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Diatom taxonomy: morphology, molecules and barcodes…

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INTRODUCTION

Diatoms are the most species rich algae, with number of species estimated to reside somewhere between 10 000 and 1 000 000 (Mann 1999, Andersen, unpublished). Until recently the basis of the most commonly practiced concept of species has been the discontinuity in expression of one or more morphological characters when compared to the known species (Round et al. 1990, Mann 1999). Indeed, the micro-architecture of the diatom frustule, the valve in particular, provides a richness of structure on which to base such distinctions. However, a great variety of characters from various other stages of the diatom life cycle are also rich in morphological detail but have not been fully explored for their taxonomic significance. Previously unappreciated features are now often being shown to be taxonomically informative (Medlin & Kaczmarska 2004, Edlund & Spaulding 2006, Sims et al. 2006). Following Mann (1999) we argue for expansion of the characters used in species delimitation beyond valve morphology.

In this paper we discuss an example of a more holistic approach to diatom taxonomy, one that integrates morphological, molecular and life history characteristics in order to evaluate the congruence between them. DNA extraction, amplification and sequencing are becoming simpler, faster and less expensive tools for examination of diatoms, including species identification. For this to succeed, a library of taxonomically sound reference sequences is needed. The planktonic diatom *Pseudo-nitzschia delicatissima* (Cleve) Heiden is an example of a species where sequence-based identification should aid in detection of taxa whose valve morphology is too subtle to routinely discriminate between them in natural samples, even with the aid of electron microscopy. Existence of cryptic diversity in the *P. delicatissima*-species complex was suspected since extensive genetic variability was documented in their nuclear rRNA gene in the LSU (Stehr et al. 2002) and later in the ITS region (Orsini et al. 2004, Amato et al. 2007) which led to description of new species (Lundholm et al. 2006). Our own data indicate also that the diversity of this sub complex has not yet been fully documented.

RESULTS & DISCUSSION

Valve morphology of 12 interbreeding monoclonal strains isolated at various times and from various locations in the Canadian Maritimes between 2004 and 2005 is summarized in Table 1. It demonstrates a considerable variance for most of the features normally used in species identification. There is clear overlap between our clone's range of quantifiable morphology and those reported for *P. delicatissima* in published reports (Lundholm et al. 2006, Amato et al. 2005, 2007), indicating that differentiation of the species based on valve morphology alone will remain difficult, if at all possible. Unlike morphology, the ITS region sequences recovered from these interbreeding clones show very little variability; only up to two nucleotides in a 561 nucleotide long fragment were found in female clones. We compared sequences divergence recovered from our sexually compatible clones to those available in GenBank for clones called *P. delicatissima* (ITS region, Table 2). ITS region sequence

comparison clearly separate our clones from most of those reported from the coast of Denmark, the Gulf of Mexico (Lundholm et al. 2006) and the Gulf of Naples considered *P. delicatissima (sensu stricto* Amato et al. 2007).

In contrast, our clones show considerable similarities to ITS sequences reported from clones isolated from Scottish waters (Fehling et al. 2006), to one Danish clone (Lundholm et al. 2006) and to clones isolated from the Pacific. The Italian clones from this group are designated as *P. delicatissima*2 by Amato et al. 2007, and might represent a new (semi)cryptic species. Even less intraspecific sequence divergence was found in a 570 bp long fragment close to the 5' end of a mitochondrial gene cytochrome c oxidase (cox1) where sequences of 7 clones (2 males and 4 females and one clone of unknown sex) were invariable except for one nucleotide substitution in one sequence. Since the cox1 sequences for *P. delicatissima* clones from the studies cited above are not available, we compared nucleotide composition in our interbreeding *P. delicatissima* clones to the range of inter- and intraspecific sequence divergence known for a few pennate diatoms represented by multiple clones (Table 3). Sequence divergence remains very low at the intraspecific level and considerably greater at the interspecific level. Therefore, this marker also may be used to discriminate between (semi)cryptic species.

Table 1. A summary of quantifiable morphometrics for clones and populations of *Pseudo-nitzschia delicatissima*.

Name/(location)	Length (µm)	Width (µm)	Fibulae (in 10 µm)	Striae (in 10 µm)	Poroids (in 1 µm)	Reference
P. delicatissima (Italy)	57–58	1.7–2.1	18–23	36–38	8–9	Lundholm et al. 2006
P. delicatissima s.s.	14–53	1.8–2.3	21–26	38–41	6–10	Amato et al. 2007
P. delicatissima2	no data	1.5–1.8	23–28	42–44	7–10	Amato et al. 2007
P. delicatissima (Bay of Fundy)	17–48	1.7–2.3	19–30	37–44	7.5–14	this study
P. delicatissima (Danish coast)	70–72	1.7–1.9	20–25	36–39	9–12	Lundholm et al. 2006
P. delicatissima (Japan)	26–35	1.4–1.8	19–23	35–38	9–10	Lundholm et al. 2006

Table 2. Comparison of intra- and interspecific sequence divergence in ITS1 and ITS2 in clones attributed to *Pseudo-nitzschia delicatissima*.

Name	Fragment length (bp)	Divergence (%)	Reference
P. delicatissima s.s.	641	2.34	Amato et al. 2007
P. delicatissima2	561	<1	Amato et al. 2007
P. delicatissima	572	<1	Fehling, unpublished
P. delicatissima	561	<1	this study

The concept of molecular barcoding as a means of species identification is still hotly debated, but a number of recent publications demonstrate the usefulness of this approach in identification of species in a wide range of animal phyla, fungi and protists (Cywinska et al. 2006, Hebert et al. 2004, Gómez et al. 2007, Seifert et al. 2007, Saunders 2005). Currently the program is testing the feasibility of using a 300-700 bp region in the cox1 gene sequence as a species-specific "barcode" or "label" in a variety of microeukaryotes, including diatoms.

The Canadian “Barcode of Life Network” shows 28,129 species barcoded and 194,654 sequences accumulated in about 4 years. Our data (albeit initial) illustrate that both the ITS region and the cox1 sequence variability hold the potential for species-specific barcoding of diatoms and may become a feasible and practical means of diatom identification in general, but morphologically cryptic diversity such as in the *P. delicatissima* species complex in particular (Table 4).

Molecular barcoding carries the potential for a rapid, globally consistent identification of species, thus liberating valuable classical taxonomic expertise to focus on taxonomic research rather than identification of known species. In GenBank there are now nearly 100,000 sequences listed for diatoms, accumulated in just 4–5 years. This number has already exceeded the estimated total number of species frequently attributed to diatoms just over a decade ago. A considerable number of sequences submitted to GenBank are unnamed, and clearly demonstrate that taxonomically sound, correctly identified reference sequences are needed for ecological studies. The benefits of such an approach may be exemplified in the identification of non-indigenous diatoms carried in ballasts of transoceanic cargo ships, protection of rare and/or endangered species or timely detection of harmful diatoms.

Table 3. Pairwise nucleotide comparison matrix for selected pennates using a 435 bp long fragment of cox1. Diatom names are abbreviated to the first three letters of genus and species plus identifying clone number.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Tha nit	0														
Fra str 1	79	0													
Fra str 2	79	0	0												
Fra str 3	79	0	0	0											
Ast glac	77	71	71	71	0										
Nit fru	86	99	99	99	89	0									
KF unk	96	90	90	90	94	77	0								
Cer clo 1	84	98	98	98	90	70	80	0							
Cer fus 1	82	93	93	93	93	69	74	43	0						
Cer fus 2	82	92	92	92	93	69	74	44	1	0					
Pse del 1	90	102	102	102	93	73	78	69	77	77	0				
Pse del 2	90	102	102	102	93	73	78	69	77	77	0	0			
Pse del 3	90	103	103	103	93	73	79	70	78	78	1	1	0		
Pse del 4	87	99	99	99	92	71	76	68	76	76	0	0	1	0	
Pse del 5	90	102	102	102	93	73	78	69	77	77	0	0	1	0	0
	Tha nit	**Fra str 1**	**Fra str 2**	**Fra str 3**	**Ast glac**	**Nit fru**	**KF unk**	**Cer clo 1**	**Cer fus 1**	**Cer fus 2**	**Pse del 1**	**Pse del 2**	**Pse del 3**	**Pse del 4**	**Pse del 5**

Table 4. Comparison of intra and interspecific divergence in the barcoding region of the cox1 gene in various protists: (1) this study, (2) