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REVISION OF THE SPECIES COMPLEX '*NERITINA PULLIGERA*' (GASTROPODA, CYCLONERITIMORPHA: NERITIDAE) USING TAXONOMY AND BARCODING

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NERITINA
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ABSTRACT. – Morphometric analyses and molecular analyses based on the COI gene were performed on nominal taxa of the genus *Neritina* supposed to represent the same species, *Neritina pulligera* (Linnaeus, 1767). The results obtained have removed any doubt about the supposed synonymies. In particular, it appears that *N. stumpffi* Boettger, 1890 and *N. knorri* (Récluz, 1841), previously considered as sub-species, are valid separate species different from *N. pulligera*. Indeed, these two taxa are sympatric, morphologically differentiable and genetically distant by more than 11 % for the COI. The same applies to *N. petitii* (Récluz, 1841), *N. canalis* Sowerby, 1825, *N. iris* Mousson, 1849, *N. asperulata* (Récluz, 1843) and *N. powisiana* (Récluz, 1843), which have been confirmed as valid distinct species from *N. pulligera*. The Indo-Pacific distribution of *N. stumpffi* is proven, that of *N. knorri* is yet to be confirmed.

INTRODUCTION

Neritidae form one of the most ancient families of gastropods (Bouchet & Rocroi 2005). This family has a huge ability to adapt and live in unfavorable and fluctuating habitats where there is little competition with other organisms (Baker 1923). In his extensive work on the Neritidae of the world, Eichhorst (2016a, b) proposed a division of this family into two subfamilies, 16 genera and more than 275 species. Unfortunately, this work is essentially based on a morphological analysis. The species of the Neritidae family occupy highly varied habitats, essentially in tropical and subtropical regions, ranging from marine to quasi-terrestrial, and including brackish and freshwater (Kano *et al*. 2002, Limpalaer 2009, Abdou *et al*. 2015, Eichhorst 2016a).

The phylogeny of this family has been studied by Quintero-Galvis & Castro (2013) based on two genes, COI and 16S rRNA. Their study confirmed the monophyly of the *Nerita* genus, the phylogeny of which, studied by Frey & Vermeij (2008) and Frey (2010) using the COI, 16S rRNA and nuclear ATPSα genes, is the most complete. Quintero-Galvis & Castro nonetheless recommend additional analyses. The genus *Theodoxus* has been relatively well studied by Bunje & Lindberg (2007), based on the COI and 16S rRNA genes, who still suggest a systematic revision. Finally, in his study on the upstream migratory behavior of amphidromous nerites, Kano (2009) performed a partial phylogenetic analysis of the genus *Neritina*, based on the COI gene. A more complete phylogeny is therefore yet to be performed.

Several nominal species of Neritidae were described in the 19th century on the basis of the morphological characteristics of the shell and operculum, mainly its form, size, color, consistency and sculpture. Subsequently, certain authors estimated that the morphological differences observed in some species were due rather to the phenotypic plasticity of the shells, well known in molluscs (Puillandre 2010), and placed them in synonymy, sometimes as a variety or sub-species. As a result, in the genus *Neritina*, according to the "Catalogue of Life" (Seddon & Rowson 2015), dozens of species have been listed as synonymous with *Neritina pulligera* (Linnaeus, 1767), a species described as from India ("*Habitat in Indiae fluviis*"), and reportedly widely distributed in the Indo-Pacific region. It is in this context that *Neritina stumpffi* Boettger, 1890 and *Neritina knorri* (Récluz, 1841), two well-known species in the Indian Ocean, have been considered by certain authors as representatives of *N. pulligera* in this region (Starmühlner 1983, Seddon & Rowson 2015, Gerlach *et al*. 2016). According to Starmühlner (1983, 1984), Haynes (1988, 1990, 2001, 2005), Bandel (2001) and Gerlach *et al*. (2016), *N. pulligera* supposedly covers practically the entire Indo-Pacific Basin (excluding French Polynesia) as far as East Africa (Australia, Fiji, Nicobar, Andaman, Guam, Malaysia, Indonesia, Micronesia, Papua-New Guinea, Philippines, New Caledonia, Solomon Islands, Vanuatu, Thailand, Comoros, Seychelles, Madagascar, Mascarene Islands, Mozambique, Kenya, Tanzania and South Africa).

This species reportedly presents a large morphological variability with several sub-species. Tryon (1888), for

example, considers that *N. petitii* (Récluz, 1841), *N. canalis* Sowerby, 1825 and *N. knorri* (Récluz, 1841), are varieties of *N. pulligera*, whereas Fischer-Piette & Vukadinovic (1974) consider that *N. knorri* is a junior synonym. Further, Eichhorst (2016b) cited *N. stumpffi* as junior synonym of *N. knorri,* and Starmühlner (1983) and Gerlach *et al*. (2016) state that they are sub-species despite the fact that the former has cited them as living in sympatry in the Seychelles and Comoros. According to Backeljau *et al*. (1986), there is doubt as to the reality of these being sub-species. Valade *et al*. (2007) agree with this opinion insofar as they were easily able to separate them in the field (no intermediate forms), and the first author of this article has often found them in sympatry in several different locations in the Comoros (Valade *et al*. 2007). Boettger (1890) had already reported this sympatry in his original description of *N. stumpffi*. Griffiths & Florens (2006) question the actual presence of *N. knorri* in the Mascarene Islands, but the Muséum national d'Histoire naturelle, Paris (MNHN) has samples of *N. knorri* from Mauritius in its historical collection (Coll. Denis 1945, identified as *N. pulligera*). Furthermore, a sample from this island was identified by Kano (2009) as *N. delestennei* Récluz, 1853, on the basis of a molecular sequence. *Neritina bruguierei* (Récluz, 1841) is cited in the World Register of Marine Species (WoRMS) as a synonym of *N. pulligera* (Rosenberg 2015, Bouchet 2016), whereas this species is considered valid by Eichhorst (2016b: 704) and is thought to be present in Melanesia in the Pacific and Comoros and Seychelles in the Indian Ocean. According to Seddon & Rowson (2015), it therefore appears likely that there are some 40 taxa considered synonyms of *N. pulligera*.

We will study here the complex *Neritina pulligera* based on samples or molecular sequences to which we have access and concerning the main taxa of this complex cited by Seddon & Rowson (2015), namely *N. pulligera, N. stumpffi, N. knorri, N. petitii*, *N. canalis, N. iris*, *N. delestennei, N. asperulata* and *N. powisiana*. The aim is to remove doubt surrounding the validity of all or some of these taxa using morphometric and genetic (COI gene) tools. Within this context, this article also claims to clarify the status of the nominal taxa *N. stumpffi* and *N. knorri*, together with their spatial distribution within the Indo-Pacific biogeographic province.

