular data

Sequences obtained in this study are summarized in Table 1. Partial COI, 16S, 18S, and Cal sequences were obtained from the two forms of *N. duplicata,* COI, 16S, and 18S sequences from *N. reclusiana,* and the COI sequence from *N. josephinia*. A COI sequence for *N. didyma* (Röding, 1798) was taken from GenBank. For reasons of simplicity, „gene sequence“ in the following refers to the sequence of the respective fragment of that gene as specified in Materials and Methods. Gene sequences of the 16S rRNA of each form of *N. duplicata* were analyzed in specimens from two different locations (Table 1). Average values are given in cases where distances were obtained from more than one specimen (Table 4). Absolute distances and % differences are mentioned in the text; for LogDet-corrected data see Table 4.

The COI sequences (355 bp) show a total of 35 differences between the two forms of *N. duplicata,* amounting to a relative distance of 9.9% (Table 4). In comparison, the COI sequence of the excavated form of *N. duplicata* shows 48.25 different positions (13.6%)

when compared to the sequence of *N. reclusiana* and 32.25 (9.9%) and 54.75 (15.4%) compared to *N. didyma* and *N. josephinia*, respectively. By comparison, the COI sequence of the typical form of *N. duplicata* shows 48.5 substitutions when compared to *N. reclusiana* (13.7%), and 37.5 (10.6%,) different positions compared to *N. didyma*. It differs in 47.25 substitutions from the sequence of *N. josephinia* (13.3%). The COI sequence of *N. reclusiana* differs in 46.0 positions (13.0%) from *N. didyma* and in 60 positions (16.9%) from *N. josephinia*, while *N. didyma* differs in 55 positions (15.5%) from *N. josephinia*. In all cases the calculated distances are even larger when the LogDet distance correction is being used. The LogDet model estimates multiple substitutions for each position in the alignment and corrects the relative data. In the phenetic tree as well as in the phylogenetic trees based on these COI values (Fig. 3A) both forms of *N. duplicata* were arranged in separate terminal taxa, each showing significant bootstrap support (100% for NJ, ML, and MP). Furthermore, the phylogenetic trees show a terminal taxon of *N. didyma* and *N. reclusiana* (bootstrap values: 45% and 43%, for ML and MP, respectively) arranged as a sister taxon of the excavated form of *N. duplicata* (bootstrap values 60/57% for ML/MP). Together, the three species are sister taxa of the typical form of *N. duplicata*. The Mediterranean *N. josephinia* is used as the outgroup for this tree due to its relative geographical isolation. Using the COI sequence of *Polinices mammilla* (Linnaeus, 1758) from Lizard Island (Queensland, Australia) for an expanded comparison generates an identical arrangement of the *Neverita* species (data not shown). Both forms of *N. duplicata* still show significant bootstrap support amounting to 100% for *N. delessertiana* and 98% for *N. duplicata* (MP).

FIGURE 3: Phenetic (left side; Neighbor Joining = NJ, LogDet calculation) as well as phylogenetic trees (right side; Maximum Likelihood = ML, and Maximum Parsimony = MP) of *Neverita* species. The ML and MP trees are exactly identical. Bootstrap analysis was performed with 10,000 replicates. In the phylogenetic trees, bootstrap values are given for both methods (ML/MP). The sequence of *N. didyma* was taken from GenBank (AF550509) and the sequence of *N. josephinia* was used as the outgroup. A: Trees based on partial mitochondrial COI gene sequences from the „typical“ form (#1: Clearwater; #2-4: Jacksonville) and the „excavated“ form (#3, #5: Clearwater; #9: Manatee County; #14: Cedar Key) of *N. duplicata* from different collecting sites on the western and eastern coasts of Florida. B: Trees based on partial mitochondrial 16S rDNA sequences from the “typical“ form (#1: Clearwater; #3: Jacksonville) and the “excavated“ form (#1, #3, #5: Clearwater; #7, #8: Manatee County) of *N. duplicata* from different colleting sites on the western and eastern coasts of Florida.

