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MORPHOLOGY AND MOLECULAR PHYLOGENY OF THE MARINE DIATOM *NITZSCHIA DENTATUM* SP. NOV. AND *N. JOHORENSIS* SP. NOV. (BACILLARIOPHYCEAE) FROM MALAYSIA

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Keywords: Girdle; *Hantzschiod*; Indented valves; Jagged; New species; Pennate diatom

Abstract

The marine diatom *Nitzschia dentatum* sp. nov. isolated from seawater samples of Kudat and *N. johorensis* sp. nov. isolated from beach sand samples of Sibu Island, Malaysia, have been described in this paper. Morphological identification, molecular phylogeny and toxin analyses were executed on the pure non-axenic algal cultures designated as KD89 and PS8, respectively. The main distinguishing feature of *N. dentatum* sp. nov. compared to other species is the jaggedcingulum structure which is only unique to this species. Meanwhile, *N. johorensis* sp. nov.is strongly characterized by the '*hantzschioid*'and '*nitzschioid*' symmetry dimorphisms; a common diagnostic feature but rarely described in other *Nitzschia* species. Identification of both strains was made based on the frustule diagnostic features and verified using the partial large ribosomal subunit DNA sequences. The results have confirmed that these two speciesare independent entities and novel species that have not been documented elsewhere. A notable finding from the Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian Index (BI) analyses have also revealed that *Nitzschia* species that have indentation in the middle of valves have been consistently grouped as same clade with high bootstrap values. The extracts of both species did not show detectable amount of domoicacid and have therefore, been classified as non-toxic. This discovery contributes to the documentation of *Nitzschia* species worldwide.

Introduction

Nitzschia Hassall is represented by 1,405 diatom species worldwide comprising the freshwater, brackish water and marine environments, with only half of it been accepted taxonomically (Guiry and Guiry, 2017). The species of *Nitzschia* are ecologically important as bioindicator (Maznah and Mansor, 2002; Trobajo*et al*., 2004; Trobajo *et al*., 2009), endosymbiont (Lee, 2011) and also as aquaculture live feed (Chu *et al*., 1996). Some *Nitzschia* species from the tropics regions are toxic. The first discovery of toxic *Nitzschia* species was identified from prawn pond samples in Vietnam (Lundholm and Moestrup, 2000) while others have been collected from estuarine sites such as in Malaysia (Suriyanti and Gires, 2015)and lagoon samples from the Southwest Mediterranean Sea (Smida *et al.*, 2014). There has been no report of harmful blooms to date that were associated with *Nitzschia*.

In Malaysia, the distribution of this genus has been listed along with other diatoms during field surveys and studies (Cleve, 1901; Nather-Khan, 1990; Shamsudin, 1990; Aishah and Nooraida, 1994; Aishah, 2005; Fareha *et al.*, 2011; Saifullah *et al.*, 2014). Taxonomical

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identification of the diatom *Nitzschia* has been highly dependent on discernable valve characters under light microscopy and the molecular data on the existing *Nitzschia* species was lacking. In molecular phylogeny of microalgae, the D1–D3 domain of LSU rDNA has been commonly used and proven as suitable marker for species delineation (Ki and Han, 2005; Sonnenberg *et al*., 2007; Lundholm *et al*., 2002). Currently, 14 species of *Nitzschia* have been compiled from Malaysian waters (Suriyanti, 2017). In the present research, two species of *Nitzschia* have been identified based on morphological and molecular characteristics and hitherto reported as new to science. This research is part of the results obtained from *Nitzschia* distribution study in Malaysia (Suriyanti, 2017).

Materials and Methods

Nitzschia cell isolate KD89 was obtained from marine net haul sample of Kudat, Borneo and isolateNPS8 from sand sediment sample of Sibu Island, Johor, Malaysia. Both isolates were established into pure non-axenic clonal cultures. All cultures were grown in silica-enriched media modified from SWII (Iwasaki, 1961) concoction adjusted to 30 Practical Salinity Unit (PSU)and maintained at 26 °C under 12:12 hour light : dark photo cycle.

The removal of organic matter by acid treatment was done according to Renberg (1990).Cultured cells were harvested by centrifugation at 8000 revolutions per minute (RPM) for 10 minutes and the supernatant was discarded. Hydrogen peroxide (30%) was added to the cell pellet and heated at 85°C for two to three hours. After oxidization, hydrogen peroxide was discarded and the samples were treated with 10% hydrochloric acid for several days at room temperature. After treatment, cells were rinsed two to three times with distilled water and stored in 70% ethanol.

The average size of valves was obtained by measuring the specimens from the first batch of clonal cultures to minimize size reduction due to mitotic division. Cleaned diatom valves were mounted on a glass slides using Naphrax mountant (Brunel Microscope Ltd., U.K.) and viewed under a light microscope (Olympus BX51TF, Japan) equipped with built-in camera (Olympus U-TV1x, Japan)at 20 × magnification. A minimum of 30 cells were randomly selected for length and width measurements by using Analy SIS Life Science Professional software version 3.0 (Build 1243).

The ultra-structural valve characteristics for species identification were observed by using electron microscopy. For scanning electron microscopy (Leo 1450 VP, United Kingdom), cleaned specimens were dried overnight on cover slips and mounted on a stub for gold-palladium coating before viewing. For transmission electron microscopy (Philips CM12, Netherland), cleaned specimens were mounted on formvar-coated copper grids. The diagnostic features for identification were the valve outlines, internal valves, valve striations, eccentricity of the raphe system and the presence of poroids in the raphe canals.

Molecular data were obtained from DNA samples of fresh specimens of pure clonal cultures. Diatom cells from the clonal cultures were harvested by centrifugation at 8000 rpm for 10 minutes. The genomic DNA was extracted using Gene JET Plant Genomic DNA Purification Mini Kit. Targeted LSU rRNA gene was amplified using primer set D1R 5'-ACC CGC TGA ATT TAA GCA TA-3' (Scholin *et al.*, 1994) and D3B 5'-TCG GAG GGA ACC AGC TAC TA-3' (Nunn *et al.*, 1996). Total PCR reaction volume of 50µl contained 0.2 g/µl bovine serum albumin, 0.2 mMdNTPs, 0.5 µM of forward and reverse primers, 1x Taq Buffer, 1.5 mM $MgCl_2$, 1.25 U Taq Polymerase (Fermentas #EP0402) and DNA template. The amplification condition was set atone initial denaturation at 94°C for 2 min, 30 cycles of 94°C for 30s, 60°C for 30s, 72°C for 30s and followed by final extension at 72°C for 2min (Lundholm *et al.*, 2002). Amplified products

were visualized by electrophoresis on a 1% agarose gel pre-casted with Red Safe nucleic acid staining solution (iNtRON Biotechnology Cat. No.21141). Purified products were sent to First Base Laboratories (Malaysia) for sequencing using the same primer set.