MATERIAL AND METHODS

Sampling: A large part of the material concerning the Indian Ocean was collected during various field missions. Such is the case for the Comoros with two campaigns in 2005 and 2006. Neritidae were collected from 30 October to 4 November 2005 on Mwali (Mohéli) and Ndzuwani (Anjouan) during an inventory mission funded by Conservation International (Keith *et al*. 2006). Another mission, led by the ARDA ('Association Réunionnaise de Développement de l'Aquaculture') took place in Maore (Mayotte) from 13 to 21 May 2006 (Valade *et al*. 2007). Additional collections were made in Mohéli on 30 and 31 December 2013. A specific mission organized jointly by UMR 7208 BOREA and USR 3278 CRIOBE, enabled us to collect samples in Moorea and Tahiti, in French Polynesia, in February 2014. Additionally, we have benefited from samples collected by BOREA members and partners in the Indo-Pacific region. In this way, we expanded our material with samples from the Seychelles, New Caledonia, Samoa, Fiji, Mayotte, Bali, Futuna and Japan (Okinawa) (Table I). These specimens were deposited in MNHN (see tables I and II).

In the field, the nerites were mainly collected on sight. They are almost all visible to the naked eye, as they live attached to blocks of rock substrate. The specimens were preserved in alcohol at 70 % or 95 %. We identified the species using the literature, original descriptions (Boettger 1890, Récluz 1841, Récluz 1853) and the collections held by the MNHN, of which: *N. knorri* (paralectotype MNHN-IM-2012-18281: see Fig. 6A), *N. delestennei* (paratypes MNHN-IM-2012-18260: see Fig. 6C) and *N. petitii* (holotype MNHN-IM-2000-32752). Morphological characteristics of the shell and operculum were used, as in original descriptions, for identification, mainly the shape, size, color, consistency and texture.

Given the conservation quality of some specimens from the Comoros, molecular analysis was only possible on a limited number of samples from the 2005 and 2006 sampling missions. Dry material from the MNHN's dry collection and material from its non-sequenced wet collection were also studied for the morphometric analysis (Table II).

Additionally, we completed our sampling by adding gene sequences from Genbank, specimens from the Pacific (see details in Table I) used for phylogenetic analysis by Kano (2009). All the locations for which we had samples and the represented taxa at each location are shown in Fig. 1.

Morphometric analyses: In all, 75 sequenced and 60 non-sequenced specimens were used for the morphometric analysis. Of these 135 specimens analyzed, 37 would appear to belong to *N. stumpffi*, 33 to *N. knorri*, 5 to *N. delestennei*, 19 to *N. pulligera*, 20 to *N. petitii* and 21 to *N. canalis* (see Tables I and II). Specimens from the MNHN historic collection were incorporated into this analysis. They come from both the Indian and Pacific oceans (Table II).

In addition to the shell dimensions, we took into account other criteria such as color and sculpture in particular, to assess the morphological variability. For this purpose, measurements were taken as indicated in Fig. 2 on specimens of the taxon of the complex '*N. pulligera*', for which we had sufficient fresh material or historic collections, using an Absolute Digimatic Caliper, model No CD-15DC, from Mitutoyo Corp, with a margin of error of 0.02 mm. Four dimensions, length (L), width (W), length of aperture (LA) and width of aperture (WA) were measured. The ratio of L/W and LA/WA were calculated. For quantitative variables, we only used the ratios L/W and LA/WA to avoid any bias that could be introduced by an allometric effect. In the case of qualitative variables, we have taken into account

Table I. – List of species used for the molecular analysis. The identification number is shown for the GenBank sequences and for material deposited at MNHN (MNHN ID). The individuals corresponding to the GenBank sequences from Kano (2009), as well as those marked (*), were not included in the morphometric analyses.

Species	No of individuals	Location	Year	MNHN ID	GenBank ID
Neritina stumpffi	3	Ndzuwani, Comoros	2005	IM-2013-62796	MF407571
				IM-2013-62797	MF407648
				IM-2013-62798	MF407639
Neritina stumpffi	2	Mwali, Comoros	2005	IM-2013-62794	MF407637
				IM-2013-62795	MF407638
Neritina stumpffi	2	Mwali, Comoros	2008	IM-2013-62792	MF407619
				IM-2013-62793	MF407620
Neritina stumpffi	14(1*)	Mwali, Comoros	2013	IM-2013-62778	MF407618
				IM-2013-62779	MF407617
				IM-2013-62780	MF407636
				IM-2013-62781	MF407622
				IM-2013-62782	MF407624
				IM-2013-62783	MF407630
				IM-2013-62784	MF407631
				IM-2013-62785	MF407626
				IM-2013-62786	MF407615
				IM-2013-62787	MF407632
				IM-2013-62788	MF407627
				IM-2013-62789	MF407633
				IM-2013-62790	MF407628
				IM-2013-62791	MF407629
Neritina stumpffi	1	Maore, Comoros	2006	IM-2013-62802	MF407642
Neritina stumpffi	3	Maore, Comoros	2014	IM-2013-62799	MF407616
				IM-2013-62800	MF407634
				IM-2013-62801	MF407635
Neritina stumpffi	3	Seychelles	2012	IM-2013-62803	MF407625
				IM-2013-62804	MF407640
				IM-2013-62805	MF407641
Neritina stumpffi	2	New Caledonia	2013	IM-2013-62806	MF407645
				IM-2013-62807	MF407623
Neritina stumpffi	1	Futuna	2014	IM-2013-62809	MF407621
Neritina sp. Kuro	2	Okinawa, Japan			AB477505
					AB477506
Neritina sp. Kuro	1	Guam, Micronesia			AB477507
Neritina asperulata	3	Okinawa, Japan			AB477472
					to AB477474
Neritina asperulata	1	Cebu, Philippines			AB477475
Neritina asperulata	2	Guam, Micronesia			AB477476
					AB477477
Neritina asperulata	4	Santo, Vanuatu	2006		AB477478
					AB477481
					to AB477483
Neritina asperulata	2	Guadalcanal,			AB477479
		Solomon Islands			AB477480
Neritina powisiana	1	Santo, Vanuatu	2006		AB477500
Neritina sp. Suji	2	Okinawa, Japan			AB477509
					AB477510
Neritina sp. Suji	2	Pohnpei, Micronesia			AB477512
					AB477513
Neritina sp. Suji	1	Guam, Micronesia			AB477511
Neritina petitii	3	New Caledonia	2013	IM-2013-62849	MF407608
				IM-2013-62850	MF407610
				IM-2013-62851	MF407646
Neritina petitii	3	Okinawa, Japan	2014	IM-2013-62852	MF407609
				IM-2013-62853	MF407614
				IM-2013-62854	MF407613

Table I. Continued.

Species	No of individuals	Location	Year	MNHN ID	GenBank ID
Neritina petitii	3	Okinawa, Japan			AB477495 to AB477497
Neritina petitii	2	Guadalcanal, Solomon Islands			AB477498 AB477499
Neritina petitii	3	Fiji	2013	IM-2013-62855	MF407647
				IM-2013-62856	MF407611
				IM-2013-62857	MF407612
Neritina iris	2	Palawan, Philippines			AB477492 AB477493
Neritina iris	2	Amami, Japan			AB477490
		Okinawa, Japan			AB477491
Neritina iris	1	Pohnpei, Micronesia			AB477494
Neritina iris	1*	Bali, Indonesia	2014	IM-2013-62858	MF407607
Neritina delestennei	2	Amami, Japan			AB477486
		Okinawa, Japan			AB477487
Neritina delestennei	1	Guam, Micronesia			AB477488
Neritina delestennei	1	Mauritius			AB477489
Neritina delestennei	3	Okinawa, Japan	2014	IM-2013-62842 to IM-2013-62844	MF407580 to MF407582
Neritina knorri	1	Maore, Comoros	2006	IM-2013-62819	MF407644
Neritina knorri	1	Mwali, Comoros	2008	IM-2013-62818	MF407643
Neritina knorri	8	Mwali, Comoros	2013	IM-2013-62810 to IM-2013-62817	MF407572 to MF407579
Neritina pulligera	2	Amammi, Japan			AB477501
		Okinawa, Japan			AB477502
Neritina pulligera	1	Guam, Micronesia			AB477503
Neritina pulligera	1	Samoa			AB477504
Neritina pulligera	1	Bali, Indonesia	2014	IM-2013-62847	MF407606
Neritina pulligera	1	Okinawa, Japan	2014	IM-2013-62848	MF407605
Neritina canalis	1	Vanuatu	2006		AB477484
Neritina canalis	1	Samoa			AB477485
Neritina canalis	5	Futuna	2014	IM-2013-62820 to IM-2013-62824	MF407598 to MF407602
Neritina canalis	1	Tahiti, Polynesia	2014	IM-2013-62825	MF407585
Neritina canalis	16(1*)	Moorea, Polynesia	2014	IM-2013-62826 to IM-2013-62830	MF407587 to MF407591
				IM-2013-62831	MF407603
				IM-2013-62832	MF407586
				IM-2013-62833	MF407597
				IM-2013-62834	MF407596
				IM-2013-62835	MF407604
				IM-2013-62836	MF407583
				IM-2013-62837	MF407584
				IM-2013-62838 to IM-2013-62841	MF407592 to MF407595
Outgroups					
Septaria porcellana	1	Okinawa, Japan			AB477515
Septaria porcellana	6	Okinawa, Japan	2014	IM-2013-62867 to IM-2013-62872	Pending
Septaria porcellana	5	French Polynesia	2014	IM-2013-62862 to IM-2013-62866	Pending
Neritilia rubida	1	Japan			AB102712
Neritilia rubida	1	Mwali, Comoros	2013	IM-2013-62859	Pending
Neritilia rubida	1	Mauritius	2013	IM-2013-62860	Pending
Neritilia rubida	1	Reunion Island	2013	IM-2013-62861	Pending
Total (genetic)	135 o/w				
Total (morphometry)	75				

Table II. – Dry and wet (in alcohol) specimens used solely for the morphometric analysis

Country	Collection/year	Identification	No of specimens	MNHN ID
Maore, **Comoros**	2006	*N. stumpffi*	3	IM-2014-6424 to IM-2014-6426
Maore, **Comoros**	2006	*N. knorri*	6	IM-2014-6418 to IM-2014-6423
Ndzuwani, **Comoros**	2005	*N. stumpffi*	3	IM-2014-6427 to IM-2014-6429
Mauritius	Denis Coll., 1945	*N. knorri*	5	IM-2012-19913
Madagascar	Soula Coll., 1968	*N. knorri*	4	IM-2012-19915
Indochina	Denis Coll., 1945	*N. pulligera*	1	IM-2012-19920
Perak		*N. knorri*	1	IM-2012-19918
Japan (Okinawa)	2014	*N. delestennei*	2	IM-2013-62845 IM-2013-62846
New Caledonia	Arnoul Coll., 1927	*N. knorri*	2	IM-2012-19916
	Caillot Coll. 2013	*N. petitii*	8	IM-2012-19922
		N. stumpffi	1	IM-2013-62808
Philippines	Marche Coll., 1882	*N. pulligera*	4	IM-2012-19919
	Cuming Coll., 1843	*N. petitii*	3	IM-2012-19921
	Portes Coll., 1860	*N. pulligera*	4	IM-2012-19910
Tahiti	Denis Coll., 1945	*N. knorri*	5	IM-2012-19917
New Guinea	Lesson & Carnot	*N. pulligera*	2	IM-2012-19914
	Denis 1945	*N. pulligera*	1	IM-2012-19911
Australia	Péron & Lesueur 1803	*N. pulligera*	5	IM-2012-19912
	Total		**60**	

Fig. 1. – Source localities for the samples studied and representation of taxa. The outline symbols represent dry material from the historical collections of the MNHN (Paris).

the dominant color of the parietal wall (CPW), its posterior part in particular, and the apertural rim (AR), according to whether it is continuous or interrupted. The color has been coded as: purplish-blue (1), red or whitish (2), russet (3), greyish-black (4), reddish (5), red-orange (6). The apertural rim is coded 0, if it is continuous, and 1 if it is interrupted. All the data was then computed using Principal Component Analysis (PCA) to evaluate morphometric variation among specimens and to identify

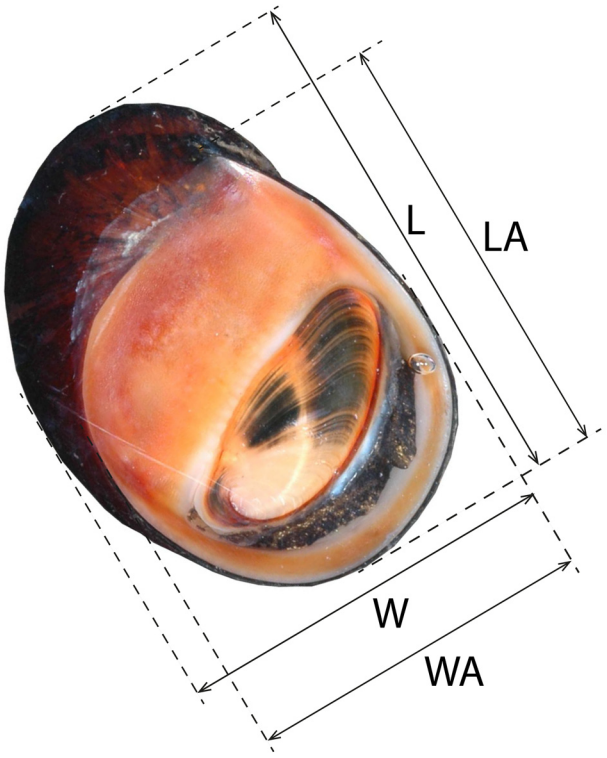

Fig. 2. – Shell measurement protocol. *N. stumpffi*.

variables contributing substantially to that variation (Konan *et al*. 2010). This statistical analysis was performed with the Statistica 10 (StatSoft 2011) software package. A linear regression was also performed for L and W, and LA and WA calculating the determination coefficient (R^2) to see if these dimensions were correlated.