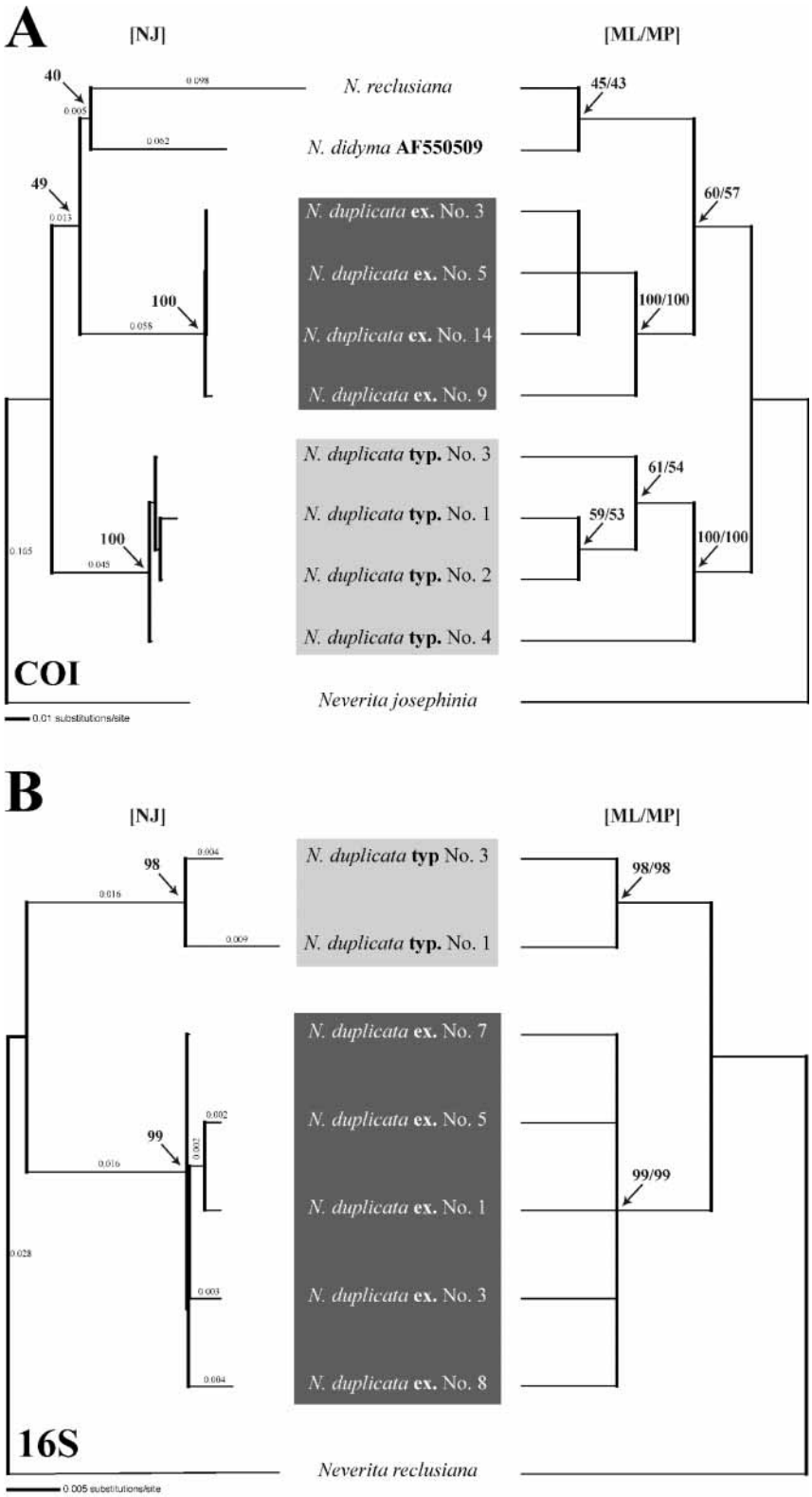


TABLE 4: Relative and absolute distances between the investigated sequences (COI, 16S rRNA, 18S rRNA, Cal) of the two different forms of *N. duplicata* („typical“ and „excavated“), *N. reclusiana*, *N. didyma*, and *N. josephinia*. Abs: absolute distances; Rel: relative distances; LogDet: corrected distances under LogDet conditions (* = average).

	N. duplicata ("typical")			*N. duplicata ("excavated")*			*N. reclusiana*			*N. didyma*		
	Abs.	**Rel.**	**LogDet**	**Abs.**	**Rel.**	**LogDet**	**Abs.**	**Rel.**	**LogDet**	**Abs.**	**Rel.**	**LogDet**
COI (355 bp)												
N. duplicata ("excavated")	35.0*	9.9*	11.9*									
N. reclusiana	48.5*	13.7*	17.0*	48.25*	13.6*	16.5*						
N. didyma	37,.5*	10.6*	13.0*	32.25*	9.9*	12.2*	46.0	13.0	16.0			
N. josephinia	47.25*	13.3*	15.6*	54.75*	15.4*	18.5*	60.0	16.9	20.5	55.0	15.5	18.7
16S (345 bp)												
N. duplicata ("excavated")	11.5*	3.35*	3.98*									
N. reclusiana	14.67*	4.31*	5.17*	14.5*	4.25*	4.59*						
18S (394 bp)												
N. duplicata ("excavated")	11	2.9	2.9									
N. reclusiana	12	3.1	3.2	5	1.3	1.3						
Cal-Intron (241 bp)												
N. duplicata ("excavated")	16	6.7	7.6									

The specimens of the two forms of *N. duplicata* analyzed were collected at different localities (Tables 1, 5). The COI sequences of typical *N. duplicata* specimens from different localities (#1: Clearwater, West Coast of Florida; #2-4: Jacksonville, East Coast of Florida) differ on average in only 4 positions (1.1%; Table 5). The sequences of the specimens #2-4 from Jacksonville, Florida, differ in merely 1.3 positions (Rel.: 0.4%; Table 5). The sequences of the four specimens of the excavated form of *N. duplicata* from different localities (#1, #5: Clearwater, Florida; #9: Manatee County, Florida; #14: Cedar Key, Florida; all west coast of Florida) are either identical (specimens #1, #5 and #14) or differ in only a single position (#9 vs. all others; 0.3%; Table 5).

The 16S rRNA (345 bp) sequences show 11.5 different positions (3%) between the two forms of *N. duplicata* (Table 4), and 14.7 differences between the typical form of *N. duplicata* and *N. reclusiana* (4.3%). The 16S rRNA sequences of the excavated form of *N. duplicata* and *N. reclusiana* are separated by 14.5 differences (4%). Once again, the phylogenetic trees (Fig. 3B) show significantly separated terminal taxa for both forms. The terminal taxon represented by the typical form of *N. duplicata* has a bootstrap value of 98%, the excavated form has one of 99%, independently of the calculation used (ML or MP). The 16S rRNA sequences of typical *N. duplicata* specimens from different localities

(#1: Clearwater, West Coast of Florida; #3: Jacksonville, East Coast of Florida) differ in 4.5 positions (1.3%; Table 5). The specimens of the excavated form of *N. duplicata* from different localities (#1, #3, #5: Clearwater, Florida; #7, #8: Manatee County, Florida) differ in 1.2 positions (0.3%; Table 5) while only a single differing position can be found in sequences of specimens from the same localities (Table 5).

TABLE 5: Intraspecific absolute differences between specimens of the excavated and typical forms of *N. duplicata*. The absolute differences for COI sequences range from 1.0 to 5.0 (Rel.: 0.3 - 1,4%; LogDet: 0.3 - 1.5%) between specimens of the typical form of *N. duplicata*, and from 0.0 to 1.0 (Rel. 0.0 - 0.3%, LogDet: 0.0 - 0.3%) between specimens of the excavated form of *N. duplicata*. The 16S sequences differ in 4.5 (Rel.: 1.3%; LogDet 1.6%) positions between specimens #1 and #3 of the typical form of *N. duplicata*, and in a range from 0.0 to 1.0 (Rel., LogDet: 0.0% - 0.3%) positions between the specimens of the excavated form of *N. duplicata*.

	N. duplicata ("typical")			N. duplicata („excavated")					
	#2	**#3**	**#4**	**#3**	**#5**	**#7**	**#8**	**#9**	**#14**
COI (355 bp)	**absolute differences**								
N. duplicata ("typical") #1	3.0	4.0	5.0						
N. duplicata ("typical") #2		1.0	2.0						
N. duplicata ("typical") #3			1.0						
N. duplicata („excavated") #1					0.0			1.0	0.0
N. duplicata („excavated") #5								1.0	0.0
N. duplicata („excavated") #9									1.0
16S (345 bp)	**absolute differences**								
N. duplicata ("typical") #1		4.5							
N. duplicata („excavated") #1				1.0	0.0	0,0	1.0		
N. duplicata („excavated") #3					1.0	1.0	1.0		
N. duplicata („excavated") #5						1.0	2.0		
N. duplicata („excavated") #7							1.0		

The 18S rRNA (394 bp) sequences of typical *N. duplicata* show 11 absolute differences (2.9%) to those of the excavated form, and 12 differences (3.1%) to the sequence of *N. reclusiana* (Table 4). The sequence of the excavated form of *N. duplicata* is separated by 5 differences from *N. reclusiana* (1.3%; Table 4). Only one specimen of each species was analyzed for this gene. Within a phylogenetic tree a terminal taxon with *N. reclusiana* and the excavated form of *N. duplicata* is distinguishable as a sister taxon of the typical form of *N. duplicata* (not shown).

The sequences of the small intron of the calmodulin gene (241 bp) show 16 differences (6.7 %; Table 4) between the two forms of *N. duplicata*. Only one specimen of each species was analyzed.

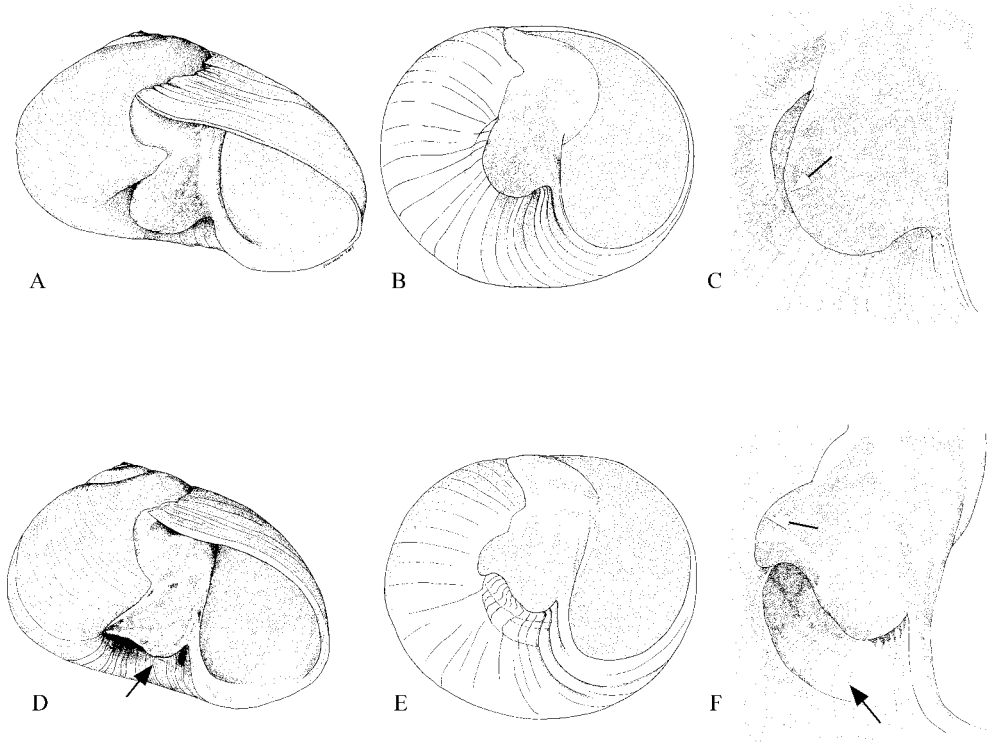


FIGURE 4: Typical shells of *Neverita duplicata* (A-C), and *Neverita delessertiana* (D-F). The umbilical areas are shown enlarged in C and F. The ridge (keel) within the umbilical channel (closed arrows in D and F) of *N. delessertiana* is present in all specimens investigated. Additionally, the shape of the umbilical callus often differs between the two species [open arrows in C and F]. The callus of *N. delessertiana* in most specimens is more triangular (F) while that of *N. duplicata* is more rounded (C).

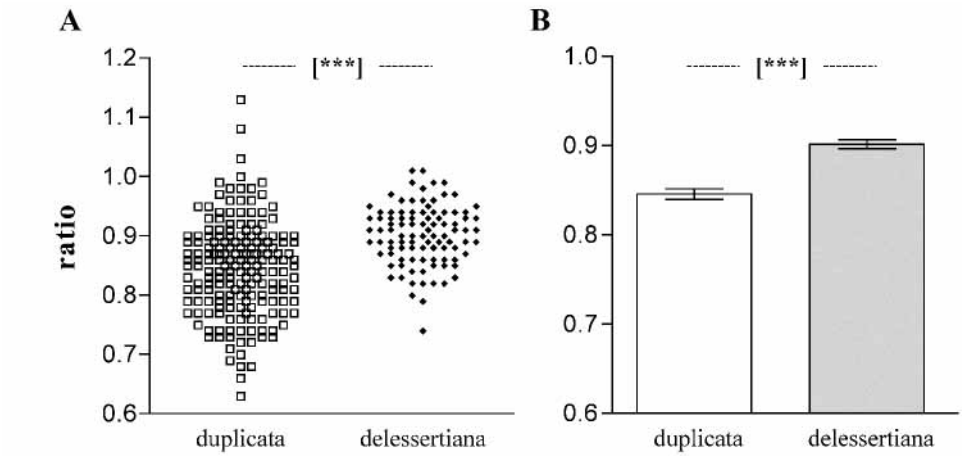


FIGURE 5: A: Size ratios (height/width) of 181 specimens of *Neverita duplicata* and 97 specimens of *Neverita delessertiana* from Massachusetts to Honduras (for localities see Table 2). The ratios range from 0.63 to 1.13 for *N. duplicata* and from 0.74 to 1.01 for *N. delessertiana*. B: The averages of the size ratios are 0.85 ± 0.006 (SEM) for *N. duplicata* and 0.90 ± 0.005 (SEM) for *N. delessertiana* ($P < 0.0001$, non-parametric two-tailed t-test).

Based on these molecular data, what was believed to be two forms of *Neverita duplicata* represents two distinct species which appear no more closely related to each other than either of them is to *Neverita reclusiana, Neverita didyma,* or *Neverita josephinia* (COI). The COI tree indicates a closer relationship between the excavated form of *N. duplicata* and *N. reclusiana* than between the typical form of *N. duplicata* and *N. reclusiana*, despite nearly identical distances. 18S rRNA sequence distances support these interpretations as do the 16S rRNA and calmodulin intron data (Table 4). We therefore re-establish the taxon *N. delessertiana* (Récluz *in* Chenu, 1843), the earliest available name for the excavated form of *N. duplicata*, as a valid species distinct from *N. duplicata* (Say, 1822). *N. fossata* (Gould, 1847) and *N. texasiana* (Philippi, 1849) are junior synonyms of *N. delessertiana*. At the same time we select a specimen from the Récluz collection at the Museum d'histoire naturelle de Geneva (MHNG 1300/48/1) that we (contra Kabat *et al.* 1997) determined to be the specimen in the type lot that best represents Figures 6, 6a on plate „Natica 4“ of Chenu 1843, to be the lectotype of *Natica delessertiana* (Figs. 1B-D). This specimen is from Lousiana, USA (original label reads: "*Hab.* La Louisiane *prés du Missisipi,* Marguier") and measures 46.5 x 46.8 mm. The designation of a lectotype is required to fix this taxon since we determined that of the two specimens figured by Récluz in the publication of *N. delessertiana* (Chenu 1843: Natica pl. 4, figs. 5, 5a, 6, 6a) only one, depicted in figs. 6, 6a (reproduced here in Fig. 1A), represents *N. delessertiana* while the other specimen, depicted in figs. 5, 5a (reproduced here in Fig. 1G), appears to represent *N. duplicata*. It is noteworthy in this context that this second figured specimen is shown in Chenu (1843) with its operculum, while no operculated specimens are present in the type lot of *N. delessertiana*. Three additional specimens from the Récluz collection present at the MHNG and considered syntypes (Kabat *et al.* 1997) become paralectotypes: MHNG 1300/47/1 (Fig. 1E), MHNG 1300/47/2 (Fig. 1F), and MHNG 1300/48/2 (Fig. 1H).

2) Morphological characters

Neverita duplicata (Say, 1822)

Natica duplicata Say, 1822, p. 247-248.
+*Natica campechiensis* Récluz *in* Chenu, 1843, Natica pl. 4, figs. 2, 2a. See Fig. 1L.
+*Natica listeri* Philippi, 1850, p. 83, pl. 12, fig. 11 [plate published in 1850, text in 1851 (Smith & England 1937)].
See Fig. 1O.
+*Natica campeachiensis* Reeve, 1855 *ex* Récluz MS, pl. 1, figs. 1a, 1b (possibly emendation of *Natica campechiensis* Récluz *in* Chenu, 1843). See Fig. 1K.
Polinices duplicatus (Say, 1822). Porter 1974, p. 187.
not *Neverita duplicata* (Say, 1822). Kabat *et al.* 1997, p. 19 [is *Neverita delessertiana* Récluz *in* Chenu, 1843].

Type localities:

Natica duplicata Say, 1822 – "Coast of the United States" (Say 1822: 247).

Natica campechiensis Récluz *in* Chenu, 1843; *Natica campeachiensis* Reeve, 1855 *ex* Récluz MS - Campeachy Bay, Gulf of Mexico (Kabat *et al.* 1997).

Natica listeri Philippi, 1850 – „e sinu Campeche – der mexikanische Meerbusen" (Philippi 1849-53: 83).

Type material:

Natica duplicata Say, 1822 – „Cabinet of the Academy and Philadelphia Museum" (Say 1822: 247). According to Virginia Orr Maes, quoted in Mikkelsen & Mikkelsen (1984), Say's collection and types, which were originally present at the ANSP, were removed in 1825 to New Harmony, Indiana, where many were later destroyed in a fire. The remainder was returned to the ANSP in 1884. However, *N. duplicata* could not be found at the ANSP (G. Rosenberg *in litt.*, 03.04.1998).

Natica campechiensis Récluz *in* Chenu, 1843; *Natica campeachiensis* Reeve, 1855 *ex* Récluz MS – 3 syntypes, BMNH 1988039 (Kabat *et al.* 1997).

Natica listeri Philippi, 1850 – Unknown. Not at ZMB (Kabat & Kilias 1991).

Description: Shell of *N. duplicata* on average thicker, larger in diameter, more compact than *N. delessertiana* (Figs. 4 A–C); color of shell light to dark grey with dark apex; surface of whorls sculptured with minute, closely arranged transverse growth striae; darker colored band revolves around the spire below the suture, becoming gradually fainter, broader towards aperture; aperture ovate, large, highly oblique; umbilicus large, wide open, centered by broad funicle ending in massive button-like funicular callus; umbilicus partly covered by big, well-rounded callus connecting to rather thin anterior lobe of parietal callus; color of funicular callus mostly brownish, occasionally white or pinkish, depending on diet (Turner 1958).

Original description of *Natica duplicata* Say, 1822

As Say's type material most likely has been destroyed (see notes above under "Type material"), in the following we provide his original description which agrees well with what is currently considered to be this species:

> „*Natica duplicata*. Shell thick, sub-globose, cinereous, with black line revolving on the spire above the suture, and becoming gradually diluted, dilated, and obsolete in its course; within brownish-livid; a large incrasseted callous of the same colour extends beyond the columella, and nearly covers the umbilicus from above; umbilicus with a profound sulcus or duplication. Greatest length about two inches. Greatest breadt rather more. Inhabits the coast of the United States." (Say 1822: 247)

***Neverita delessertiana* (Récluz *in* Chenu, 1843)**

Natica delessertiana Récluz *in* Chenu, 1843, Natica pl. 4, figs. 5,5; 6,6 [figures only; no description]. See Figs. 1A-H.

+*Natica fossata* Gould, 1847, p. 263; Gould 1862, p. 202.

+*Natica texasiana* Philippi, 1849; Philippi 1849a [March], p. 158 [description only; no figures]*;* Philippi 1849-53, Syst. Conchyl. Cab., p. 37, pl. 5, f. 3 [plate published in 1849, text in 1852 (Smith & England 1937)], see Fig. 1M*;* Philippi 1849b, p. 457 [description only; no figures].

+*Natica texasiana var.* Philippi, 1849; Philippi 1849-53, Syst. Conchyl. Cab., p. 80, pl. 12, f. 10. See Fig. 1N.

+*Neverita duplicata* (Say, 1822). Kabat *et al.* 1997, p. 19 [the four syntypes of *Natica delessertiana* Récluz *in* Chenu, 1843 at the MHNG].

Type localities:

Natica delessertiana Récluz *in* Chenu, 1843 – "La Louisiana, pré du Missisipi", USA (Kabat *et al.* 1997: 19).

Natica fossata Gould, 1847 – „Florida Coast", USA (Gould 1847: 263).

Natica texasiana Philippi, 1849 – „Galveston", Texas, USA (Philippi 1849a: 159).

Natica texasiana var. Philippi, 1849 – „die Küste von Texas bei Galveston", USA (Philippi 1849-53: 38).

Type material:

Natica delessertiana Récluz *in* Chenu, 1843 – 4 syntypes, 1300/48.1, and 1300/48.2, MHNG 1300/47.1, 1300/47.2 (Figs. 1B-D, H, E, and F, respectively).

Natica fossata Gould, 1847 – Unknown (Johnson 1961).

Natica texasiana Philippi, 1849 – Unknown. Not at ZMB (Kabat & Kilias 1991).

Natica texasiana var. Philippi, 1849 – Unknown. Not at ZMB (Kabat & Kilias 1991).

Shell: Shell moderately small, more compact than *Neverita duplicata*; proportionately higher (see Fig. 4D); light to medium grey, apex dark, area below suture often decorated with reddish-brown band; aperture ovate, large, less oblique than in *N. duplicata*; umbilical callus triangular, brownish, crossing umbilicus to connect to body whorl (Figs. 4D-F); umbilicus deep, centered by moderately small umbilical cord terminating in a funicle; deeply excavated, longitudinally striated umbilical canal anterior to umbilical cord, separated from wall of body whorl by distinct ridge or keel; umbilical channel often retains periostracum.