In the analysis of multiple sequence alignment, the quality of DNA sequences were checked manually using Bioedit version 7.0.9.0 (Hall, 1999). Reverse sequences were reverse-complemented with the forward sequences using optimal GLOBAL pairwise alignment. Trimmed sequences were saved in FASTA file and uploaded to NCBI online database for query using BlastX (Zhang *et al.*, 2000). Other *Nitzschia* LSU sequences were downloaded from the http://www.ncbi.nlm.nih.gov/ websiteas well. Multiple sequence alignment was executed using Muscle in MEGA Version 6 (Tamura *et al.*, 2013) and Clustal X version 1.81 (Thompson *et al.*, 1997) and saved in NEXUS format.

Phylogenetic analyses were performed using ML and MP algorithm in PAUP* Version 4.0b10 (Swofford, 2003). For ML analysis, the best model GTR+G+I was generated using Modeltest3.7 (Posada and Crandall, 1998). Heuristic search was used in the MP analysis. Tree reliability was estimated using bootstrap method with 1000 replicates of data set for ML and MP. Bayesian Analysis was used to generate the best phylogenetic tree using prior probability Monte Carlo Markov Chains (MCMC) Method with 490000 generation in Mr Bayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). Distance Analysis was generated using PAUP* Version 4.0b10 (Swofford, 2003).

Results and Discussion

Nitzschia dentatum Suriyanti S.N.P. & G.Usup, **sp. nov.** **(Figs 1A–L).**

Diagnosis: The outline and valve characteristics of *N. dentatum* sp. nov. is compatible to species categorized under the section of *Lanceolatae*. Translated from Cleve and Grunow 1830 (as cited in Mann, 1978), the section *Lanceolatae* is defined as "lanceolate-linear or rarely oval, highly eccentric keels, keel puncta not prolonged". Many species in this section have cell dimensions close to *N. dentatum* sp. nov. Due to the high variability of the sizes, other features such as the shape of valve and presence of central interspace were used in combination to select the most proximate species from its allies. *Nitzschia dentatum* sp. nov.has slender and narrow cell outlines, most similar with *N. inconspicua* Grunow, *N. frustulum* (Kutzing) Grunow and *N. pusilla* Grunowin this section (Table 1). *N. dentatum* sp. nov.lack the central interspace, different from *N. frustulum* and *N. pusilla* except *N. inconspicua*. *N. dentatum* sp. nov. differs from *N. inconspicua* and the rest of it allies by its relatively high density of striae (78 in 10 µm); whereby other species only have average maximum number of 30–40 striae in 10µm. The main distinguish features that are only present in this species is the cingulum structure that is jagged which resembles the 'teeth' which could be easily distinguished from the cingulum of other species of *Nitzschia.* This feature is similar with the lateral extensions of closed copula in *Rhabdonema* sp. (Round *et al.*, 1990), with the exception that *N. dentatum* sp. nov. has an open-type girdle bands.

Type: MALAYSIA. Kudat, Sabah, seawater sample, 6° 51' N, 116° 51' E, collected 8 December 2013, isolated by capillary washing technique on 10 December 2013, Suriyanti and Usup.

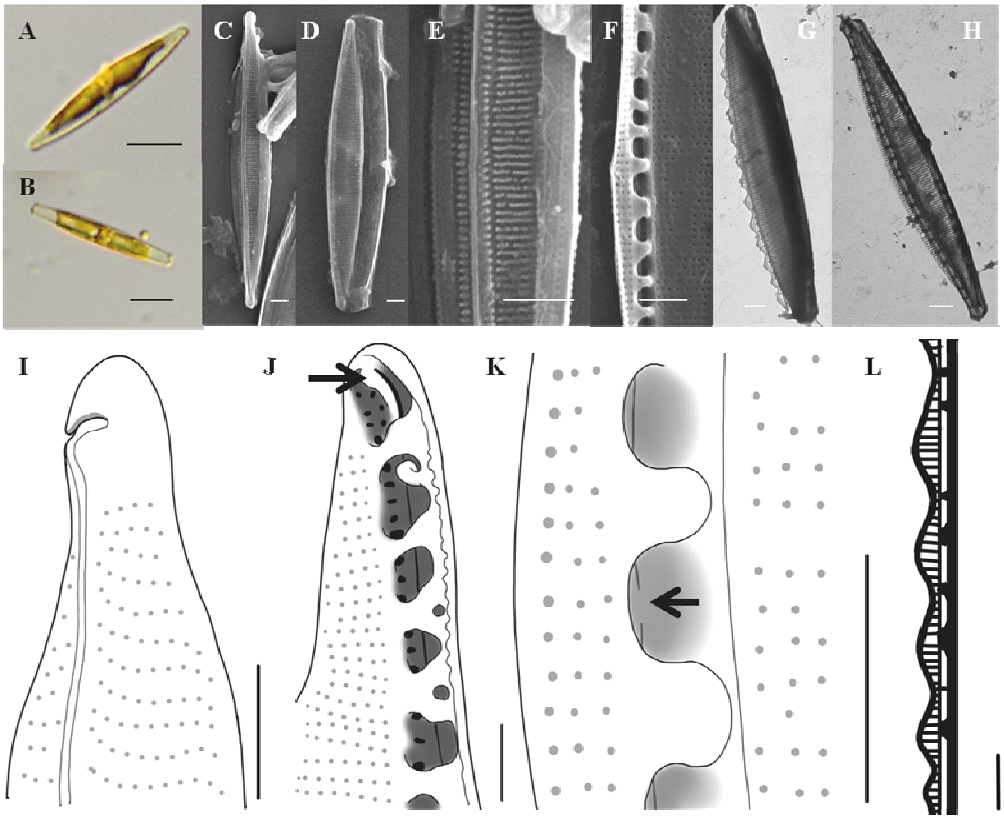
Holotype: Voucher # KD89, deposited in the Marine Microbes and Biotechnology Laboratory, Universiti Kebangsaan Malaysia.