Genetic analyses: Our study concerned 135 specimens analyzed using the coding mitochondrial gene for the first subunit of the cytochrome oxidase (COI). For each individual selected, we sampled several milligrams of foot muscle tissue. The total genomic DNA was extracted using the semi-automatic Eppendorf epMotion 5075 robot in accordance with the NucleoSpin® 96 Tissue Core kit from Macherey-Nagel. The DNA was amplified by PCR using the universal primers developed by Folmer *et al*. (1994), LCOI1490 (5'-GGTCAACAAATCATAAAGATATTGG–3') and HCO2198 (5'–TAAACTTCAGGGTGACCAAAAAATCA–3'). The amplification was performed in a final volume of around 21 µl containing approximately 12.94 µl of sterile water, 2 µl of Taq buffer, 1 µl of DMSO (dimethyl sulfoxide), 1 µl of BSA (bovine serum albumin), 1.5 µl of $MgCl_2$, 0.80 µl of dNTP (deoxynucleotide triphosphate) mix at 6.6 mM, 0.32 µl of each primer, 0.12 µl of Qiagen Taq polymerase and 1 to 1.5 µl of DNA. The PCR was performed using a thermocycler Bio-Rad C1000 Touch™ , in accordance with the following programme: 3 min of initial denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, hybridization 40-42 °C for 30 s, elongation at 72 °C for 45 s, followed by 5 min final elongation at 72 °C. The PCR products were displayed by electrophoretic migration on agarose gel at 1.5 %, prepared with TBE (Tris/Borate/EDTA) buffer and a DNA intercalation agent, ETB (ethidium bromide). The sequencing of the PCR products was performed by Eurofins (http://www.eurofins.fr) using the same primers.

The partial sequences of the COI gene were checked and cleaned, and the chromatograms of the two strands (forward and reverse) were compared using the CodonCode Aligner 5.0.2 software (Codon Code Corporation). The sequences were aligned by ClustalW using the MEGA 6.0 software (Tamura *et al*. 2013). All sequences have been deposited in Genbank (accession numbers MF407571-MF407648). We enriched our sampling with sequences AB477472 to AB477514, available in DDBJ/EMBL/Genbank, which were used for a phylogenetic analysis of certain species of the genus *Neritina* (Kano 2009), and we used *Septaria porcellana* and *Neritilia rubida* as outgroup (see Table I). The best nucleotide substitution method, with distribution by codon position, was selected by JModelTest 2.1.1 (Darriba *et al*. 2012). This is the HKY+G+I model. Our molecular data was analyzed using the Bayesian inference (BI) method, using the MrBayes 3.2 software (Ronquist *et al*. 2012). The BI involved two separate analyses of 4 Markov chains, using the Monte Carlo method, of 10 million generations, sampled at the rate of one tree every 200 generations. The resultant consensus tree was calculated after eliminating 10 % of the trees generated as burn-ins while making sure that stationery phase had been reached.

The Automatic Barcode Gap Discovery (ABGD) tool: ABGD, developed by Puillandre *et al*. (2012a), is a method based exclusively on the genetic distance between the DNA sequences (Puillandre *et al*. 2012b). Using the barcode gap, it is possible to provide primary hypotheses of species delimitations from several genetic sequences. The details of the method are provided by Puillandre *et al*. (2012a). We used the online version (http://wwwabi.snv.jussieu.fr/public/abgd/) to analyze our COI gene data, using the default ABGD parameters.

RESULTS

Morphometric analysis

The linear regressions, concerning the length and width of the shell on the one hand, and the length and width of aperture of the shell on the other hand, both reveal a strong correlation between the two dimensions for the 6 taxa of the genus *Neritina* analyzed, with a determination coefficient (R^2) of 0.98 for L and W ($y = 0.7457x - 0.186$) and 0.97 for LA and WA ($y = 0.8934x - 0.158$). This coefficient is also high for each taxon considered separately (Table III).

The multifactorial principal components analysis (PCA), plotted on the graphs in Fig. 3 and 4, clearly revealed a morphological distinction of the taxa. As shown in Table IV, the first three principle components (PC) explain 88.11 % (41.16 % for PC1, 25.79 % for PC2

Table III. – Determination coefficient (R^2) of each of the 6 taxa of the genus *Neritina*, concerning the width and length of the shell and its aperture.

	N. stumpffi	*N. knorri*	*N. delestennei*	*N. pulligera*	*N. petitii*	*N. canalis*
R^2 for L and W	0.98	0.99	0.81	0.97	0.97	0.97
R^2 for LA and WA	0.93	0.97	0.91	0.91	0.97	0.96

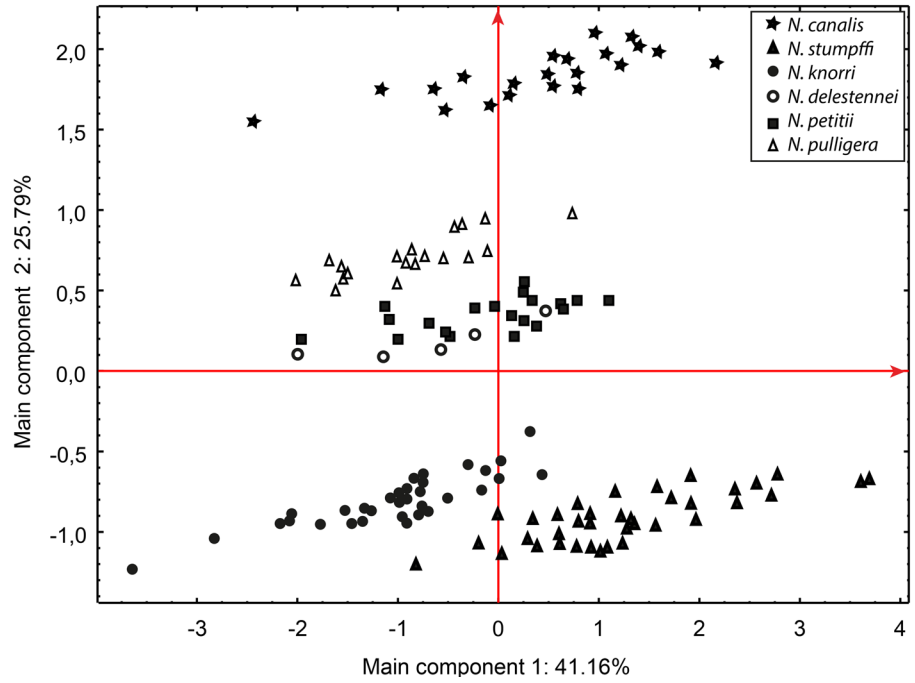

Fig. 3. – Cloud of points for the first main two components of the Principal Component Analysis (PCA) of 135 individuals of *Neritina stumpffi*, *N. knorri*, *N. canalis*, *N. petitii*, *N. pulligera* and *N. delestennei*.

