

Preliminary molecular phylogeny of the diatom genus *Nupela* with the description of a new species and consideration of the interrelationships of taxa in the suborder Neidiineae D.G. Mann sensu E.J. Cox

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Abstract: Molecular investigation of genera *Nupela* and *Brachysira* is conducted using strains from Indonesia and Vietnam. New species from the genus *Nupela indonesica* sp. nov. is described using combined approach. *Nupela lesothensis* (Schoeman) Lange–Bertalot is investigated using molecular data too. Phylogenetic analysis shows that *Nupela* and *Brachysira* are not closest genera. Morphology of *Nupela* and its differences from *Brachysira* is discussed. The genus *Nupela* differs from all other diatom taxa by having coalescent hymenes outside of areolae but not inside. Facultative development of raphe between different *Nupela* species is discussed.

Key words: *Brachysira*, diatoms, morphology, molecular investigation, *Nupela*, phylogeny, taxonomy

INTRODUCTION

Nupela is a freshwater diatom genus established by VYVERMAN & COMPÈRE (1991) with *N. giluwensis* Vyverman et Compère, as the type species. The new genus and species were described originally from high elevation ponds in Papua New Guinea. VYVERMAN & COMPÈRE (1991, p. 178) postulated that this very interesting species and new genus “could find its place in the suborder Neidiineae, somewhere near or in the families Brachysiraceae or Diadesmidiaceae”. *Nupela* shares some morphological features with both *Brachysira* and *Diadesmis* such as transapically elongated areolae, relatively few areolae per striae, presence of ridge or a hyaline area at the junction of valve face and mantle and simple or inconspicuous proximal raphe fissures. This genus was described as a naviculoid biraphid taxon (VYVERMAN & COMPÈRE 1991).

Since its original description, many new species were transferred to *Nupela* mainly on the basis of striae and areolae morphology. Lange–Bertalot with coauthors (LANGE–BERTALOT 1993; LANGE–BERTALOT & MOSER 1994; METZELTIN & LANGE–BERTALOT 1998;

MOSER et al. 1998; LANGE–BERTALOT & GENKAL 1999; RUMRICH et al. 2000) transferred to the genus *Nupela* many monoraphid species from the previously catch-all genus *Achnanthes* sensu lato. Presence of unique morphological features allowed them to place biraphid and monoraphid taxa in the same genus, even though this was not common practice. Well-known species such as *Nupela impexiformis* (Lange–Bertalot) Lange–Bertalot, *N. subrostrata* (Hustedt) Potapova, *N. lapidosa* (Krasske) Lange–Bertalot, *N. pennsylvanica* (R.M. Patrick) Potapova, *N. poconoensis* (R.M. Patrick) Potapova, *N. biconfusa* (Van Landingham) C.E. Wetzel et Ector and many others were transferred to *Nupela*. From the biraphid diatom, species previously described as *Anomoeoneis* Pfitzer, *Brachysira* Kützing, *Navicula* Bory, *Neidium* Pfitzer, *Rhoicosphaenia* Grunow and *Stauroneis* Ehrenberg, were also transferred to this genus. At present, there are less than 100 species reported in this genus; 48 species records are listed in Catalogue of Diatom Names (FOURTANIER & KOCIOLEK 2011), and 80 have been flagged as accepted taxonomically (GUIRY & GUIRY 2020).

The characteristics of the genus *Nupela* are small frustule size, generally less than 25 µm, valves are narrowly

elliptical with strongly capitate ends, slightly curved transapically, the central area is asymmetric, and may extend nearly to the valve margin. Although Vyverman and Compere (1991, p. 178) indicate that, “An inner, centrally perforated hymen is present in each areola” it is generally considered that the areolae of *Nupela* species are covered by hymenes externally. Other features of the genus include large external and small internal openings of the areolae, the areolae are arranged in striae of variable length, raphe fissures slightly undulate, raphe endings inconspicuous, terminal fissures sharply curved into one side of valve, proximal endings T-shaped located in a moderately inflated central nodule, the external terminal raphe endings in most cases form a double hook or occasionally a single hook, valves have obvious siliceous thickenings on the external surface, and either biraphid or monoraphid frustules (VYVERMAN & COMPERE 1991; POTAPOVA et al. 2003; SIVER et al. 2007). The genus *Nupela* does not show substrate preferences and it has been found on rocks, wood and saturated soils; does not show a pH preference, being present both in slightly acidic (POTAPOVA 2013) and alkaline waters (BAHLS 2011).

From this long list of features, the main morphological characteristics that diagnose this genus are the morphology of striae and areolae with hymenes, that are covered externally, distinguishing them from for all other naviculoid and achnanthoid genera (KULIKOVSKIY et al. 2016). Areolae are broader externally versus internally (POTAPOVA 2011).

The suborder Neidiineae was proposed by D.G. MANN in ROUND et al. (1990), for a group of naviculoid diatoms (in the order Naviculales Bessey), that included a number of new families. Amongst the families D.G. Mann included in his new suborder are Berkeleyaceae, Cavinulaceae, Cosmioneidaceae, Scolioneidaceae, Diadesmidiaceae Amphipleuraceae, Brachysiraceae, Neidiaceae and Scoliotropidaceae; only the latter two had been previously described. In his description of this diverse suborder (MANN in ROUND et al. 1990, p. 657), many features of the group were described as “various”, while the feature of internal hymenate occlusions was specified.

Nupela was described the year following publication of ROUND et al. (1990), and while there is ample evidence that the hymenate occlusions of *Nupela* species are positioned on the exterior of the valves (POTAPOVA et al. 2003). E. Cox, in her revised classification of the diatoms (COX 2015), assigned *Nupela* to the family Brachysiraceae. This assignment was made even though nearly all of the features listed for the family are not found in *Nupela*.

The aims of this publication include 1) to describe a new species of *Nupela* from Indonesia, 2) to use molecular data from this new species and a second species transferred to *Nupela* to assess the phylogenetic position of the genus and 3) to reassess the Neidiineae to see how the suborder reflects a natural classification of the taxa assigned there.

MATERIALS AND METHODS

Samples from Indonesia were collected by I.I. Ivanov and from Vietnam by M. Kulikovskiy and E. Gusev. Water mineralization and temperature measurements were performed using the Hanna Combo (HI 98129) device, Hanna Instruments, Inc., USA. List of all strains examined in this study with their GenBank accession numbers and geographic position of sample areas with measured ecological parameters presented in Table 1. Data that were not determined are indicated with N.D.

A subsample of each collection was added to WC liquid medium (Guillard & Lorenzen 1972). Monoclonal strains were established by micropipetting single cells under an inverted microscope. Non-axenic unialgal cultures were maintained in WC liquid medium at 10 °C in a growth chamber with a 12:12 h light:dark photoperiod.

Strains for LM and SEM investigations have been processed by means of a standard procedure involving treatment with 10% HCl and concentrated hydrogen peroxide (\approx 37%) to oxidize organic matter. After treatment, the sample was washed with deionized water four times at 12 h intervals. Permanent diatom preparations have been mounted in Naphrax®. Light microscopic (LM) observations have been performed by means of a Zeiss Axiovert and Zeiss Axioscope A1 microscope equipped with an oil immersion objective (100/n.a. 1.4, DIC). Valve ultrastructure was examined with a JSM-6510LV field emission scanning electron microscope and EMS FF200-Cu-50 (USA) transmission electron microscope (Borok, Russia). The stubs were sputter coated with 50 nm of Au by means of a Eiko IB 3. Samples, cultures and slides are deposited in the collection of Maxim Kulikovskiy at the Herbarium of the Institute of Plant Physiology Russian Academy of Science, Moscow, Russia.

Total DNA of monoclonal cultures of *Nupela* strains was extracted using InstaGene™ Matrix according to the manufacturer’s protocol 2.2 “DNA preparation from cultured mammalian cells”. Fragments of 18S rDNA (306–433 bp, including V4 domain), and partial rbcL plastid genes (1062–1129 bp) were amplified using primers D512for and D978rev from ZIMMERMAN et al. (2011) for 18S rDNA fragments and rbcL40+ from RUCK & THERIOT (2011) and rbcL1255– from ALVERSON et al. (2007) for rbcL fragments.