NCBI Accession no.: KX839243.

Notes: Marine habitat; cells solitary; each cell contains two yellow-brown chloroplasts; valves small and narrow; cell outline lanceolate and tapering towards the apices; rectangular in girdle view; length 17.0–18.0 µm, width 2.5–4.0 µm; apices are slightly capitate; wide pervalvar axis; raphe more or less eccentric; raphe continuous in external view; rectangular fibulae, not widely

separated in the middle of valve; jagged structure of the girdle band was observed in each valve; fibulae on the diagonal side of valves (*nitzschioid*); terminal fissures are slightly hooked to the sameside; 13 fibulae in 10µm; terminal fissures bent towards the same side which end in a large *helictoglossa* internally; raphe ending at the centre only observable from internal view; it is raised in a simple shallow raphe canal which contains poroids; interstriae are raised externally, striae not interrupted by laternal sterna;78 striaein 10µm;single row of round to rectangular poroids, occluded by simple-type hymen perforation, 9–10 in 1 µm across; single strip of jagged cingulum structure with striae is observed for each valve; 1 row of poroid in the cingulum; jagged strip is lined by striaethat containporoids.

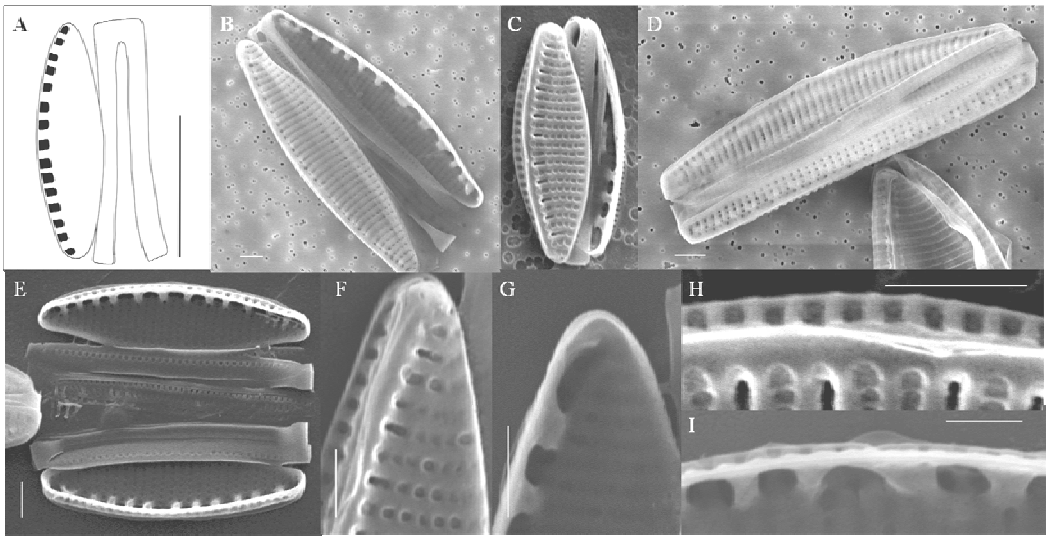
Etymology: Named after the unique jagged structure of the girdle band. 'Dentatum' is Latin for 'toothed'.



Figs 1A–L. LM, SEM, TEM and drawings of *N. dentatum* sp. nov. voucher #KD89. LM (A–B). A: Whole cell in valve view showing two yellow-brown chloroplasts; B: Rectangular in girdle view. (Scale bar = 10 µm) SEM (C–F). C: Valve view showing slightly capitate ends; D: Wide pervalvar axis of intact valves, showing the position of the jagged cingulum in girdle; E: Continuous raphe slit from the external view; F: arrangement of the fibulae, without large central interspace. TEM (G–H). G: Partially intact dental-like cincture at the margin of the valve; H: Fibulae on the diagonal side of the valves ('*nitzschioid*' symmetry). Scale bar = 1 µm. Illustration (I–L): I: Terminal fissure bent towards the same side in both ends; J: Terminal raphe ends in *helictoglossa* internally. K: central raphe ending observed from the internal view; L: close-up of the jagged structure of the girdle with horizontal striae. (Scale bar = 1 µm).

Nitzschia johorensis Suriyanti S.N.P. & G. Usup, **sp. nov**. **(Figs 2A–I).**

Diagnosis: *Nitzschia johorensis* sp. nov.is relatively small in size and best fit into the section *Lanceolatae*. After acid-treatment, the prominent girdle attachment of the epivalves and hypovalves remained intact, compared to other species whereby the frustules completely disintegrated after the same procedures of acid treatment. Observation of the valve symmetry in this species was made possible this way. Only small-sized *Nitzschia* species in this section were included for comparison. Overall, *N. johorensis* sp. nov. is most similar with *N. fonticola* except for habitat origin whereby the latter is strictly confined to the freshwater (Foged, 1971; Tudesque *et al.*, 2008), has a central interspace and only exist in *nitzschioid* form (Mann, 1978). On the other hand, the valve size of *N. johorensis* sp. nov.is compatible to the measurements of *N. tropica* Hustedt (Tudesque *et al.*, 2008) and *N. costei* Tudesque, Rimet *et.* Ector but differs in the shape of valve ends. In addition, *N. tropica* has widely separated fibulae in the middle (Tudesque *et al.*, 2008), a feature not present in *N. johorensis* sp. nov (Table 1). *N. johorensis* sp. nov. can be distinguished from *N. costei*by its thickened and imperforated siliceous marginal wall along the keel towards the apices, whereas *N. costei*has a double row of striae near the keel. The main feature in *N. johorensis* sp. nov. not found in other samples in this study is the *nitzschioid* and *hantzschioid* dimorphisms. It is rarely documented in other *Nitzschia* species (Mann, 1978; Round *et al.*, 1990), but it has been noted insome species such as the heterotrophic *N. alba* J.C. Lewin and R. A. Lewin (Lauritis *et al.*, 1967), the polar species *N. frigid* Grunow (Medlin and Hasle, 1990) and the middle-constricted *N. dubia* Smith (Mann, 1978).None of those species matched the description of *N. johorensis* sp. nov. morphologically and ecologically.



Figs 2A–I. Drawings and SEM micrographs and of *N. johorensis* sp. nov. isolate PS8. Illustration (A) A: Lanceolate valve outline. Scale bar = 10 µm. SEM (B–I). B: Irregular fibulae arrangement and width; C: Thick interstriae and margin, poroids more elongated near margin; D: Rectangular in girdle view of the intact valves; E: '*hantzschioid'* symmetry of the raphe; closed-type of girdle band with single row of poroid; F: Terminal fissure, valve margin near apical ends without perforation; G: Terminal fissure ends in helictoglossa internally; H: Raphe is interrupted in the middle; I: raised raphe canal with pores. (Scale bar = 1µm).

Table 1. Morphometric data of species that have the closest similarity to *Nitzschia dentatum* sp. nov. and *N. johorensis* sp. nov. (n.d. = no data).

Species	Length (μm)	Width (μm)	Fibulae (10 μm)	Striae (10 μm)	Central interspace (+/–)	Reference
*N. costei*Tudesque, Rimet & Ector	8–45	2.5–4.5	9–12	23–27	+	Tudesque *et al.* (2008)
N. dentatum sp. nov. (n>30)	17.0–18.0	2.5–4.0	11–13	70–78	–	Present study
N. fonticola (Grunow) Grunow	10.0–55.0	2.5–4.5	n.d.	24–27	+	Kociolek (2011)
	13.5–22.0	4.0–5.0	9–11	22–26	n.d.	Foged (1971)
	7.0–46.0	2.5–5.5	10–12	26–30	+	Tudesque *et al.* (2008)
N. frustulum (Kutzing) Grunow	n.d.	n.d.	8–10	28–30	+	Mann (1978)
	10.8–34.0	3.0–3.9	13.3–15	26.6–30	+	Trobajo *et al.* (2013)
	12.0–14.0	3.0–4.0	14–15	45–52	+	Present study
N.inconspicua Grunow	4.1–15.3	2.3–3.1	8.9–17	23.7–30.4	+	Trobajo *et al.* (2013)
	12.1–14.4	2.0–3.2	3–4	28–31	–	Present study
*N. johorensis*sp. nov. (n>30)	7.1–11.8	1.8–3.5	11–12	30–33	–	Present study
N. pusilla Grunow	18.0–20.0	3.5–8.0	15–16	40–46	–	Coste and Ricard (1980)
	7.2–9.7	1.8–3.5	>9	>37	–	Present study
N. tropica Hustedt	14.5–44.6	3–3.7	8–10	23–25	+	Tudesque *et al.* (2008)
Nitzschia sp. 1 (PgMky44)	25.7–32.8	2.8–4.6	7–8	n.d.	+	Present study
*Nitzschia*sp.2 (KD90)	9.2–13.4	2.2–3.5	8–10	n.d.	–	Present study

Type: Malaysia. Pulau Sibu (Sibu Island), Johor, beach sand sediment, 2° 12‘ N, 104° 04’ E, collected 5 June 2012, isolated by capillary washing technique on 7 June 2012, Suriyanti and Usup.

Holotype: Voucher no.PS8,deposited in the Marine Microbes and Biotechnology Laboratory, Universiti Kebangsaan Malaysia.

NCBI Accession no.: KX839235

Notes: Two chloroplasts at both ends; valve lanceolate, length 7.1–11.8 μm, width1.8–3.5 μm; slightly capitated ends; rectangular in girdle views; valve mantel is a one-row height of elongated poroids; fibulae are either on the same side (*hantschioid)* or on the diagonal side (*nitzschioid)* of the complimentary valve; fibulae coarse and wide; irregularly spaced; 11 in 10 μm; interstriae are raised externally, smooth on the internal surface; 33 striae in 10 μm; poroids are round to rectangular; more elongated towards the valve margins; terminal fissure hooked

towards the valve face; valve near the raphe and apices thickened without perforations; *helictoglossa* ending internally; 1 row of poroids, 3–5 in 1 μm; 3–4 bands of semi-closed girdle type; single row of poroids in the copulae; raphe canal is raised with pores; central nodule in raphe slit; central interspace absent.

Etymology: The species epithet is named after the state (Johor) from where it was found.

Phylogenetic analyses: The LSU rDNA sequences used in the phylogenetic analyses were obtained from 11 *Nitzschia* species (Table 2) out of 14 total marine *Nitzschia* species recorded from Malaysia (Suriyanti, 2017). Amplification of the LSU rDNA region yielded product length of ca. 800 basepairs. Taxa relationship of *Nitzschia* was inferred by the placement of *Pseudo-nitzschia americana* (Hasle) Fryxell and *Fragilariopsis kerguelensis* (O'Meara) Hustedt as out groups. These two genera were previously originated as two subsections in the genus *Nitzschia* beforeclassified as separate genera. The bootstraps (1000 replicates) were shown next to branch. The sequences used for the ML (Fig. 3), MP (Fig. 3), Bayesian Analysis (Fig. 4) and Distance Analysis (Table 3) were obtained from *Nitzschia* spp. recorded from Malaysia and retrieved from the Genbank BLAST query database. Those analyses have included 21 partial LSU nucleotide sequences including two out groups.

Table 2. *Nitzschia* culture strains used in the phylogenetic analyses.