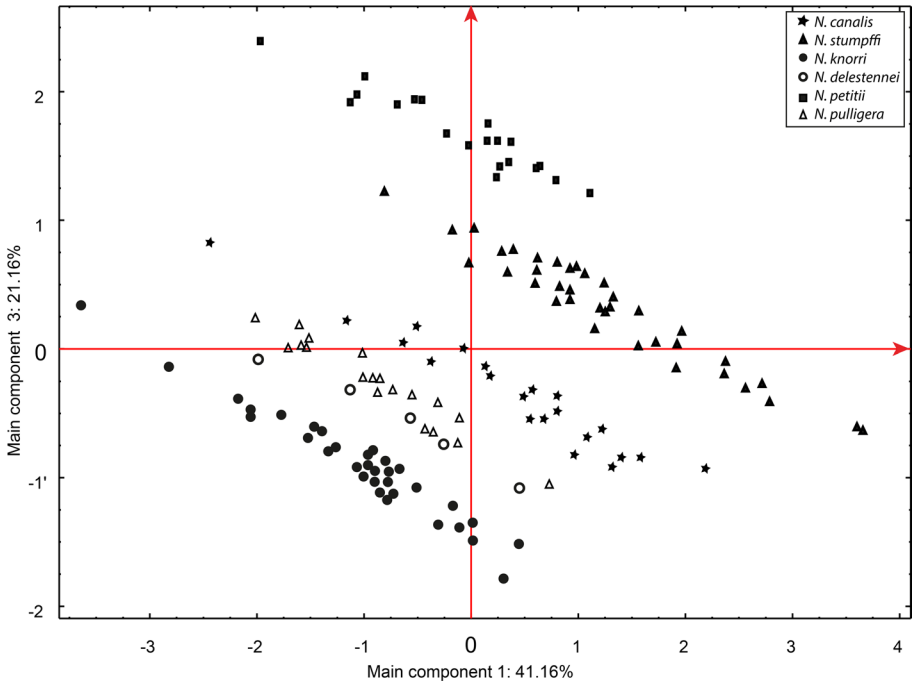

Fig. 4. – Cloud of points for the first and the third main components of the Principal Component Analysis (PCA) of 135 individuals of *Neritina stumpffi*, *N. knorri*, *N. canalis*, *N. petitii*, *N. pulligera* and *N. delestennei*.

and 21.16 % for PC3) of the total variance. The first two PCs (1 and 2) explain 66.95 % (Fig. 3), and PCs 1 and 3 explain 62.32 % (Fig. 4) of the total variability. PC1 is mainly defined by L/W and LA/WA ratios of 0.85 and 0.80, respectively (Table IV). For this component, the individuals located essentially on the right belong to the species *N. stumpffi* and *N. canalis*. They indeed have an oblong shape that is relatively more elongated

Table IV. – Factorial coordinates, specific values or percentages of the total variance explained by the four factorial axes of the analysis of the main components of *N. stumpffi, N. knorri, N. delestennei, N. pulligera, N. petitii* and *N. canalis*.

Variables	Main component 1	Main component 2	Main component 3	Main component 4
L/W ratio	0.85	0.00	-0.17	-0.50
LA/WA ratio	0.80	0.17	-0.35	0.46
CPW	0.08	0.92	0.38	-0.04
AR	-0.53	0.40	-0.74	-0.12
Specific values	1.65	1.03	0.85	0.47
Cumulative specific values	1.65	2.68	3.53	4.00
Total variance (%)	41.16	25.79	21.16	11.89
Cumulative total variance (%)	41.16	66.95	88.11	100.00

Table V. – Average genetic distance, on the COI gene, between the groups identified by the ABGD tool. G1: *N. petitii*, G2: *N. pulligera*, G3: *N. asperulata* 1, G4: *N. asperulata* 2, G5: *N. iris*, G6: N. sp. Suji, G7: *N. stumpffi* 1, G8: *N. stumpffi* 2, G9: *N. canalis*, G10: *N. knorri*, G11: *N. delestennei*. In bold, the genetic distances between the two double-groups A and B; shaded, the distances between *N. pulligera* and the other taxa. S: sympatric, nS: not sympatric, M = Morphologically identical, M ≠ Morphologically different.

Distinct groups/ clades	G1/D	G2/H	G3/B1	G4/B2	G5/E	G6/C	G7/A	G8/A	G9/I	G10/G	G11/F
Taxon names	*N. petitii*	*N. pulligera*	*N. asperulata*	*N. asperulata*	*N. iris*	*N. sp. Suji*	*N. stumpffi*	*N. stumpffi*	*N. canalis*	*N. knorri*	*N. delestennei*
G1/D		7.82 %	11.89 %	11.90 %	6.77 %	3.11 %	11.77 %	12.82 %	8.02 %	7.04 %	7.47 %
G2/H	S, M≠		10.75 %	10.82 %	5.46 %	7.56 %	11.68 %	11.41	4.10 %	5.99 %	5.68 %
G3/B1				**9.04 %**	9.67 %	12.04 %	11.86 %	11.87 %	11.37	10.34 %	10.92 %
G4/B2			nS, M=		9.67 %	12.03 %	13.33 %	13.19 %	10.64 %	10.14 %	10.92 %
G5/E						6.96 %	10.90 %	11.24 %	4.67 %	4.95 %	5.09 %
G6/C							12.07 %	13.38 %	7.67 %	7.33 %	7.94 %
G7/A		S, M≠						**2.61 %**	11.16 %	11.03 %	10.54 %
G8/A		S, M≠					S, M=		11.21 %	11.94 %	11.66 %
G9/I		S, M≠					S, M≠			5.09 %	5.08 %
G10/G		nS, M≠					S, M≠	S, M≠	nS, M≠		3.14 %
G11/F		S, M≠					S, M≠		nS, M≠	nS, M≠	