Amplifications of the 18S rDNA fragments and partial rbcL gene fragment were carried out using the premade mix ScreenMix (Evrogen, Russia) for the polymerase chain reaction (PCR). The conditions of amplification for 18S rDNA fragments were: an initial denaturation of 5 min at 95 °C, followed by 35 cycles at 94 °C for denaturation (30 s), 52 °C for annealing (30 s) and 72 °C for extension (50 s), and a final extension of 10 min at 72 °C. The conditions of amplification for partial rbcL were: an initial denaturation of 5 min at 95 °C, followed by 45 cycles at 94 °C for denaturation (30 s), 59 °C for annealing (30 s) and 72 °C for extension (80 s), and a final extension of 10 min at 72 °C.

The resulting amplicons were visualized by horizontal agarose gel electrophoresis (1.5 %), colored with SYBR Safe (Life Technologies, United States). Purification of DNA fragments was performed with the ExoSAP-IT kit (Affimetrix, USA) according to the manufacturer’s protocol. 18S rDNA fragments and partial rbcL gene were decoded from two sides using forward and reverse PCR primers and the Big Dye system (Applied Biosystems, USA), followed by electrophoresis using a Genetic Analyzer 3500 sequencer (Applied Biosystems).

Editing and assembling of the consensus sequences were carried out by comparing the direct and reverse chromatograms

Table 1. List of all strains examined in this study with their GenBank accession numbers. Geographic position of samples and measured ecological parameters indicated.

Strains	Slide No	Sample locality	Collection of date	Coordinates	t (°C)	pH	Substratum	GenBank accession number, SSU rDNA, partial	GenBank accession number, rbcL, partial
<i>Nupela lesothensis</i> Ind168	01713	Bali Island, Indonesia	04.11.2014	8°14'283" S 115°8'280" E	24.6	6.88	phytoplankton	KU951583	KU951590
<i>Nupela lesothensis</i> Ind170	01688	Bali Island, Indonesia	04.11.2014	8°14'283" S 115°8'280" E	24.6	6.88	phytoplankton	KU951584	KU951593
<i>Nupela indonesica</i> Ind121	01802	Sulawesi Island, Indonesia	07.11.2014	2°54'585" S 119°53'014" E	N.D.	7.26	phytoplankton	MT949704	MT951629
<i>Nupela indonesica</i> Ind219	01858	Bali Island, Indonesia	04.11.2014	8°14'346" S 115°8'264" E	23.8	8.33	phytoplankton	MT949705	MT951630
<i>Brachysira</i> sp. VN374	00374	Con Ho lake, Vietnam	07.10.2010	10°46'579" N 106°41'454" E	24.1	N.D.	phytoplankton	KU951588	KU951597

using the Ridom TraceEdit program (ver. 1.1.0) and Mega7 (KUMAR et al. 2016). Newly determined sequences and DNA fragments from 161 other diatoms, which were downloaded from GenBank (taxa, strain numbers (if available) and Accession Numbers are given in Supplementary table 1), were included in the alignments. Four centric diatom species were chosen as the outgroups.

The nucleotide sequences of the 18S rDNA and rbcL genes were aligned separately using the Mafft v7 software and the E-INS-i model (KAROH & TOH 2010). For the protein-coding sequences of the rbcL gene, we checked that the beginning of the aligned matrix corresponded to the first position of the codon (triplet). The resulting alignments had lengths of 552 (18S rDNA) and 1254 (rbcL) characters.

A phylogenetic tree was inferred by Bayesian inference (BI) using MrBayes version 3.2.7 (RONQUIST et al. 2012). For each of the alignment partitions, the most appropriate substitution model was estimated using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.10 (DARRIBA et al. 2012). This BIC-based model selection procedure selected the following models, shape parameter α and a proportion of invariable sites (pinvar): TrN+G, $\alpha = 0.2830$ for 18S rDNA; TIM1+I+G, $\alpha = 0.5220$ and pinvar = 0.5780 for the first codon position of the rbcL gene; TIM1ef+I+G, $\alpha = 0.2620$ and pinvar = 0.6680 for the second codon position of the rbcL gene; TVM+I+G, $\alpha = 0.7750$ and pinvar = 0.0760 for the third codon position of the rbcL gene. We used the HKY model of nucleotide substitution instead of TrN and TIM1ef, GTR instead of TIM1 and TVM, given that they were the best matching model available for Bayesian inference method. The parameters—estimated convergence, effective sample size (ESS) and burn-in period were checked using the software Tracer ver. 1.7.1. (DRUMMOND & RAMBAUT 2007). Three “hot” and one “cold” Markov chains were run for 1×10^6 cycles in two repetitions with the selection of each 200th generated tree. The initial 25% of the trees were removed, the rest retained to reconstruct a final phylogeny. The phylogenetic tree and posterior probabilities of its branching were obtained on the basis of the remaining trees, having stable estimates of the parameter models of nucleotide substitutions and likelihood. Maximum Likelihood (ML) analysis was performed using the program RAXML (STAMATAKIS et al. 2008). The nonparametric bootstrap analysis with 1000 replicas was used. The statistical support values were visualized in FigTree ver. 1.4.4 and Adobe Photoshop CC (19.0).

RESULTS

Nupela indonesica Kulikovskiy, Maltsev, Glushchenko et Kocielek sp. nov. (Figs 1–41)

Description: Valve outlines lanceolate with more or less protracted ends, subrostrate to subcapitate. Valves biraphid. Length 8.6–10.8 μm , breadth 3.5–4.6 μm . Valves with filiform raphes lying in a narrow, almost linear axial area, becoming conspicuously expanded from distal parts towards the central area. Distal raphe ends present on valve face and slightly curved or hooked to mantle. Striae difficult to discern in LM. Striae radiate throughout, 46–50 in 10 μm , areolae 68–70 in 10 μm . Striae consist of many (greater than five) areolae. The large lanceolate central area is irregularly rugose. Areolae larger externally than internally.

In the SEM, external views (Figs 13–16, 33–36) frustules can be heterovalvate, with some valves having more elongate (e.g. Fig. 33) or shorter (Figs 13, 34) raphe systems. Axial area narrowly lanceolate, widening to form a variably-shaped and sized central area that can extend unidirectionally to the margin with (Figs 13, 14, 33) or without (Fig. 34) isolated areolae being present. Raphe is filiform, more or less straight to weakly undulate. Proximal raphe ends straight (Figs 13, 14) or bent slightly in the same direction (Figs 33, 35). Distal raphe ends may be deflected onto the mantle (Figs 15, 33, 36) or terminate before reaching the apex of the valve (Figs 16, 34, 35). Striae are radiate the entire length of the valve, quadrate or ellipsoidal in shape. The striae are interrupted between the valve face and mantle by a narrow hyaline area on the valve margin. On the mantle striae are composed of a single areola.

Internally (Figs 37–41) the central nodule can be small and round (e.g. Figs 37, 38) or extended and shallower (Fig. 39). Proximal raphe ends are straight, (Figs 40, 41) and distal ends terminate as small but distinct helictoglossae (Figs 37–39). Areolae are ellipsoidal, smaller than their external expressions.

Holotype (designated here): slide no 01802b (Fig. 7 represents the holotype) in herbarium of MHA, Main Botanical Garden Russian Academy of Science, Moscow, Russia.

Type strain: Strain Ind 121, isolated in from the sample S1/1, deposited at the culture collection of the Laboratory of molecular systematics of aquatic plants, Timiryazev Institute of Plant Physiology Russian Academy of Science, Moscow, Russia.

Type locality: Indonesia, Sulawesi Island, sample S1/1, phytoplankton (2°54'585"S 119°53'014"E), leg. I.I. Ivanov, coll. date 07.11.2012.

Etymology: The specific epithet “*indonesica*” refers to the name of the country where this species was observed.

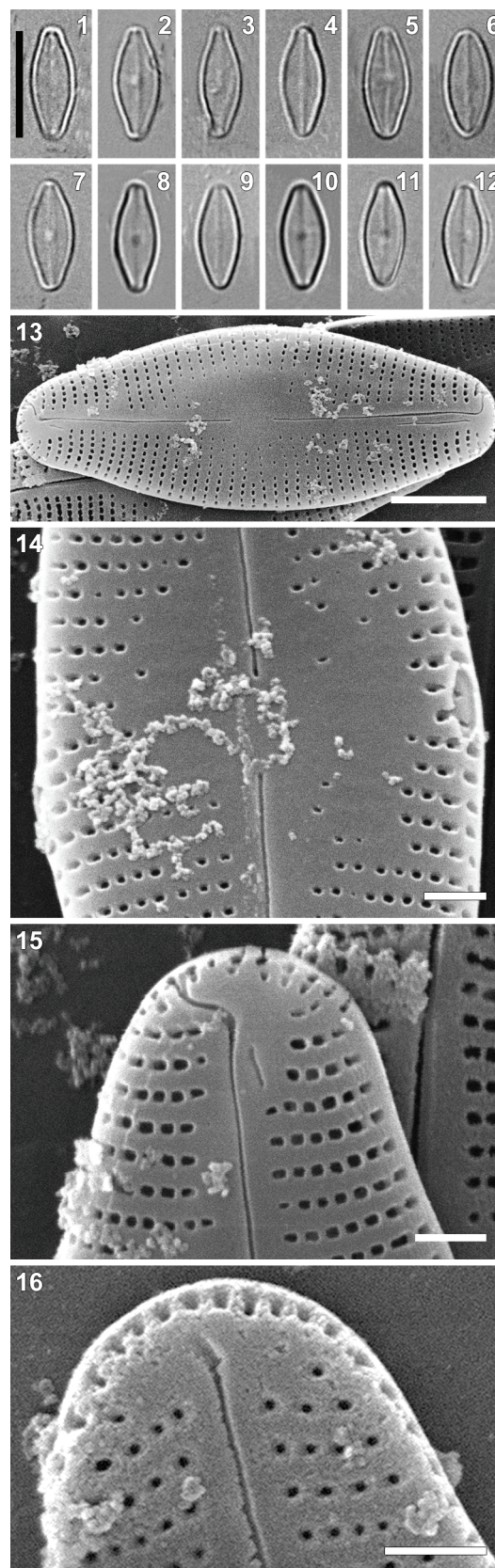
Distribution. Indonesia, Sulawesi Island (type locality) and Bali Island (strain Ind219, slide no 01858).

***Nupela lesothensis* (Schoeman) Lange–Bertalot 2000 (Figs 42–72, 78–85)**

Basionym: *Rhoicosphenia lesothensis* Schoeman 1973.

Description: Valves elliptical–lanceolate, with protracted, rostrate apices. Length 11–15.5 µm, breadth 3.8–5 µm. Striae not evident the light microscope.

In the SEM, the valve exterior (Figs 54–56, 78–81) has a filiform raphe that is straight, with straight proximal ends (Figs 54, 55, 78–70) and hooked to “?”-shaped distal ends that curve onto the mantle (Figs 54, 56, 78, 79, 81). The axial area is narrow at the ends, widening towards the center to form an unidirectionally-expanded central area. Striae 45–46 in 10 µm, are radiate at the center of the valve, becoming convergent at the ends. Areolae are quadrate to ellipsoidal in shape. Areolae



Figs 1–16. *Nupela indonesica* Kulikovskiy, Maltsev, Glushchenko et Kociolek sp. nov. Strain Ind 121. Slide no 01802. (1–12) LM, DIC, size diminution series. (13–16) SEM, external views, (13) the whole valve, (14) central area, (15, 16) valves ends. Figure 7 is of the holotype. Scale bars 10 µm (1–12), 2 µm (13), 0.5 µm (14–16).

Table 2. Comparison of species and strains of *Nupela* from studied material with similar taxa.

Species	Frustule	Length (µm)	Width (µm)	Valve outline	Valve apices	Central area	Proximal raphe ends	Number of striae in 10 µm	Number of areolae in 10 µm	Number of longitudinal rows of areolae	Locality	References
<i>Nupela indonesica</i> Kulikovskiy, Maltsev, Glushchenko et Kocotolek sp. nov. strain Ind121*	biraphid, hetero-valvar	9.5–10.6	3.6–4.2	broadly lanceolate	rounded	apical oval, a one-sided fascia	straight, small, distinct	45–46	60–65(70)	3–10	Sulawesi Island, Indonesia	this study
<i>Nupela indonesica</i> sp. nov. strain Ind219	biraphid, hetero-valvar	8.1–9.2	3.1–3.9	lanceolate	rounded	apical oval, a one-sided fascia	straight, small, distinct	45–46	60–70	4–8	Bali Island, Indonesia	this study
<i>Nupela lesiothensis</i> (Schoeman) Lange-Bertalot strain Ind168	biraphid, hetero-valvar, the raphe shorter on the second valve	9.7–11.4	3.6–3.9	elliptic or narrowly elliptic	broadly capitate	apical oval, one-sided fascia extending to the valve margin	straight, small, distinct	40–43	50	2–6	Bali Island, Indonesia	this study
<i>Nupela lesiothensis</i> strain Ind170	biraphid, hetero-valvar, the raphe shorter on the second valve	14.2–15.0	4.2–5.0	narrowly elliptic	capitate	apical oval, one-sided fascia extending to the valve margin	straight, small, distinct	40–41	50–70	3–6	Bali Island, Indonesia	this study
<i>Nupela lesiothensis</i>	biraphid, the raphe shorter on the second valve	4.5–13.5	2.3–3.3	elliptical to lanceolate	broadly rounded	apical oval, one-sided fascia extending to the valve margin	straight, small, distinct	42–46	50–68	4–5	Paballong, Lesotho, Africa	SCHOEMAN 1973
<i>Nupela gilawensis</i> Vyverman et Compere	biraphid, iso-valvar	13–16	2.0–4.5	narrowly elliptical	strongly capitate	rectangular	T-shaped	42	20–25	1–3	Papua New Guinea	VYVERMAN & COMPERE 1991
<i>Nupela biconfusa</i> (Van Landingham) C.E. Wetzel et Ector	monoraphid	8.2–12.4	3.0–3.7	lanceolate	substrate	apical oval	oval, distinct	45	50	3–5	Sumatra, Java, Indonesia	WETZEL & ECTOR 2018
<i>Nupela praecipuoides</i> Tremarin et T. Ludwig	monoraphid, heterovalvar	9.2–21.5	4.1–5.5	lanceolate	slightly protracted to substrate	apical oval	expanded	36–38	35–39	2–8	Paraná, Brazil	TREMARIN ET AL. 2015

* - at least 30 valves were counted. Measurements were carried out on the clonal cultures.

Table 3. Percent similarity (p -distance) matrix of 21 strains on the basis of the partial 18S rRNA gene including the V4 barcoding subregion (433 bp).