Species	Location	Coordinate	Strain	GenBank
N. amabilis	Teluk Kumbar, Penang	5°17'4"U, 100°14'22''T	TK47	KX839238
Nitzschia sp. 1	Teluk Kumbar, Penang		PgMky44	KX839237
N. sigma	Kuala Selangor, Selangor	3°20'20"U, 101° 14'41"T	KS58	KX839241
N. lorenziana	Kuala Selangor, Selangor		KS55	KX839240
N. navis-varingica	Sungai Pendas, Johor	1°23'3"U, 103°37'30"T	P22C7	KX839243
N. johorensis sp. nov.	Pulau Sibu, Johor	2°13'34"U, 104°3'44"T	PS8	KX839235
N. pusilla	Pulau Tioman, Pahang	2°47'37"U, 104°12'7"T	TMN26	KX839236
N. dentatum sp. nov.	Kudat, Sabah	6°53'12"U, 116°49'31"T	KD89	KX839243
Nitzschia sp. 2	Kudat, Sabah		KD90	KX839242
N. frustulum	Kudat, Sabah		KD92	KX839245
N. inconspicua	Simpang Mengayau, Sabah	7°1'26"U, 116°44'34"T	TOB54	KX839239

All reconstructed phylogenetic trees showed almost identical topologies but differed in bootstrap values. The ML and MP phylogenetic tress were only distinguished in the placement of *Nitzschia* sp. 1. Two distinct clades were generated in the trees in which *N. Soratensis*E. A. Morales *et* M. L. Vis and*N.* cf. *Fonticola* (Grunow) Grunow formed as sister clade to other *Nitzschia* spp. *N. dentatum* sp. nov. dan *N. johorensis* sp. nov. formed isolated branches on each phylogenetic tree and were supported by bootstrap 63 dan 57 in ML and 66 dan 61in MP, correspondingly. The prior probability in Bayesian inference was 0.9939 for*N. dentatum* sp. nov. and 0.716 for*N. johorensis* sp. nov. The least genetic distance to delineate these species was estimated at 4.86% (Table 3). *N. dentatum* sp. nov. was grouped into the same clade as *N.* cf. *promare* Medlin, *N. pellucida* Grunow, *N. navis-varingica* N. Lundholm and Ø. Moestrup, *N. amabilis* Suzuki, *Nitzschia* sp. 1dan *N. lecointei* van Heurck. On the other hand, *N. johorensis* sp. nov. formed single branch and did not cluster with any other clades. Consistent grouping was

observed in a clade comprising *N.* cf. *promare*, *N. pellucida*, *N. navis-varingica* and *N. amabilis* in all trees and supported by high bootstrap values (ML: 98, MP: 100, BI: 1).

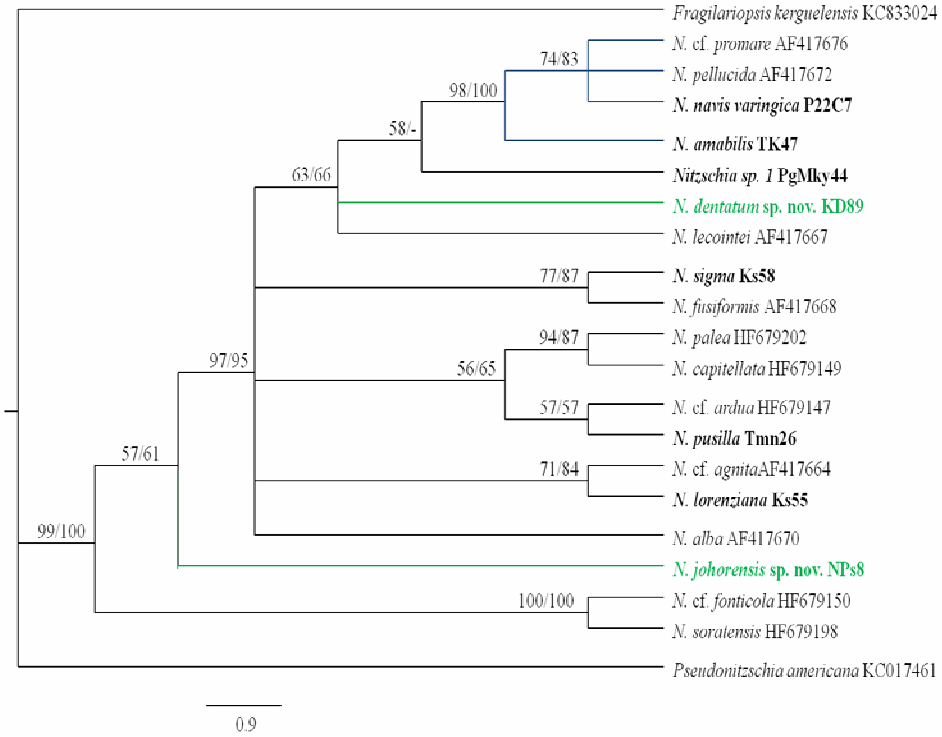


Fig. 3. Phylogenetic trees of *Nitzschia* spp. reconstructed based on the D1–D3 gene region of LSU rDNA using Maximum Likelihood and Maximum Parsimony with *P. americana* and *F. kergeulensis* as outgroups. Bold-lettered strains were obtained from this study (black) and proposed species (green). Species that are constricted in the middle valve grouped into the same clade consistently in all tress (blue). Bootstrap values with 50% majority are shown (ML/MP).

This study proposes two *Nitzschia* species into the section *Lanceolatae* based on morphological diagnoses and molecular evidence using LSU rDNA genes. As taxonomic conclusion should not solely rely on the valve characters, molecular characterisation based on the LSU rDNA regions was done to verify the phylogeny placement of the two new species. The D1–D3 region of LSU rDNA is a highly variable and a suitable marker for species identification (Ki and Han 2005; Sonnenberg *et al.*, 2007; Lundholm *et al.*, 2002) amongst the 12 more conserved D-domains. Highly conserved SSU marker on the other hand is less desirable in taxonomic purposes, but is helpful in depicting the original lineage of diatom (Zimmermann *et al.*, 2011; Smida *et al.*, 2014).

Phylogenetic tree reconstruction showed that the genus *Nitzschia* is not monophyletic, in agreement with its great variationin morphology. Based on the tree, *N. dentatum* sp. nov. is closely related to *N.* cf. *promare*, *N. pellucida*, *N. navis-varingica*, *N. amabilis*, *Nitzschia* sp. 1 and *N. lecointei*.None of these species has jagged girdle bands except for the proposed species *N. dentatum* sp. nov. Girdle character is one of the important features for species delineation (Mann, 1978; Round *et al.*, 1990; Lundholm and Moestrup, 2000). Furthermore, those species are genetically diverged at 7%–11%, respectively (Table 3).

Table 3. Genetic distance matrix of 21 *Nitzschia* spp. analyzed based on LSU rDNA partial gene sequences with *P. americana* and *F. kerguelensis* as outgroups. The distance was calculated using Kimura-two-model (Kimura, 1980).