than the 4 others (Figs 3, 4), essentially located to the left of PC1. They might be confused by shape or color, but *N. stumpffi* has a characteristic operculum with a very dark brown, nearly black outer surface (Boettger 1890, Starmühlner 1983), and a densely granulated parietal wall. Some samples have a yellowish arc-strip from the operculum nucleus stretch, which is divided Y-like (see Fig. 2). *N. canalis* has a "channel" between the parietal edge and the upper end of the lip (apertural rim interrupted), which the former does not have. The 3 taxa *N. pulligera*, *N. petitii* and *N. delestennei*, proportionally the largest in size and of comparable dimensions, are close to each other, but other criteria relating to color and aperture, in particular, can be used to distinguish them. *N. petitii* is mainly distinguished from the two others by the absence of the channel between the upper end of the lip and the wall (apertural rim continuous), but also by its reddish to yellowish aperture, dilated towards the columellar margin. *N. delestennei* is distinguishable from *N. pulligera* by its uniform brown color, its aperture bordered by a russet wall calloused at the rear and flat and inclined at the front, and a lip bordered internally with a white area, under which the colour is dark orange or olive-greenish. The shell of *N. pulligera* is dark brown, almost black, or sometimes olive-ish; its parietal wall is flat, black-greyish, but may vary towards yellowish; a wide orange band, parallel with the lip, provides the color of its aperture. *N. knorri* is distinguishable from the others by its relatively smaller size compared to the first three, its uniformly black periostracum, its purplish-blue parietal wall that is slightly convex towards the rear, and its internally saffron-red lip. PC2 is highly associated with the color of apertural wall (CPW) and PC3 is mainly defined by the apertural rim (AR), for which the factorial coordinates are 0.92 and –0.74, respectively (Table IV). The projection of the individuals on the factorial plane PC1 × PC3 is represented by Fig. 4. The taxa *N. stumpffi* and *N. petitii*, the apertural rim of which is continuous, are situated on the positive side of the axis; those whose apertural rim is discontinuous are on the negative side. Of similar size (Fig.3), *N. pulligera*, *N. delestennei* and *N. petitii* are here separated, the first two having an interrupted apertural rim. However, they are distinguished by other criteria relating to color and sculpture, in particular.

Molecular analyses

The analyses concerned 135 partial sequences (609 base pairs) of the COI gene. The ABGD method identified 11 groups (G1 to G11), whatever the prior value used, whereas the Bayesian inference (BI) revealed a distribution of 9 clades (A to I), represented in Fig. 5. The average of the values *a posteriori* of the parameters is (respectively for the first, the second and the third position of the codons): TL = 26.499818; alpha = 0.046403; 0.043755; 7.598073; pinvar = 0.026786; 2.845089; 0.128126. Two clades, A (*N. stumpffi*) and B (*N. asperulata*), each corresponded to two groups defined using the ABGD tool. For clade A, ABGD identifies two groups of '*N. stumpffi*' (G7 and G8), genetically distant by 2.61 %, without any geographic pattern being observed. For clade B, ABGD identified two groups of '*N. asperulata*' (G3 and G4), diverging by 9.04 %, one comprised of specimens from the western Pacific (Micronesia, Japan and the Philippines), the other comprised of specimens from the central Pacific (Vanuatu and the Solomon Islands). Table V shows the genetic distances between the various groups in the analysis.

The seven other clades, C (*Neritina* sp. Suji), D (*N. petitii*), E (*N. iris*), F (*N. delestennei*), G (*N. knorri*), H (*N. pulligera*) and I (*N. canalis*), correspond perfectly to the ABGD method groups. All the clades defined by BI are supported by a posterior probability (PP) at least equal to 97 %, four out of nine being at 100 %. Thus, our samples are in theory comprised of at least 10 different taxa belonging to the complex *N. pulligera* (*N. pulligera, N. stumpffi, N. knorri, N. petitii*, *N. canalis, N. iris*, *N. delestennei, N. asperulata, N. powisiana* and *N.* sp. Suji).

DISCUSSION

The results of the morphometric analysis, confirmed by the molecular analysis of the COI gene, both by BI and the ABGD method (Fig. 5), mean we can remove the doubt about the taxonomic status of certain species and clarify the situation of the complex *N. pulligera*. Thus, *N. stumpffi* (clade A) and *N. knorri* (clade G) turn out to be valid species, contrary to what Stahrmülner (1983), Eichhorst (2016b) and Gerlach *et al*. (2016) thought. These two species are easily separated in the field, where they live in sympatry, as there are no intermediate forms. Indeed, *N. knorri* is distinguishable by its uniformly black periostracum, its purplish-blue parietal wall that is slightly convex towards the rear, and its internally saffron-red lip. *N. stumpffi* is characterized by a very dark brown, nearly black operculum, an oblong form and granular, red or whitish parietal wall.

Clade A of *N. stumpffi* (Fig. 6E, F) includes individuals from the Pacific and Indian oceans mixed together. Individuals from both ABGD groups (G7 and G8), displaying a mitochondrial COI divergence of 2.61 %, are

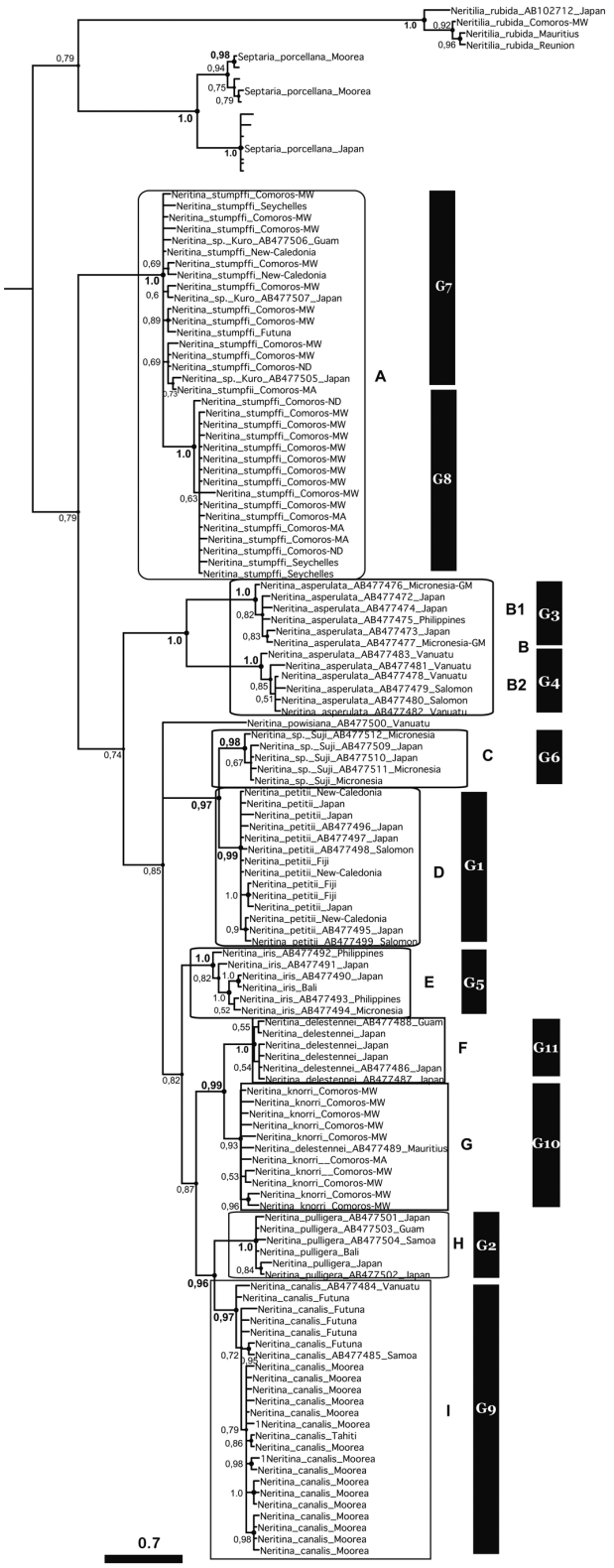

Fig. 5. – Phylogenetic tree from a Bayesian analysis of the gene of the sub-unit 1 of the Cytochrome oxidase (COI) for 119 individuals of the genus *Neritina*. Numbers on the nodes represent posterior probabilities.