Distinctive characters to separate *N. duplicata* from *N. delessertiana* morphologically: ratio of height to width, shape of umbilical area; less consistently, shape of umbilical callus.

Original descriptions of *Natica fossata* Gould, 1847

As no type material of *N. fossata* is known to still exist, and since the species was never figured, we in the following provide the original description to illustrate just how well this

description matches the original figure (see Fig. 1A) of *N. delessertiana*:

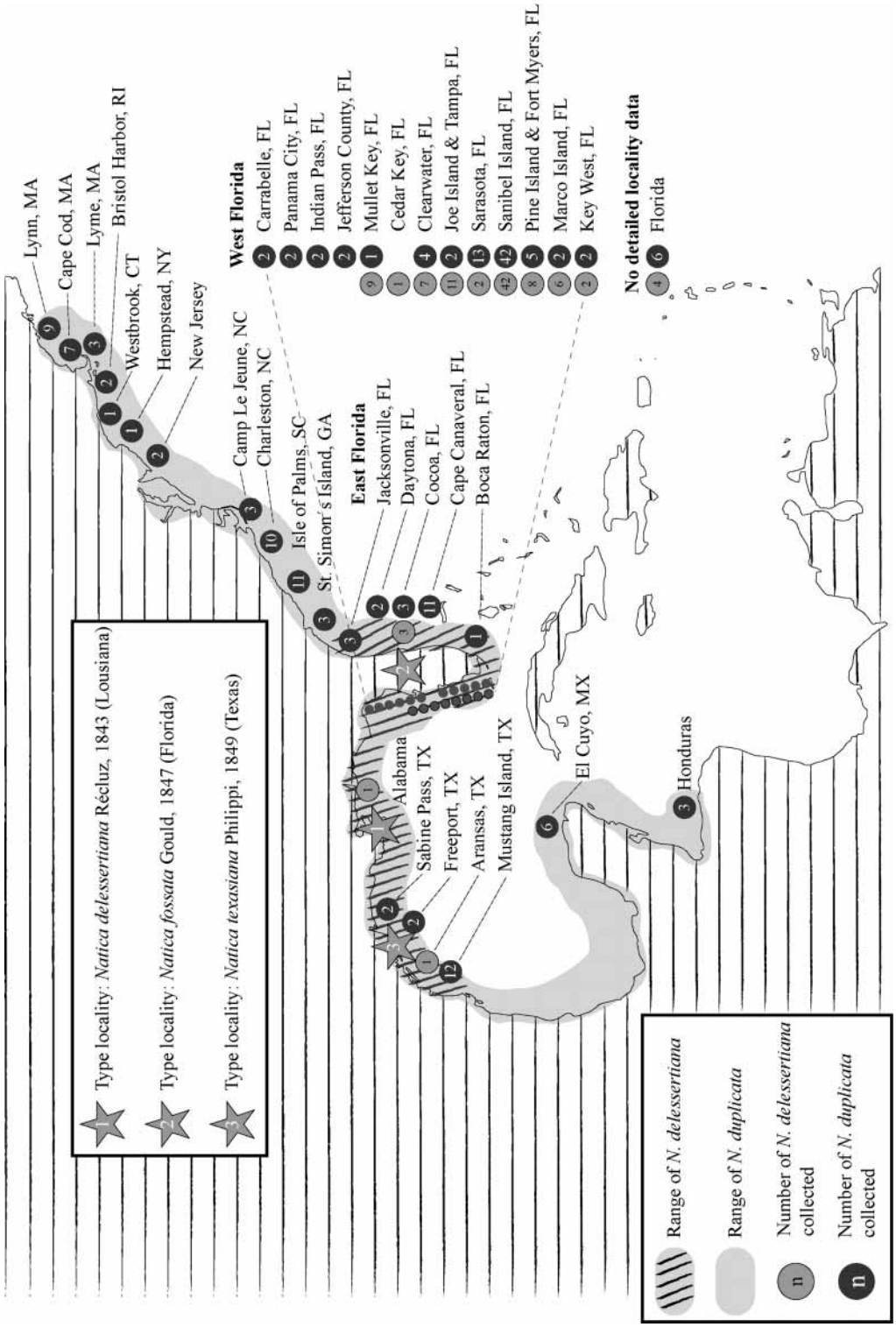
> "*Natica fossata*. Depressed shell, conically-rounded, solid, very finely striated, ashgrey-white, purplish above the suture, pale below; spire with 5 convex whorls, sloping, slightly angulated at the periphery; aperture semilunate, mouth light-brown, columellar callus halfways overhanging the umbilicus, chestnut-brown; umbilicus large, deep, circumscribed by a deep, precipitous channel which is covered with a straw-colored epidermis. Width 1 3/4 (44.31 mm), height 1 1/4 (31.65 mm) pollex. Habitat: Florida Coast." (Gould 1847: 263; first part, originally in Latin)
>
> „This would not at first sight be distinguished from *N. duplicata*, Say, which it precisely resembles in form and coloring. But the umbilical region is entirely different; and the deep, wide channel leading to the umbilicus and covered with epidermis is in striking contrast with the pale, polished region adjacent. In this aspect it is like *N. lamarckiana*." (Gould 1847: 263; second part, in English)

Original description of *Natica texasiana* Philippi, 1849

As no type material of *N. texasiana* could be located, in the following we provide an English translation of the original description which shows how closely this description resembles the original figure (see Fig. 1A) of *N. delessertiana*:

> ''*Natica texasiana* Ph. Semiglobular shell, obliquely conical, solid, light-weight, whitish brown-yellow and bluish; spire half the size of the aperture; umbilicus wide; brown, undivided callus, on the outside not separated from the last whorl, filling half the umbilicus. Height from apex to base of the aperture 23''' [50.1 mm]; diameter 26''' [56.7 mm]; external height of aperture 19''' (41.4 mm). Habitat: Galveston.
>
> Whorls quite convex; umbilical callus not really semicircular, but clearly triangular, on the outside not limited by a sulcus. It reminds one of *N. duplicata*, of which it is actually easily distinguished by, among other things, the more closed umbilicus. Variation α) shell a little more depressed, umbilicus simple; β) shell a little higher, umbilicus striated, on the outside bordered by a prominent margin, somewhat like a keel, a belt. Aperture more chestnut-colored on the inside, white at the base, as in the others.'' (Philippi 1849a: 158-159, originally in Latin)

FIGURE 6: Pattern of distribution of *Neverita duplicata* (grey area) and *Neverita delessertiana* (hatched grey area) based on the locality data of the specimens analyzed here (Table 2). The circles represent the locality and number of collected specimens of each species. Specimens of *N. duplicata* were collected from Massachusetts to Honduras. By contrast, *N. delessertiana* is found only on the eastern and western coasts of Florida and on the US Gulf coast from Florida to Texas. The stars mark the collecting sites of the type specimens of the synonymous taxa *N. delessertiana* (Récluz *in* Chenu, 1843), *Neverita fossata* (Gould, 1847), and *Neverita texasiana* (Philippi, 1849).



Morphological characteristics and geographical distribution

The shells of *N. delessertiana* and *N. duplicata* show many similarities (Figs. 1, 4). However, the two forms display several distinct differences. Separating characters are the umbilical channel, which is deeply excavated only in *N. delessertiana,* the shape (ratio of height to width) of the shell, which is proportionally heigher in *N. delessertiana,* the transition from the walls of the body whorl to the umbilicus, which is concave and discontinuous with a distinct ridge leading into the umbilical channel in *N. delessertiana* but convex and smooth in *N. duplicata*, and the funicular callus, which is triangular and overhanging the umbilicus from above in *N. delessertiana* but well-rounded and not spanning the umbilicus in *N. duplicata*. Both species have undistinguishable corneous opercula. Figure 4 shows line drawings of typical shells of *N. duplicata* and *N. delessertiana* which emphasize their distinct morphological characteristics also evident in Récluz´ original figure (Chenu 1843; pl. Natica, fig. 6, 6a). The shell morphologies and collecting localities of 278 specimens of *N. duplicata* from 37 localities (one lot had no detailed locality data) in the collection of the senior author were examined. Using the excavated umbilicus as the separating criterion, 97 specimens were determined to represent the excavated form, *N. delessertiana*, while all other specimens were determined as *N. duplicata sensu stricto* (n =181) (Table 2).

Neverita delessertiana is more globose than *N. duplicata* (Fig. 4). The average ratio of width to height of the shell is 0.90 ± 0.005 (SEM) for *N. delessertiana* and 0.85 ± 0.006 (SEM) for *N. duplicata* (Fig. 5). The difference is significant in a non-parametric two-tailed t-test including 97 pairs (p-value < 0.0001). On average, the specimens of *N. delessertiana* are smaller (mean: height = 26.67 mm; width = 29.37) than those of *N. duplicata* (mean: height = 31.6 mm; width = 36.51 mm). Thus, the mean height of *N. delessertiana* is 15.6% smaller than that of *N. duplicata* (not significant; p = 0.110), while the mean width is smaller by 19.6% (significant; p < 0.01). However, despite the statistical significance of the differences in the mean width and the ratio of width to height, individual specimens from the area of sympatric distribution (Fig. 6) can not be identified unambiguously based on size alone, due to the overlapping size distribution (Fig. 5).

The collecting sites of all investigated specimens were plotted on a map (Fig. 6, Table 2). *Neverita duplicata* is distributed from Massachusetts to Honduras (grey area in Fig. 6) while shells identified as *N. delessertiana* were only found on the eastern and western coasts of Florida, in Alabama, Lousiana, and Texas (hatched grey area in Fig. 6). Of the 52 specimens collected at 11 different localities north of Florida all 52 were *N. duplicata*. On the east coast of Florida, out of 23 specimens available for study 20 were *N. duplicata* (collected at 5 different localities) while only three specimens were *N. delessertiana* from one of the 5 localities (Cocoa, Florida). By contrast, out of 166 specimens from 13 different localities on the west coast of Florida (Table 2, Fig. 6), 88 specimens from 9 different localities were identified as *N. delessertiana* while 78 specimens were *N. duplicata,* collected from 12 different sites. In eight cases, both species were collected

from the same locality (Table 2, Fig. 6). One lot has no detailed locality data but the shells were also found in Florida. Single records of *N. delessertiana* can also be shown for Alabama (no detailed locality) and Texas (Aransas), defining the Western end of the distribution of *N. delessertiana*. By contrast, *N. duplicata* was also collected at El Cuyo (Mexico) and was additionally obtained from Honduras (no detailed locality data).

The distribution pattern of *N. delessertiana* as derived from the specimens studied suggests its occurance at the east coast of Florida and the US Gulf coast from Florida to Texas. The type localities of the three taxa *N. delessertiana*, *N. fossata*, and *N. texasiana*, indicated by stars in Figure 6, cover the same geographical range, supporting our conclusion from morphological characters that these taxa are synonymous.