	Species	1	2	3	4	5	6	7	8	9	10	11	12
1	AF417676 *Nitzschia* cf. *promare*	-											
2	*Fragilariopsis kerguelensis*	0.097	-										
3	KX839241 *N. sigma*	0.089	0.112	-									
4	AF417672 *N. pellucida*	0.022	0.105	0.095	-								
5	HF679150 *N.* cf. *fonticola*	0.109	0.107	0.131	0.117	-							
6	KC017461 *Pseudonitzschia americana*	0.092	0.027	0.098	0.095	0.099	-						
7	KX8392358 *N. johorensis*	0.110	0.090	0.127	0.120	0.123	0.090	-					
8	HF679202 *N. palea*	0.076	0.112	0.083	0.086	0.120	0.103	0.104	-				
9	AF417664 *N.* cf. *agnita*	0.080	0.111	0.086	0.086	0.118	0.096	0.104	0.067	-			
10	KX839243 *N. dentatum*	0.076	0.112	0.079	0.083	0.114	0.110	0.113	0.071	0.074	-		
11	HF679147 *N.* cf. *ardua*	0.085	0.120	0.085	0.095	0.111	0.114	0.097	0.055	0.085	0.082	-	
12	AF417668 *N. fusiformis*	0.082	0.105	0.063	0.082	0.117	0.096	0.109	0.083	0.073	0.080	0.078	-
13	KX839238 *N. amabilis*	0.034	0.107	0.085	0.038	0.109	0.101	0.116	0.074	0.072	0.073	0.092	0.082
14	AF417667 *N. lecointei*	0.055	0.094	0.076	0.062	0.115	0.094	0.101	0.071	0.068	0.052	0.073	0.063
15	KX839240 *N. lorenziana*	0.085	0.115	0.097	0.088	0.123	0.108	0.118	0.064	0.052	0.081	0.069	0.090
16	HF679198 *N. soratensis*	0.118	0.099	0.120	0.122	0.065	0.088	0.112	0.104	0.100	0.103	0.115	0.106
17	AF417670 *N. alba*	0.083	0.110	0.087	0.086	0.129	0.109	0.114	0.069	0.077	0.083	0.080	0.082
18	HF679149 *N. capitellata*	0.076	0.107	0.074	0.080	0.117	0.099	0.099	0.026	0.070	0.070	0.056	0.074
19	KX839236 *N. pusilla*	0.080	0.121	0.079	0.086	0.111	0.111	0.097	0.044	0.067	0.076	0.049	0.074
20	KX839243 *N. navisvaringica*	0.019	0.094	0.092	0.027	0.111	0.094	0.110	0.088	0.083	0.074	0.089	0.082
21	KX839237 *Nitzschia* sp1	0.059	0.110	0.089	0.061	0.117	0.109	0.112	0.064	0.063	0.064	0.079	0.086

Table 3 contd. right side

	Species	13	14	15	16	17	18	19	20	21
13	KX839238 *N. amabilis*	-								
14	AF417667 *N. lecointei*	0.064	-							
15	KX839240 *N. lorenziana*	0.078	0.076	-						
16	HF679198 *N. soratensis*	0.106	0.107	0.119	-					
17	AF417670 *N. alba*	0.086	0.074	0.089	0.118	-				
18	HF679149 *N. capitellata*	0.076	0.061	0.066	0.104	0.071	-			
19	KX839236 *N. pusilla*	0.080	0.071	0.069	0.103	0.069	0.044	-		
20	KX839243 *N. navisvaringica*	0.037	0.053	0.087	0.118	0.086	0.077	0.086	-	
21	KX839237 *Nitzschia* sp1	0.056	0.046	0.070	0.116	0.074	0.068	0.071	0.062	-

In the other hand, *N. johorensis* sp. nov. formed an isolated branch giving 9%–13% genetic divergence from other *Nitzschia* species (Table 3). The most morphologically similar species *N. Fonticola*also clustered into another clade and is 12.3% divergence from *N. johorensis* sp. nov. Apart from that, the dimorphic resemblance of *N. alba* did not show clear relationship with *N. johorensis* sp. nov. and there were no genetic data for *N. dubia* and *N. frigida*.

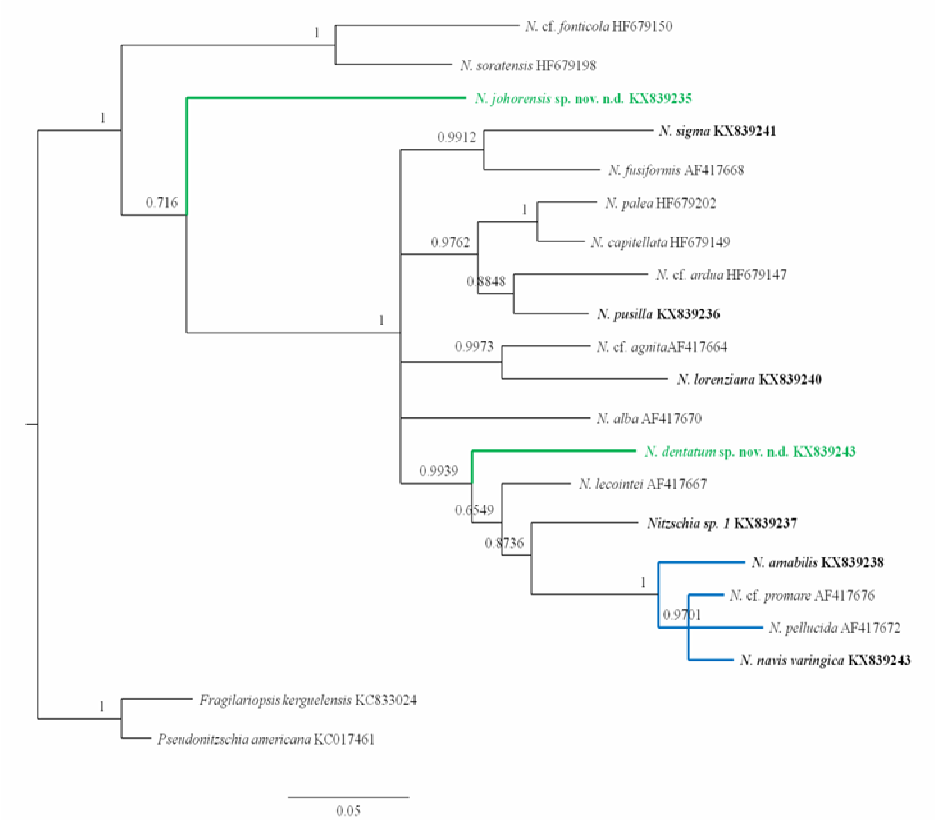


Fig. 4. Phylogenetic trees of *Nitzschia* spp. reconstructed based on the D1–D3 gene region of LSU rDNA using Bayesian Analysis with *P. americana* and *F. kergeulensis* as out groups. Bold-lettered strains were obtained from this study (black) and proposed species (green). Species that are constricted in the middle valve grouped into the same clade consistently in all tress (blue). Prior probability values are shown.