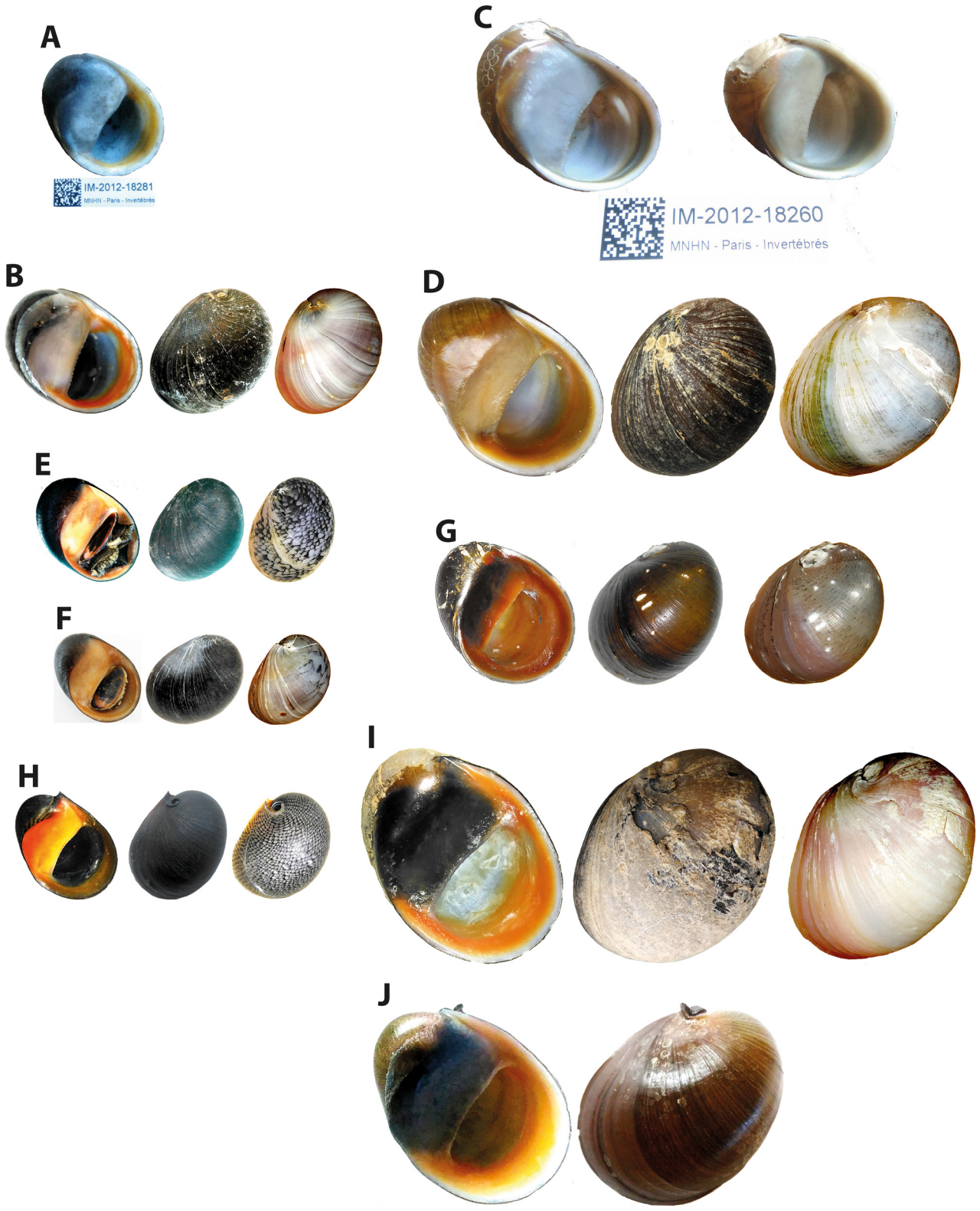

Fig. 6. – Several fresh water *Neritina* used for this study. The second dorsal view, on the right, shows the shell without its periostracum following treatment with bleach; this view shows the shell's real motifs and colour, produced in the external calcite layer. **A**: Paralectotype of *Neritina knorri*, MNHN-IM-2012-18281 (Madagascar, 25.3 mm). **B**: *N. knorri*, MNHN-IM-2013-62819 (Comoros, 24.31 mm). **C**: Paratypes of *N. delestennei*, MNHN-IM-2012-18260 (35 mm and 31 mm). **D**: *N. delestennei*, MNHN-IM-2013-62842 (Japan, 34.57 mm). **E**: *N. stumpffi*, MNHN-IM-2013-62796 (Comoros, 20 mm). **F**: *N. stumpffi*, MNHN-IM-2013-62806 (New Caledonia, 17.18 mm). **G**: *N. petitii*, MNHN-IM-2013-62854 (Japan, 27.75 mm). **H**: *N. canalis*, MNHN-IM-2013-62829 (French Polynesia, 21.10 mm). **I**: *N. pulligera*, MNHN-IM-2013-62848 (shell covered with an incrustation, probably calcareous. Japan, 41.61 mm). **J**: *N. pulligera*, MNHN-IM-2007-31091 (Vanuatu, 37 mm).

found in sympatry. Since there are no morphological differences and no clue about any ecological difference, the two ABGD groups are not supported as distinct species by integrative taxonomy approaches. Further, the PCA (Figs 3, 4) sets *N. stumpffi* morphometrically apart from the other species studied, without distinguishing the sub-groups G7 and G8 that are morphologically identical (Table V). In the sub-group G7, individuals from the Pacific and Indian oceans present no morphological difference. We also note that *Neritina* sp. Kuro, corresponding to *Neritina* sp. 1 in the article by Kano (2009), is found in clade A and so is genetically identical to *N. stumpffi*. Clade A appears to represent the species *N. stumpffi*, with two different groups G7 and G8. *N. stumpffi* appears to be morphologically very similar to *N. bruguierei*, a species described from New Caledonia and likely present in the Philippines, Vanuatu, and even Comoros and Seychelles (Eichhorst 2016b). In any case, the samples from Mayotte used by Eichhorst (2016b: 704; figs 1-3, p.731) to illustrate *N. bruguierei* undoubtedly belong to *N. stumpffi*. The important work of this author being based solely on a morphological analysis, it is essential to obtain molecular sequences of *N. bruguierei* to be able to decide on the synonymy or not of these two taxa.

Clade F concerns *N. delestennei* (Fig. 6C, D). While morphometric analysis associates it with *N. petitii* and *N. pulligera*, genetic analysis brings it closer to *N. knorri* (clade G) (Fig. 6A, B), which is morphologically very different. *N. delestennei* is relatively larger and more elongated than *N. knorri,* and has a russet parietal wall. Although the two G10 and G11 sister groups are genetically distant of only 3.14 %, the fact that the samples come from the Indian Ocean, for one, and the Pacific, for the other, is also an argument in favor of the distinction of the two species. They do not live in sympatry and are morphologically different (Table V). We assume, here, that beyond 3 % of genetic distance, species are different. The specimen from Mauritius clearly belongs to *N. knorri*, and we therefore consider the identification made by Kano (2009), which attributed it to *N. delestennei*, to be erroneous. However, if we consider unlikely the synonymy between these two taxa, the presence of *N. knorri* in the Pacific Ocean is feasible, for at least two reasons. First, Récluz, who described this species in 1841, with its type locality in Madagascar, also indicated its presence in Mindanao in the Philippines in 1850, before describing *N. delestennei* in 1853 without specifying the locality. Second, the samples in the MNHN historic collection that we consulted, from Tahiti, New Caledonia and Perak (Malaysia), are morphologically closer to *N. knorri* than *N. pulligera*, a species which moreover does not seem to exist in French Polynesia (Pointier & Marquet 1990). The fact remains that it has not yet been possible to find in this region genetically usable samples for confirmation by molecular analysis. *N. knorri* differs from *N. pulligera* by its uniformly black periostracum, its purplish-blue and the slightly convex posterior of its parietal wall, its internally saffron-red lip, which is never terminated with a spiral auricle, unlike *N. pulligera*, and by the fact that the dark spiral strips of its operculum are scattered and less pronounced. In any event, molecular sequences are necessary to remove doubt about the status of the species, for which the description is only based on morphology, and which could turn out to be synonymous species. This is the case, for example, of *N. janetabbasae*, a species newly described by Eichhorst (2016b: 712) and whose illustrations (page 736) are entirely compatible with *N. knorri*, in particular figs 2 and 3 showing specimens of Borneo, and fig. 6 representing a relatively fresh shell from Mayotte in the Comoros, and which could thus be a synonym of *N. knorri*.

N. pulligera sensu stricto (clade H: see Fig. 6I, J) and *N. canalis* (clade I: see Fig. 6H), clearly differ for both our morphological analyses (Figs 3, 4) and our genetic analyses (PP of 100 % and 97 %, respectively) and are thus considered as valid species. They cannot be confused morphologically in size and color. *N. canalis* is dark brown to black, smaller in size with a red-orange parietal wall. That of *N. pulligera* is greyish-black, possibly varying towards yellowish, and the peristome is colored with a broad orange band parallel with the lip. The supposed synonymy between *N. pulligera* and *N. petitii* (G1: see Fig. 6G), *N. iris* (G5) and *N. knorri* (G10) (Tryon 1888; Fischer-Piette & Vukadinovic 1974), for which the clades are supported in our analysis by a PP of at least 99% and significant genetic distances (Table V), is not borne out. There is a clear discrimination between these species, as identified by Kano (2009) and our morphological analysis (Figs 3, 4).

Within clade I (*N. canalis*), the individuals from Futuna, Vanuatu and Samoa are separated from those from Moorea and Tahiti. These are genetically structured populations that have already been documented by Crandall *et al*. (2010). It should be noted that the *N. canalis* 'channel' can be more or less apparent or absent for certain samples, that could be considered as belonging to another species. This is the case of *Neritina* sp. d, defined by Eichhorst (2016b: 747), and illustrated by a specimen from Tahiti (plate 218, Figs 4 to 6), which clearly belongs to *N. canalis*.

Finally, clade B *Neritina asperulata* is comprised of two sister subclades B1 and B2, corresponding to groups G3 and G4 in the ABGD method and separated by a genetic divergence of more than 9 %. We have no knowledge of the individuals corresponding to the sequences analyzed by Kano (2009), but according to Kano (2009), they are morphologically identical. We note however that they are not sympatric (Table V); their geographic distribution separates them, as B1 comes from Southeast Asia and Micronesia, and B2 from Melanesia. This geographic distribution, combined with such a high divergence rate (9.04 %), reveals the existence in this clade of a probable

cryptic species that may be the B2 species, the type locality for *N. asperulata* being the island of Luzon in the Philippines, a locality present in B1. However, in the absence of morphological analysis, this remains to be verified.

CONCLUSION

We are aware that the use of COI as a barcoding gene for the Neritidae has recently been criticized (Chee *et al*. 2015), mostly on the grounds that processes such as hybridization and incomplete lineage sorting cannot be detected. But in our study, such cases are unlikely to occur, given the contrasting results obtained with respect to the geographic distribution of taxa.

We are also aware of the low number of specimens for some species, but the sampling however seems sufficient as the results obtained are significantly marked: this study has enabled us to clear doubt about the taxonomic status of most of the species in the complex *Neritina pulligera*. Indeed, we can now assert that the nominal taxa *N. stumpffi* and *N. knorri* represent different valid species that are clearly distinguishable from *N. pulligera*, and that the former, until now only reported in the western Indian Ocean, is also present in the Pacific Ocean, especially in New Caledonia, Guam, Futuna and Japan. This difference had already been noted by the first author (pers obs) and expressed by Backeljau *et al*. (1986) and Valade *et al*. (2007). As a consequence, the presence of *N. pulligera* in the western Indian Ocean and East Africa (Brown, 1994) is not proven. We can also confirm with Kano (2009) that *N. petitii*, *N. canalis*, *N. iris*, *N. asperulata* and *N. powisiana* are valid species different from *N. pulligera*. On the other hand, *N. knorri* and *N. delestennei*, morphologically differentiable and genetically close, should be the subject of further study. Concerning the nominal taxon *N. asperulata*, it is undeniably different from *N. pulligera*, but seems to designate two species, one of which is probably cryptic, for which the morphological characteristics need to be studied in order to distinguish between them.

Work is therefore still needed to finalise the complete phylogeny of the genus *Neritina*, which will probably result in new taxonomic revisions. For this purpose, the analysis of a nuclear gene and other mitochondrial genes, in addition to the COI, is needed.

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