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 <i>Nupela indonesica</i> Ind121	–																				
2 <i>Nupela indonesica</i> Ind219	99.6	–																			
3 <i>Nupela lesiothensis</i> Ind168	98.5	98.7	–																		
4 <i>Nupela lesiothensis</i> Ind170	98.4	98.7	100	–																	
5 <i>Neidium affine</i> UTEX FD127	91.3	90.3	90.1	89.6	–																
6 <i>Neidium amphigomphus</i> JAR89 G6Run12	91.4	90.3	90.0	89.4	97.7	–															
7 <i>Neidium bisulcatum</i> UTEX FD417	91.0	90.1	89.9	89.4	98.6	98.0	–														
8 <i>Neidium dilatatum</i> JAR70 A4Run13	91.2	90.2	89.8	89.3	98.1	98.4	98.2	–													
9 <i>Neidium fossum</i> JAR83 D5Run12	91.0	90.0	89.6	89.2	99.5	97.6	98.3	98.1	–												
10 <i>Neidium hitchcockii</i> JAR89 G4Run12	91.8	90.5	90.4	89.8	97.9	98.4	97.7	98.7	97.7	–											
11 <i>Neidium longiceps</i> CANA:108131 D5R18	91.3	90.3	90.0	89.5	99.7	97.9	98.7	98.4	99.5	97.9	–										
12 <i>Neidium saccoense</i> CANA:108122 ASR17	91.0	89.9	89.6	89.1	99.4	97.7	98.4	98.2	99.3	97.7	99.5	–									
13 <i>Diploneis</i> cf. <i>cheronensis</i> GU44AY-6 Dip. cf. crab-2	92.7	92.1	92.9	92.5	90.4	90.2	90.3	90.0	90.5	90.5	90.7	90.4	–								
14 <i>Diploneis</i> cf. <i>smithii</i> KSA2015-30 oval-B6	93.0	92.5	93.1	92.9	89.2	89.9	89.4	89.7	89.5	90.1	89.8	89.5	94.5	–							
15 <i>Diploneis</i> cf. <i>smithii</i> GU44AY6	92.5	92.2	92.2	91.9	87.4	88.5	88.2	88.3	88.5	88.6	88.3	93.7	97.4	–							
16 <i>Diploneis parca</i> KSA2015-49 oval-ED-2	92.9	92.6	92.7	92.5	88.8	88.4	88.7	88.8	88.8	89.1	89.1	88.8	93.6	97.3	95.2	–					
17 <i>Diploneis</i> sp. PackChannelPlank Diplo-1	93.2	92.5	93.0	92.6	89.9	90.1	90.1	90.1	90.2	90.1	90.4	90.2	94.9	94.5	93.4	93.5	–				
18 <i>Diploneis</i> sp. KSA2015-14 oval-J31	92.8	92.5	92.8	92.5	88.8	89.4	89.1	89.2	89.2	89.6	89.5	89.2	93.7	97.2	95.4	97.6	93.9	–			
19 <i>Diploneis</i> sp. 21IV14-2A oval penn-B1	93.1	92.7	93.1	92.8	89.3	89.9	89.5	89.9	89.6	90.2	89.9	89.6	94.4	97.6	95.8	97.1	94.1	97.4	–		
20 <i>Diploneis subovalis</i> UTEX FD282	91.6	91.2	91.4	91.2	88.4	88.8	88.8	89.0	88.8	89.5	89.0	88.7	93.0	95.9	94.2	96.0	92.6	95.5	95.5	–	
21 <i>Diploneis vacillans</i> KSA2015-11 peanut-E14	93.0	92.5	93.1	92.8	89.8	90.4	90.1	90.5	90.2	90.7	90.5	90.2	94.4	97.8	96.6	96.9	94.4	96.6	97.9	95.8	–

Table 4. Percent similarity (p -distance) matrix of 21 strains on the basis of the partial ribulose-1,5-bisphosphate carboxylase, large subunit (377 amino acids).

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 <i>Nupela indonesica</i> Ind121	–																				
2 <i>Nupela indonesica</i> Ind219	99.7	–																			
3 <i>Nupela lesiothensis</i> Ind168	98.1	97.7	–																		
4 <i>Nupela lesiothensis</i> Ind170	98.0	97.7	100	–																	
5 <i>Neidium affine</i> UTEX FD127	94.8	94.9	94.1	93.8	–																
6 <i>Neidium amphigomphus</i> JAR89 G6Run12	94.5	94.4	93.6	93.2	99.7	–															
7 <i>Neidium bisulcatum</i> UTEX FD417	94.5	94.6	93.9	93.5	99.7	100	–														
8 <i>Neidium dilatatum</i> JAR70 A4Run13	94.7	94.7	93.9	93.5	100	99.7	99.7	–													
9 <i>Neidium fossum</i> JAR83 D5Run12	94.7	94.7	93.9	93.5	100	99.7	99.7	100	–												
10 <i>Neidium hitchcockii</i> JAR89 G4Run12	94.5	93.8	93.6	93.2	98.9	98.6	98.6	98.9	98.9	–											
11 <i>Neidium longiceps</i> CANA:108131 D5R18	94.7	94.7	93.9	93.5	100	99.7	99.7	100	100	98.9	–										
12 <i>Neidium saccoense</i> CANA:108122 ASR17	94.7	94.7	93.9	93.5	100	99.7	99.7	100	100	98.9	100	–									
13 <i>Diploneis</i> cf. <i>cheronensis</i> GU44AY-6 Dip. cf. crab-2	93.4	92.7	94.1	93.8	93.0	92.8	92.8	93.1	93.1	92.8	93.1	93.1	–								
14 <i>Diploneis</i> cf. <i>smithii</i> KSA2015-30 oval-B6	94.5	93.8	95.2	94.9	93.5	93.4	93.4	93.6	93.6	93.4	93.6	93.6	96.5	–							
15 <i>Diploneis</i> cf. <i>smithii</i> GU44AY6	93.1	92.3	94.4	93.8	92.1	91.8	91.8	92.2	92.2	91.3	92.2	92.2	95.7	98.7	–						
16 <i>Diploneis parca</i> KSA2015-49 oval-ED-2	93.6	92.9	93.8	93.5	92.4	92.1	92.1	92.4	92.4	92.1	92.4	92.4	96.5	98.1	96.0	–					
17 <i>Diploneis</i> sp. PackChannelPlank Diplo-1	95.1	94.4	95.5	95.2	93.5	93.4	93.4	93.6	93.6	93.4	93.6	93.6	95.5	97.1	95.2	96.7	–				
18 <i>Diploneis</i> sp. KSA2015-14 oval-J31	94.0	93.2	94.1	93.8	93.0	92.8	92.8	93.1	93.1	92.8	93.1	93.1	96.8	98.7	97.0	99.5	96.8	–			
19 <i>Diploneis</i> sp. 21IV14-2A oval penn-B1	93.7	92.9	94.4	94.1	93.3	93.1	93.1	93.4	93.4	93.1	93.4	93.4	97.6	98.4	97.0	98.6	96.5	99.2	–		
20 <i>Diploneis subovalis</i> UTEX FD282	92.6	92.1	92.8	92.7	91.9	91.4	91.5	91.7	91.7	91.4	91.7	91.7	96.3	97.3	96.1	97.0	95.7	97.1	96.8	–	
21 <i>Diploneis vacillans</i> KSA2015-11 peanut-E14	94.8	94.1	95.5	95.2	94.1	93.9	93.9	94.2	94.2	93.9	94.2	94.2	96.8	99.2	99.1	97.8	96.8	98.4	98.7	96.8	–

60–68 in 10 μ m. A narrow hyaline area separates the striae on the valve face from the mantle.

Internally (Figs 82–85), the raphe has straight proximal raphe ends that terminate at an indistinct central nodule, and distal ends that terminate as small helictoglossae.

Areolae are round to ellipsoidal, smaller in size from the external openings.

Molecular investigation

Studied strains presented low similarities to *Diploneis* species, and the evolutionary distance matrix based on

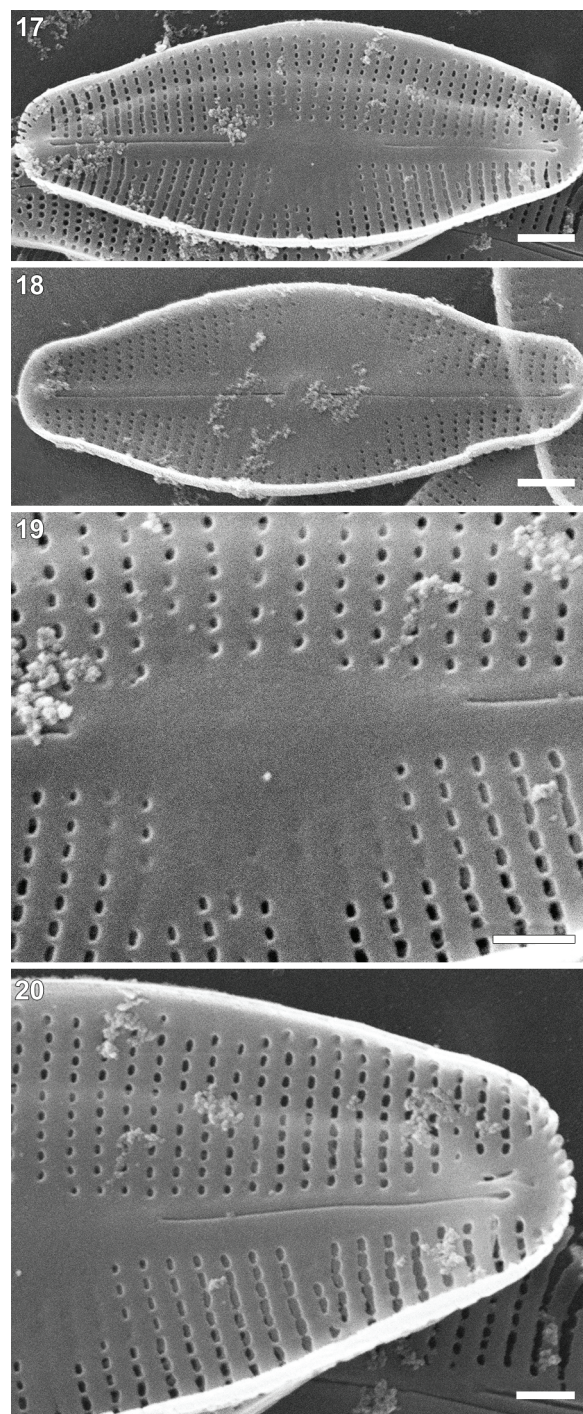
the partial 18S rRNA gene including the V4 barcoding subregion showed that two *Nupela indonesica* strains shared 99.6% similarity with each other, but they shared 98.4%–98.7% similarities with *Nupela lesothensis* strains, 91.2%–93.1% similarities with *Diploneis* species, and 89.5%–91.8% similarities with *Neidium* species (Table 3). Simultaneously, the evolutionary distance matrix showed that *Neidium* species shared 97.7%–99.7% similarity with maximum value for *Neidium affine* (Ehrenberg) Pfitzer and *Neidium longiceps* (W. Gregory) R. Ross, 93.0%–97.9% similarity for *Diploneis* strains with maximum for *Diploneis* sp. 21IV14–2A oval penn–B1 and *Diploneis vacillans* KSA2015–11 peanut–E14 (Table 3).

Pairwise comparisons with putatively related taxa showed that strains of *Nupela indonesica* were 99.7% similar, however they shared 97.7%–98.1% similarities with *Nupela lesothensis* strains and less than 95.1% amino acids similarity of ribulose–1,5–bisphosphate carboxylase with other diatoms in the analysis (Table 4). The percent similarity among aligned amino acids between *Neidium* species was available with a range of 98.6–100%.

In the phylogenetic tree the four strains of *Nupela* investigated here are more closely related to each other than to any other taxa, and this single branch has high statistical support (see Fig. 86). This clade was identical in the Maximum Likelihood (Supplement S3) and Bayesian (Fig. 86) trees. This lineage of *Nupela* taxa is most closely related to the monophyletic group of *Diploneis* taxa, and together the lineage of *Nupela* and *Diploneis* are more closely related to a lineage comprised of two strains of *Biremis*. However these branches have poor statistical support. The monophyletic group of *Nupela* + *Diploneis* + *Biremis* is sister to a monophyletic group that includes *Neidium* + *Scoliopleura* (considered in the family Neidiaceae, Cox 2015) + *Brachysira* + *Luticola* strains + *Diadismis sensu stricto* (considered in the family Diadesmidae, Cox 2015). The Brachysiraceae is not recovered as a monophyletic group in this analysis. Molecular investigation of the new strain from the genus *Brachysira* is included to analysis (LM pictures see Figs 73–77). Other families that have in the past been considered part of the suborder Neidiineae D.G. Mann, represented here by the family Amphipleuraceae (with species *Frustulia* spp., *Amphipleura*) and Berkeleyaceae (with species *Berkeleya* spp., and *Climaconeis* spp.) are widely separated from suborder in this analysis (see Fig. 86).

DISCUSSION

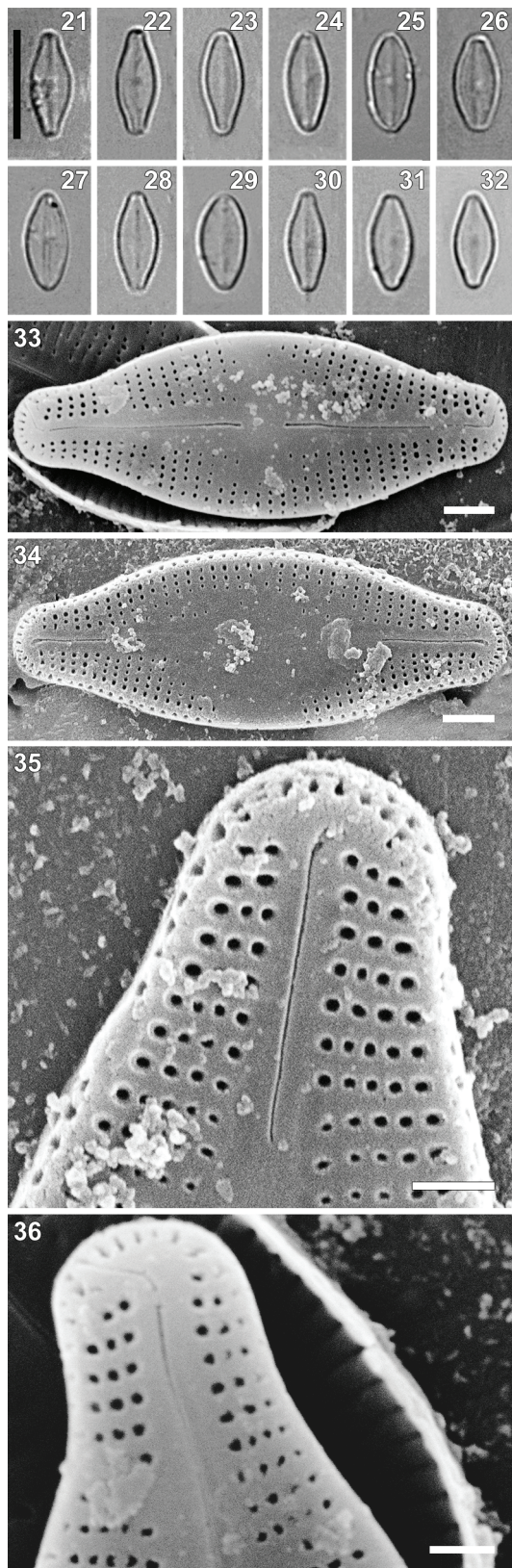
Two *Nupela* species are included in this investigation. These are *Nupela indonesica*, described here as a new species, and *Nupela lesothensis* (Schoeman) Lange–Bertalot (see Figs 42–72, 78–85). These two species are similar in having lanceolate valves with protracted ends, but they can be distinguished from one another on the basis number of striae and number of areolae in 10 μ m



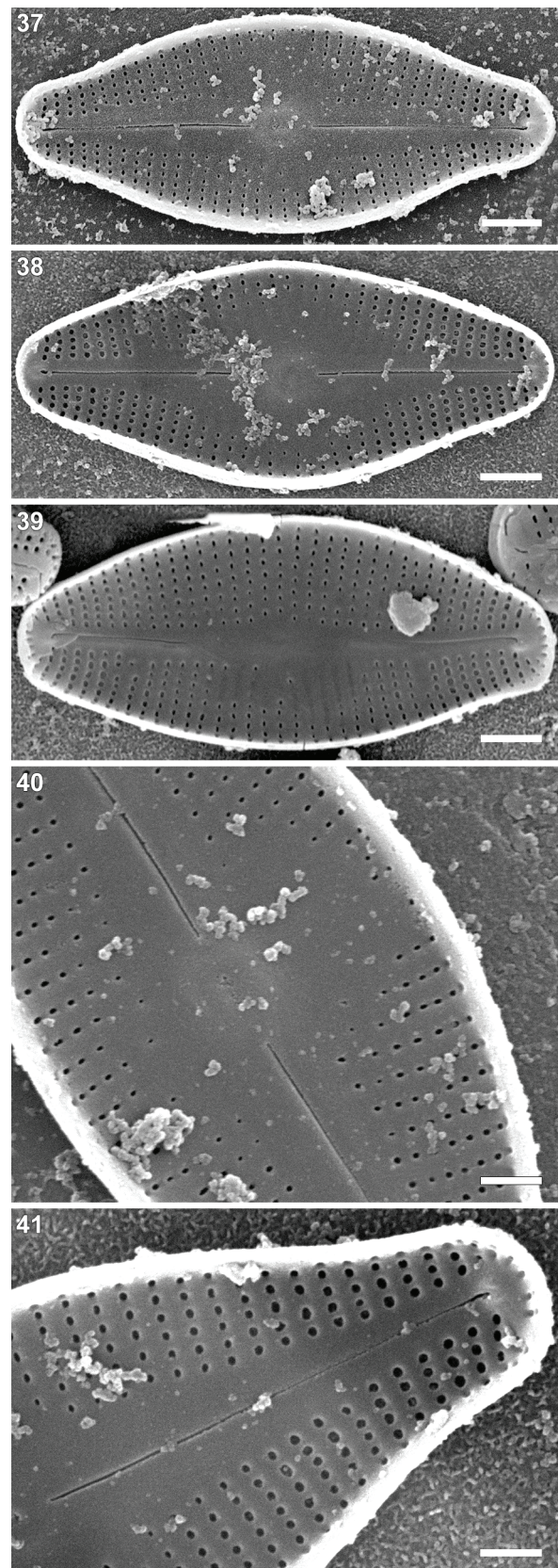
Figs 17–20. *Nupela indonesica* Kulikovskiy, Maltsev, Glushchenko et Kociolek sp. nov. Strain Ind 121. SEM, internal views. (17, 18) the whole valve; (19) central area; (20) valve end. Scale bars 1 μ m (17, 18), 0.5 μ m (19, 20).