From our observation, entities that have similar outlines tend to group together in the tree. For instance, cells with middle indentations of valves i.e. *N. navis-varingica*, *N. amabilis*, *N. promare*, *N. pellucida* and *N. laevis* clustered together as a group. To our knowledge, there are no other cells that have middle indentations located outside of that clade in the tree. *N. amabilis*is the new nomination of *N. laevis* (Suzuki *et al.*, 2010) and hence is the same species. *N. amabilis* branches out separately in the phylogenetic tree but it differs from *N. laevis* (syn. *N. amabilis*) genetically by 4%. There was no strong evidence to prove them as separate species. *N. polaris* Grunow*ex* Cleve and *N. neglecta* Hustedt (Medlin and Hasle, 1990) are among others that have indentation in the middle, but there were no DNA sequences available.

The behavior of colony formation also reflects the clustering but was very weakly supported (Lundholm *et al.,* 2002). The strains' habitat, localities, and toxicity were not resolved in the phylogenetic tree. Freshwater species *N. palea* (Kützing) W. Smith and *N. sigma* (Kützing) W. Smith (Aishah and Nooraida, 1994) grouped with other marine species. Likewise, *N. amabilis* was isolated from tropical water (Suriyanti, 2017) while *N. promare* is a polar species (Medlin and Hasle, 1990). In terms of toxicity, the DA producers *N. bizertensis* Smida, Lundholm, Sakka and Hadj Mabrouk and *N. navis-varingica* Lundholm*et*Moestrupwere also not closely related (Smida *et al.,* 2014). Both of the newly proposed species in this study are non-toxic.

It is indeed very difficult to identify the key traits for the phylogenetic grouping of the genus *Nitzschia*. Lengths and widths are not stable characters to differentiate among *Nitzschia* species (Suriyanti, 2017) due to measurements that mostly overlap among species. Other ultra-structural features such as presence of central nodules, densities of striae and fibulae as well as rows of poroids did not show any distinguishable pattern either (Lundholm *et al.,* 2002). The valve outlines, raphe arrangements and presence of central interspace seemed to be persistent in species delineation in this genus (Mann, 1978; Round *et al.,* 1990; Lundholm and Moestrup, 2000). Inconsistent taxa placement in the tree is observed in species such as *N. pusilla*, even though most of the strains from Genbank have been verified by experts (Lundholm *et al.,* 2002). This could indicate the existence of cryptic and pseudo-cryptic species. Further studies are required to develop a better insight of relations between the genetic encoding and the morphology of *Nitzschia*.

From the morphology and genetic data, it is therefore to affirm that *N. dentatum* sp. nov. and *N. johorensis* sp. nov. have not been described elsewhere. The phylogenetic trees inferred usingLSU rDNA gene sequences also revealed *Nitzschia* species that have indentation in the middle of valve were grouped into the same clade consistently. Further genetic analyses are necessary to clarify the natural groupings within the genus *Nitzschia*.

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References

Aishah, S. 2005. Phytoplankton. *In*: Sasekumar, A. and Chong, V.C. (Eds),Ecology of Klang Strait. University of Malaya Press, Kuala Lumpur, Malaysia, pp. 133–167.

Aishah, S. and Nooraida, A.R. 1994. Diatoms of the River Kanching, Templer Park, Malaysia. Diatom**9**: 73–81.

Chu, W.L., Phang, S.M. andGoh, S.H. 1996. Environmental effects on growth and biochemical composition of *Nitzschia inconspicua* Grunow. J. Appl. Phycol. **8**: 389–396.

Cleve, P.T. 1901. Plankton from the Indian Ocean and the Malay Archipelago. K. Svenska Vetenskapsakademiens Handl. **35**(5): 1–58.

Coste, M. and Ricard, M. 1980. On some interesting finely striated *Nitzschia* observed under light and electron microscopes. Systematic and ecological Aspects. *In*: Ross, R. (Ed.),Proceedings of the 6th Symposium of Recent Fossil Diatoms.Otto Koeltz Science Publishers, Koenigstein, pp. 191–202.

Fareha, H., Leaw, C.P. and Lim, P.T. 2011. Morphological observation of common pennate diatoms (Bacillariophyceae) from Sarawak estuarine waters. Ann. Microsc. **11**: 12–23.

Foged, N. 1971. Freshwater Diatoms in Thailand. Cramer, Lehre, pp. 1–102.

Guiry, M.D. and Guiry, G.M. 2017. Algae Base. <http://www.algaebase.org>. National University of Ireland, Galway. Retrieved on 29 August 2017.

Hall, T.A. 1999. Bioedit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/Nt. Nucl. Acids. Symp. Ser.**41**: 95–98.

Iwasaki, H. 1961. The life-cycle of *Porphyra tenera in vitro*. Biol. Bull.**121**: 173–187.

Ki,, J.S. andHan,M.S. 2005. Molecular analysis of complete SSU to LSU rDNA sequence in the harmful dinoflagellate *Alexandrium tamarense* (Korean Isolate, Hy970328m). Ocean Sci. J. **40**(3): 43–54.

Kociolek, P. 2011. *Nitzschia fonticola*. Diatoms of the United States. <http://westerndiatoms. colorado. edu/ taxa/species/nitzschia_fonticola>. Retrieved on14 December 2015.

Laurittis, J.A., Hemmingsen, B.B. and Volcani, B.E. 1967. Propagation of *Hantzschia* sp. Grunow daughter cells by *Nitzschia alba* Lewin & Lewin. Phycol. **3**: 236–237.