Discussion

The morphological differences between the two forms of *Neverita* up to now were generally not considered sufficient for consistent species separation. Indeed, *N. delessertiana* is very similar to *N. duplicata* as mentioned by Tryon (1886). However, the excavation of the umbilical channel in virtually every case allows classification into one of two groups, representing *N. duplicata* and *N. delessertiana*. Other features such as the shape of the umbilical callus, overall size, and the height-to-width ratio are less reliable indicators for one of the two species.

The molecular differences support a clear separation between the specimens of *N. delessertiana and N. duplicata*. To circumvent the problem of potential molecular differences due to sex differences or different alleles, two mitochondrial sequences were analyzed in addition to the two nuclear gene fragments. The COI sequences of *N. delessertiana* and *N. duplicata* differ by 9.9% while both species show nearly identical differences (13.6% and 13.7%, respectively) to the COI sequence of *N. reclusiana*. Additionally, the 16S rRNA sequences, while being less divergent than the COI sequences, also show differences between *N. delessertiana* and *N. duplicata* (3%) which are similar for their distances from *N. reclusiana* (4%). Both genes, 16S rRNA and COI, are encoded in the mitochondrial genome which is inherited maternally. The clonal uniparental inheritance of the mitochondrial genome allows a direct reconstruction of bifurcating trees because the paternal mitochondrium is not retained by the zygote (Curole 1999). Mitochondrial gene sequences from all investigated specimens (Table 1) of the two species, *N. delessertiana* and *N. duplicata*, were found to be virtually identical within each species (Table 5) while differing across species (Table 4). On average, the COI sequences of distinct congeneric molluscan species have been reported to differ by 11.1%, while only 3.1% of those COI sequence pairs show less than 2% differences (Hebert 2003). In comparison, the COI sequences divergence of *N. delessertiana* and *N. duplicata* is 9.9%, similar to the COI sequence divergence of most (67.5%) congeneric molluscan species, which according to Hebert (2003) display 8-16% COI sequence divergence. The COI

sequences of specimens from *N. duplicata* from different localities differ in only 1.3%, and the COI sequence of *N. delessertiana* specimens from different localities on the western coast of Florida are either identical or differ in only a single position. Preliminary studies (Avise 2000; Hebert, 2003) indicate that intraspecific divergence is rarely larger than 2%, and most often less than 1%. The very small intraspecific distances recognized in this study between specimens from different localities (COI: 0.0-1.4%; 16S: 0.0-0.3%) may be interpreted as geographical differences, or may indicate polymerase errors. Thus, our data set leaves no doubt that the analyzed specimens of *N. delessertiana* and *N. duplicata* are two distinct species within the gastropod family Naticidae.

The bifurcating tree calculated from the COI sequences shows that *N. delessertiana* is arranged together with *N. didyma* and *N. reclusiana* in a terminal taxon while *N. duplicata* is arranged paraphyletic to these species. Like *N. reclusiana*, *N. didyma* is a Pacific species and both species are clustered together in a terminal taxon in a COI-based tree. Based on the present data of mitochondrial COI sequences, the Mediterranean *N. josephinia*, which we used as the outgroup, seems to be more closely related to *N. duplicata* than to *N. delessertiana*. This result further supports the interpretation that the sequences isolated here belong to two different species. Furthermore, our data, particularly our analysis of mitochondrial sequences, suggests that the morphological variation observed is not linked to sex differences as advocated by Dall (1892) and Jacobson (1973).

Nuclear genes (18S, Cal), which were also analyzed, corroborate the mitochondrial data. Both genes show considerable differences between *N. delessertiana* and *N. duplicata*, which may be taken as supporting evidence for the conclusion previously reached based on COI and 16S sequence data. The 18S sequences of both, *N. delessertiana* and *N. duplicata*, show more differences among each other than to *N. reclusiana*, which means that the 18S sequence of *N. delessertiana* is more closely related to the 18S of *N. reclusiana* than to the 18S of *N. duplicata*. The Cal sequences of *N. delessertiana* and *N. duplicata* show 6.7% differences, which in other caenogastropod groups (e.g., Conidae) is a level of difference usually observed between two distinct species (Duda 2001). In contrast to the mitochondrial DNA, the nuclear genes are inherited biparental and thus may be multiallelic. However, taking together the nuclear and mitochondrial data leaves no doubt that *N. delessertiana* and *N. duplicata* are two distinct species.

The geographical data of our specimens show a wide distribution range for *N. duplicata* from Massachusetts to Honduras and correspond with literature data compiled by Rosenberg (2005). By comparison, the pattern of distribution of *N. delessertiana* is quite different. This species occurs at the eastern and western coasts of Florida, and the US Gulf coast to Texas. Only in this area *N. duplicata* and *N. delessertiana* live sympatrically. The geographical data presented show that the two species have clearly different distributions. This further corroborates that the morphological variation observed is not explained by sex differences. The significant difference of the height-to-width ratios of *N. delessertiana* and *N. duplicata* also supports the existence of two different species. However, this morphological character cannot be used for direct species identification due

to an overlapping Gaussian distribution (Fig. 5A).

In summary, while the morphological data alone were not persuasive enough to unambiguously prove the existence of two separate species, the molecular data presented here support and validate the separation of *N. delessertiana* and *N. duplicata* at the species level.

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