(see Table 2). Calculated evolutionary distances for the barcoding subregion V4 of the 18S rRNA gene and amino acid sequence of ribulose–1,5–bisphosphate carboxylase provides strong evidence for *Nupela indonesica* being in a separate species from *Nupela lesothensis*, since for example the intraspecific percent dissimilarity for *Neidium* species being 0.3%–2.3% (V4) or <1.4% (rbcL).

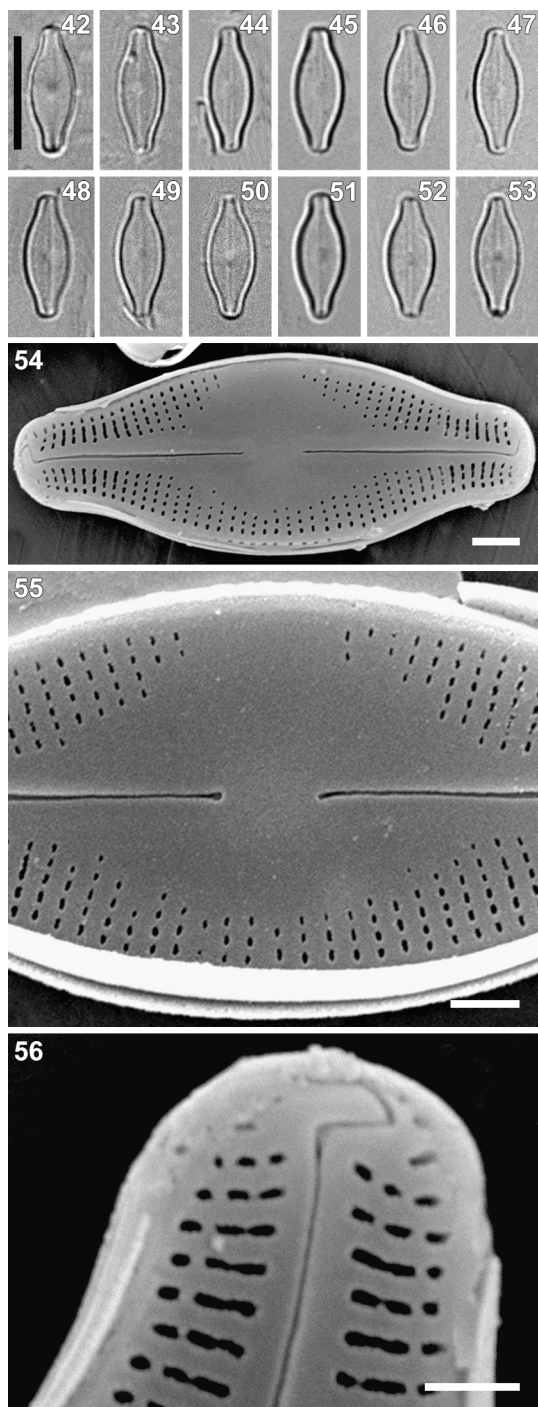
These species were isolated from Bali and Sulawesi



Figs 21–36. *Nupela indonesica* Kulikovskiy, Maltsev, Glushchenko et Kociolek sp. nov. Strain Ind 219. Slide no 01858. (21–32) LM, DIC, size diminution series, (33–36) SEM, external views, (33, 34) the whole valve, (35, 36) valves ends. Scale bars 10 μ m (21–32), 2 μ m (33, 34), 0.5 μ m (35, 36).

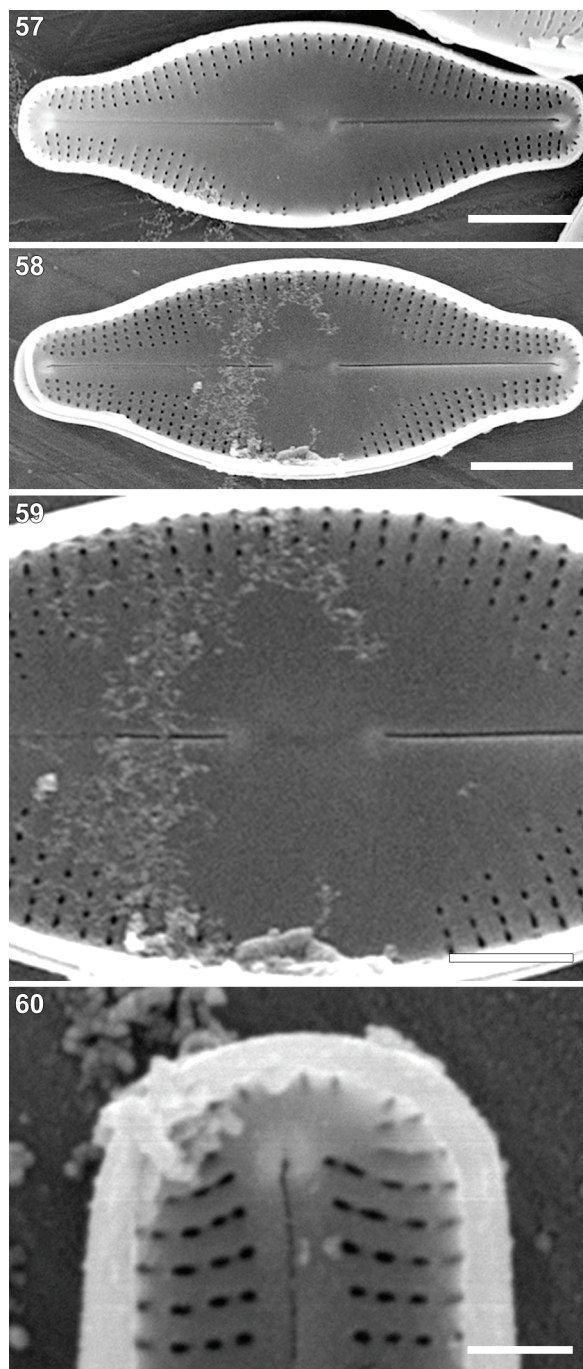


Figs 37–41. *Nupela indonesica* Kulikovskiy, Maltsev, Glushchenko et Kociolek sp. nov. Strain Ind 219. SEM, internal views. (37–39) the whole valve; (40) central area; (41) valve end. Scale bars 1 μ m (37–39), 0.5 μ m (40, 41).



Figs 42–56. *Nupela lesothensis* (Schoeman) Lange–Bertalot. Strain Ind 168. Slide no 01713. (42–53) LM, DIC, size diminution series; (54–56) SEM, external views, (54) the whole valve, (55) central area, (56) valve end. Scale bars 10 µm (42–53), 1 µm (54, 55), 0.5 µm (56).

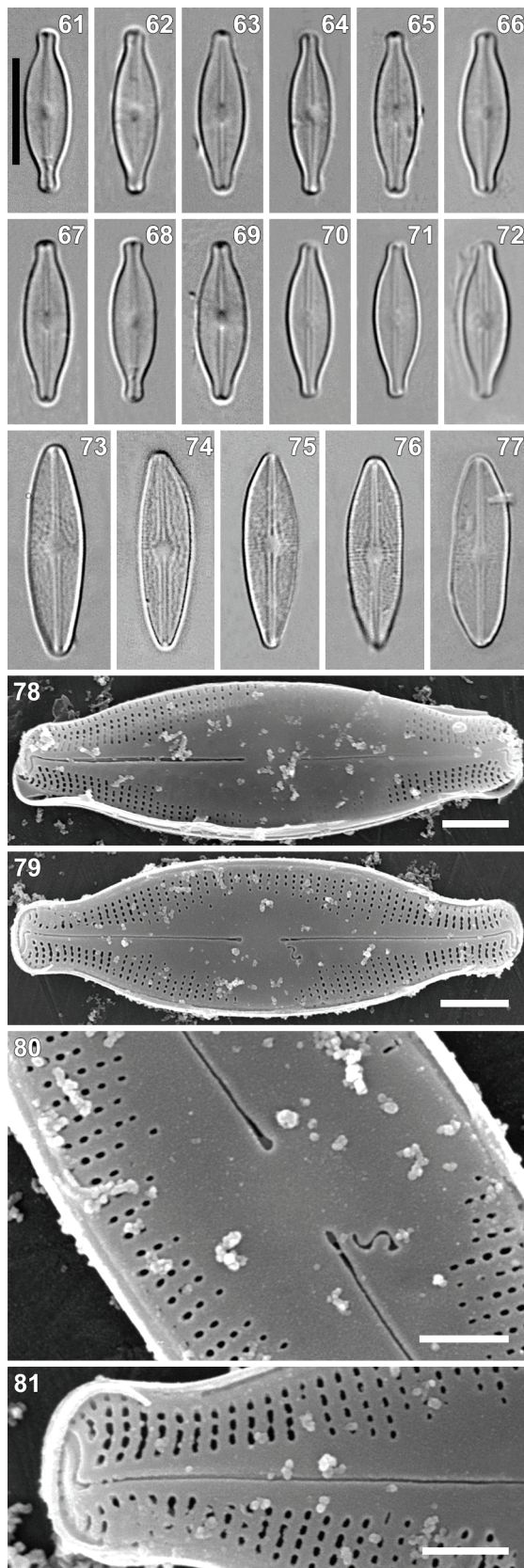
Islands in Indonesia. *N. indonesica* sp. nov. is distinguished from other known species of *Nupela*, such as the morphologically–similar species *Nupela giluwensis*, *N. biconfusa* and *N. praecipuoides*, on the basis of the shape of valves and especially number of striae, areolae in 10 µm and number of areolae per stria (see Table 2). Specimens of *N. lesothensis* from our material conform to the type description of this species given by SCHOEMAN



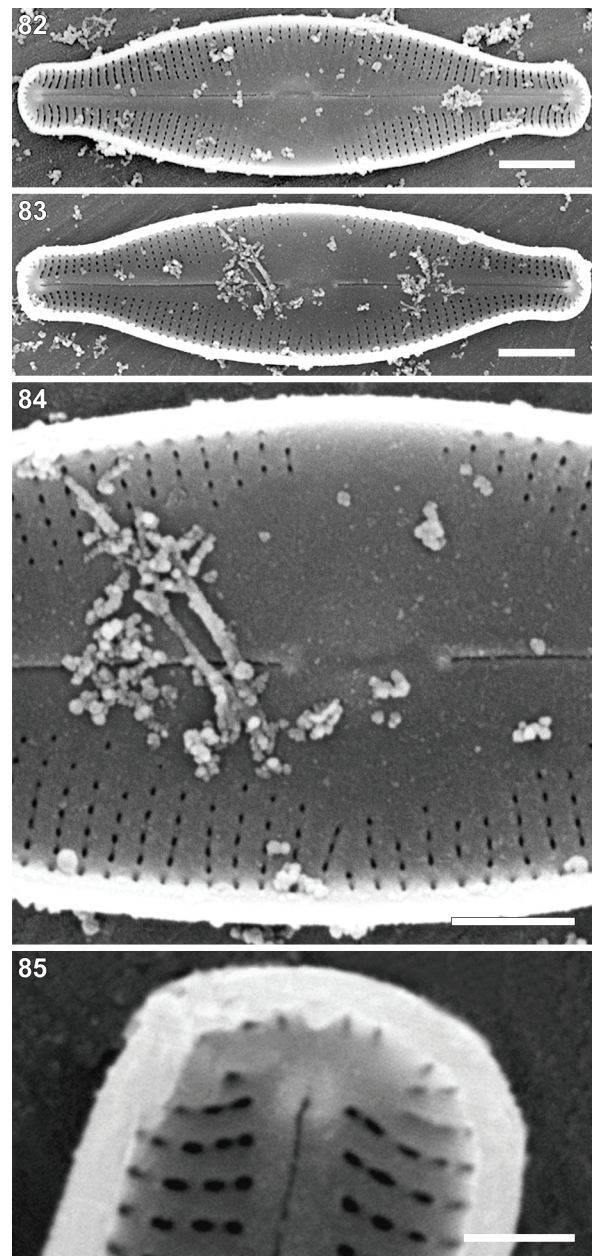
Figs 57–60. *Nupela lesothensis* (Schoeman) Lange–Bertalot. Strain Ind 168. SEM, internal views. (57, 58) the whole valve; (59) central area; (60) valve end. Scale bars 2 µm (57, 58), 1 µm (60), 0.5 µm (59).

(1973) on the basis of shape as well as striae and areolae density. This species was originally described by Schoeman from a spring and its effluent stream at Paballong near Mamathe in the Berea district of Lesotho, and known also from Italy, Seychelles, Africa southern and south–western, Germany, Munich, Botanical Gardens and South America (see WOJTAŁ 2009; RUMRICH et al. 2000; SALA et al. 2014). These data show that this species is widespread.

The phylogenetic position of *Nupela*, as represented



Figs 61–81. *Nupela lesothensis* (Schoeman) Lange–Bertalot (Strain Ind 170, slide no 01688) and *Brachysira* sp. (Strain VN 374, slide no 04582). (61–77) LM, DIC, size diminution series; (78–81) SEM, external views, (78, 79) the whole valve, (80) central area, (81) valve end. Scale bars 10 µm (61–77), 2 µm (78), 1 µm (79, 80).



Figs 82–85. *Nupela lesothensis* (Schoeman) Lange–Bertalot. Strain Ind 170. SEM, internal views. (82, 83) the whole valve; (84) central area; (85) valve end. Scale bars 2 µm (82, 83), 1 µm (84, 85).

by these two taxa from SE Asia, suggest they are part of a lineage that includes *Diploneis* and *Biremis*, rather than any group assigned to the Neidiineae. Strains of *Nupela* occur in one cluster that is sister to a branch of *Diploneis* species (Fig. 86). *Diploneis* as a genus that belongs to the family Diploneidaceae D.G. Mann and suborder Diploneidineae D.G. Mann (Cox 2015). And *Biremis*, which had previously been assigned to Scoliotropidaceae (ROUND et al. 1990), also does not appear to be part of the Neidiineae. Our data do not support the idea of Cox (2015) that *Nupela* as a genus is closely to *Brachysira*, another group that had been included in the Neidiineae, but that falls outside the Neidiineae, and is not closely related to *Nupela*. In other words, the only way for the

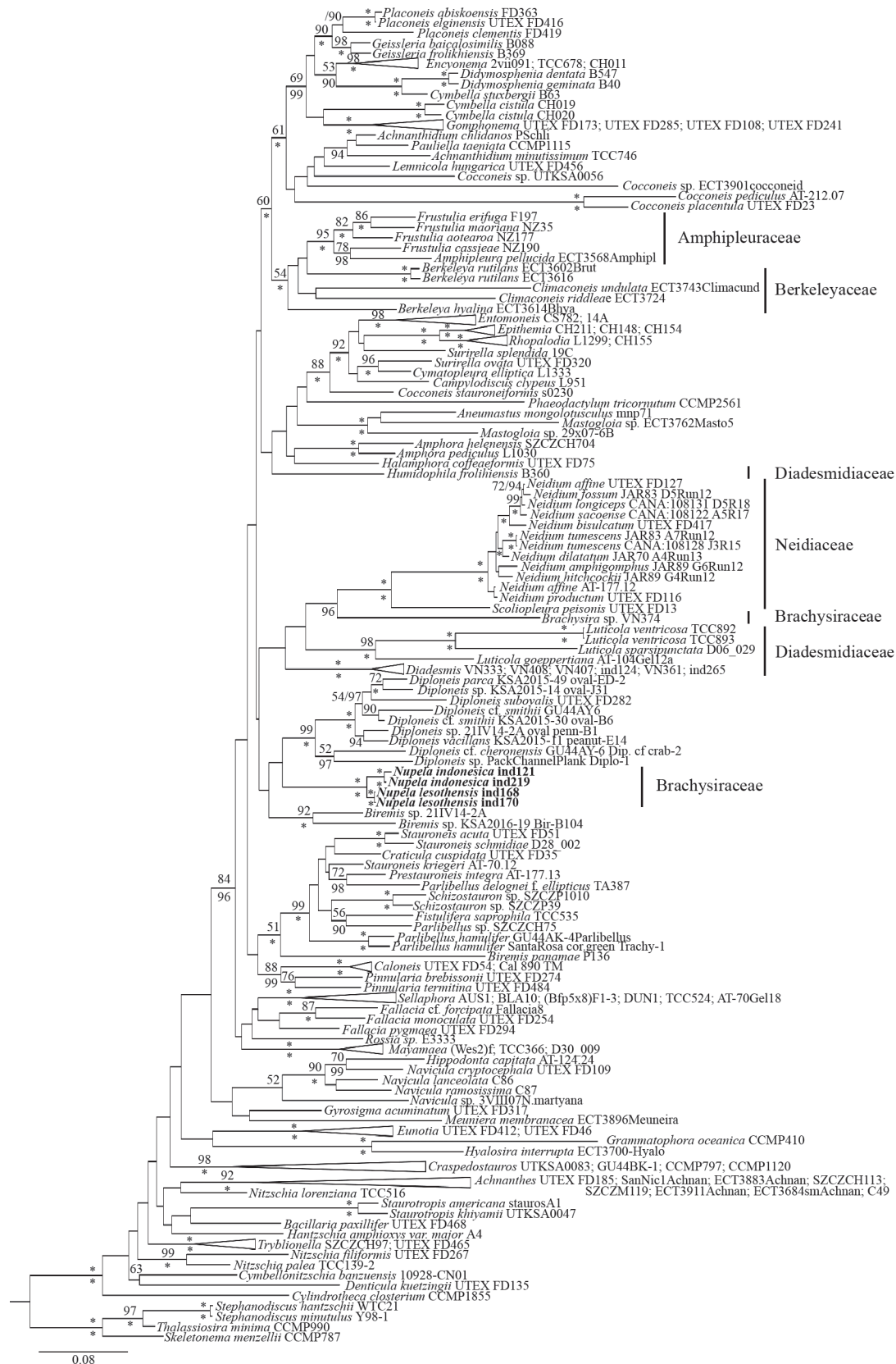


Fig. 86. Bayesian tree of *Nupela* species (indicated in bold) constructed from a concatenated alignment of 163 partial rbcL and partial 18S rDNA sequences of 1806 characters. Values above the horizontal lines (slash) are bootstrap support from RAxML analyses (<50 are not shown); values below the horizontal lines (slash) are Bayesian posterior probabilities (<90 are not shown). Species from the centric diatoms were used as an outgroup. * is 100% statistical support. Families indicated according Cox (2015).

Neidiineae to be a monophyletic group and to include *Nupela* in it, would be to include *Diploneis* and *Biremis* in this suborder. Our investigation does not support idea that *Nupela* belongs to the family Brachysiraceae sensu Cox (2015). Of course, increased taxon sampling of both *Brachysira* and *Nupela*, as well as other raphid diatom groups, is needed, but the current molecular analysis presented here shows that *Nupela* is distantly related to *Brachysira*.

Features that diagnose the genus *Nupela* are related to the morphology of areolae and pore occlusions, with areolae being larger externally than internally and covered by hymenes externally. Presence of external hymenes is known only for this genus among the pennate diatoms; it is absent from other pennate diatoms (POTAPOVA 2011). However, *Nupela* and *Brachysira* do have a similar structure of hymenes that POTAPOVA (2011) identified as coalescing hymenes (see POTAPOVA 2011; ROUND et al. 1990). This type of hymen exceeds the boundary of individual areolae and is organized into a total belt with more elongated shape inside the areolae in *Brachysira* and more kidney-like on the exterior of the areolae in *Nupela* (see POTAPOVA 2011; ROUND et al. 1990).

This analysis shows that several families assigned to the suborder Neidiineae sensu Cox are not part of a monophyletic group to the exclusion of other families. For example, both the Amphipleuraceae and Berkeleyaceae are sister taxa and combined to form independent lineage without high statistical support; they do not form a monophyletic group with members of the Neidiineae. Another cluster that is part of a monophyletic lineage that includes the Neidiaceae, has both the Brachysiraceae (including just the genus *Brachysira*) and Diadesmidiaceae (with genera *Luticola* and *Diadensis*). Interestingly, *Humidophila* and *Diadensis* are not sister genera on the basis molecular investigation, and the phylogenetic position of first genus needs investigation in the future with a greater number of strains. Thus, we can conclude that families originally assigned to the suborder Neidiineae as proposed by D.G. Mann actually represent at least four independent lineages. Several families as of yet do not have molecular sequence data represented for them (e.g. Cavinulaceae, Cosmioneidaceae).

Nupela indonesica sp. nov. is very interesting on the basis heterovalvy with respect to raphe development on a frustule. In our material strains of *N. indonesica* sp. nov. produced frustules with one valve with a fully-developed raphe and a short central nodule (see Figs 13, 18, 33) and the other valve with a short raphe with longer central nodule (see Figs 20, 34, 35, 39). Presence of reduced raphes in the genus *Nupela* is well documented, especially from Lake Baikal (Kulikovskiy et al. 2015, 2016). These data suggest that presence or absence of a raphe is very facultative feature; the genus *Sinoperonia* (LIU et al. 2018) may be the most extreme example of this phenomenon. From a phylogenetic point of view, these data provide evidence that instability in raphe development occurred both early in the development

of raphid diatom phylogeny (e.g. Eunotiales), as well as through secondary loss in taxa that belong now to Stauroneiaceae, Achnanthaceae and Achnanthidiaceae (COX 2015; KOCIOLEK et al. 2019; KULIKOVSKIY et al. 2019).

Presence of the monoraphid condition between suborder Neidiineae was shown perfectly by Cox (2006, 2012) who investigated raphe instability in *Diadensis gallica* that now transferred to a new genus *Humidophila* (LOWE et al. 2014). Cox (2015) combined *Humidophila* and *Diadensis* to a joint family Diadesmidiaceae. However, in our molecular investigation *Humidophila* is organized as an independent branch with lower statistical support with the genera *Amphora* and *Halampora* (see Fig. 86), and phylogenetic position of *Humidophila* will need more careful investigation in future with more available strains. The same results about phylogeny of the genus *Humidophila* was shown by NAKOV et al. (2019) who showed it in an unresolved position between the Stauroneiaceae and Neidiaceae. These data support the idea that raphe instability, in terms of size, structure and number is common among different evolutionary lineages of pennate diatoms.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Taxa and DNA sequence data used in phylogenetic analysis.

This material is available as part of the online article (<http://fottea.czechphycology.cz/contents>)

Genetic data and underlying taxa and GenBank sources of diatoms used in phylogenetic analysis for the diatom genus *Nupela* [Data set]. Zenodo. <http://doi.org/10.5281/zenodo.3862169>

Supplement S2. Final alignment of 2–gene DNA sequence data used for phylogenetic analysis in FASTA format.

Supplement S3. Maximum Likelihood tree of *Nupela* species (indicated in bold) constructed from a concatenated alignment of 163 partial *rbcL* and partial 18S rDNA sequences of 1806 characters. Values near the horizontal lines (slash) are bootstrap support from RAXML analyses (<50 are not shown). Species from the centric diatoms were used as an outgroup. Families indicated according Cox (2015).

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