Lee, J.J. 2011. Diatoms as endosymbionts. *In*: Seckbach, J. and Kociolek, J.P. (Eds), The diatom world. Springer Science & Business Media, New York, pp. 439–463.

Lundholm, N., Daugbjerg, N. and Moestrup, Ø. 2002. Phylogeny of the Bacillariaceae with emphasis on the genus *Pseudo-nitzschia* (Bacillariophyceae) based on partial LSU rDNA. Eur. J. Phycol. **37**(1): 115–134.

Lundholm, N. and Moestrup, Ø. 2000. Morphology of the marine diatom *Nitzschia navis-varingica* sp. nov. (Bacillariophyceae), Another producer of the neurotoxin domoic acid. J.Phycol. **36**: 1162–1174.

Mann, D.G. 1978. Studies in the Family Nitzschiaceae (Bacillariophyta) Vols**1 & 2**. Xxxiii + 386 pp. + 146 Plates. Ph.D. Dissertation, Universiti of Bristol, United Kingdom.

Maznah,W.W.O.and Mansor, M. 2002. Aquatic pollution assessment based on attached diatom communities in the Penang River Basin, Malaysia. Hydrobiologia**487**: 229–241.

Medlin, L.K. andHasle, G.R. 1990. Some *Nitzschia* and related diatom species from fast ice samples in the Arctic and Antarctic. Polar Biol.**10**: 451–479.

Nather-Khan, I.S.A. 1990. Assessment of water pollution using diatom community structure and species distribution -A case study in a Tropical River Basin. Int. Rev.Hydrobiol.**75**(3): 317–338.

Nunn, G.B., Theisen, B.F., Christensen, B. and Arctander, P. 1996. Simplicity-correlated size growth of the nuclear 28s ribosomal RNA D3 expansion segment in the Crustacean Order Isopoda. J. Mol.Evol. **42**: 211–223.

Posada, D. and Crandall, K.A. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics **14**(9): 817–818.

Renberg, I. 1990. A procedure for preparing large sets of diatom slides from sediment cores. J. Paleolimnol. **4**: 87–90.

Ronquist, F. and Huelsenbeck, J.P. 2003. MrBayes3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19**: 1572–1574.

Round, F.E, Crawford, R.M. and Mann, D.G. 1990. The Diatoms: Biology and Morphology of the Genera. Cambridge University Press, New York, USA, 747pp.

Saifullah, A.S.M, Abu Hena, M.K., Idris, M.H., Halimah, A.R. and Johan, I. 2014. Diversity of phytoplankton from mangrove estuaries of Sarawak, Malaysia. World Appl. Sci. J. **31**(5): 915–924.

Scholin, C.A., Herzog, M., Sogin, M. and Anderson, D.M.1994. Identification of group- and strain-Specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II. Sequence analysis of a fragment of the LSU rDNA. J. Phycol. **30**: 999–1011.

Shamsudin, L. 1990. Diatom marin di perairan Malaysia. Percetakan Dewan Bahasadan Pustaka, Kuala Lumpur, Malaysia, 260 pp.

Smida, D.B., Lundholm, N., Kooistra, W.H.C.F., Sahraoui, I., Ruggiero, M.V., Kotaki, Y., Ellegaard, M., Lambert, C., Mabrouk, H.H. and Hlaili, A.S. 2014. Morphology and molecular phylogeny of *Nitzschia bizertensis* sp. nov. - A new domoic acid-producer. Harmful Algae **32**: 49–63.

Sonnenberg, R., Nolte, A.W. and Tautz, D. 2007. An evaluation of LSU rDNA D1–D2 sequences for their use in species identification. Front. Zool. **4**: 6.

Suriyanti, S.N.P. 2017. Kajiantaksonomi, filogenidanketoksikandiatom marin *Nitzschia* (Bacillariophyceae) di perairan Malaysia. PhD Thesis. Universiti Kebangsaan Malaysia, Bangi, Malaysia.

Suriyanti, S.N.P. and Gires, U. 2015. First report of the toxigenic *Nitzschianavis-varingica* (Bacillariophyceae) isolated from Tebrau Straits, Johor, Malaysia. Toxicon**108**: 257–263.

Suzuki, H., Nagumo, T. and Tanaka, J. 2010. *Nitzschiaamabilis* nom. nov., a new name for the marine species *N. Laevis* Hustedt. Diatom Res. **25**(1): 223–224.

Swofford, D.L. 2003. PAUP*: Phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sinauer Associates, Sunderland.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. **30**: 2725–2729.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. 1997. The clustalX windows interface: Flexible strategies for multiple sequence alignment aided by the quality analysis tools. Nucleic Acid Res. **24**: 4876–4882.

Trobajo, R., Cox, E.J. and Quintana, X.D. 2004. The Effects of some environmental variables on the morphology of *Nitzschia frustulum* (Bacillariophyta), in relation to its use as a bioindicator. Nova Hedwigia**79**: 433–445.

Trobajo, R., Clavero, E., Chepurnov, V.A., Sabbe, K., Mann, D.G., Ishihara, S. and Cox, E.J. 2009. Morphological, genetic and mating diversity within the widespread bioindicator *Nitzschiapalea* (Bacillariophyceae). Phycologia **48**(6): 443–459.

Trobajo, R., Rovira, L., Ector, L., Wetzel, C.E., Kelly, M. and Mann, D.G. 2013. Morphology and identity of some ecologically important small *Nitzschia* species. Diatom Res. **28**(1**)**: 37–59.

Tudesque, L., Rimet, F. andEctor, L. 2008. A new taxon of the Section *Nitzschiae Lanceolatae* Grunow: *Nitzschia costei* sp. nov. compared to *N. fonticola* Grunow, *N. macedonia* Hustedt, *N. tropica* Hustedt and related species. Diatom Res. **23**(2): 483–501.

Zhang, Z., Schwartz, S., Wagner, L. and Miller, W. 2000. A greedy algorithm for aligning DNA sequences. J.Comput. Biol. **7**(1–2): 203–14.

Zimmermann, J., Jahn, R. and Gemeinholzer, B. 2011. Barcoding diatoms: Evaluation of the V4 Subregion on the 18s rRNA Gene, including new primers and protocols. Org. Divers. Evol. DOI 10.1007/s13127-011-0050-6

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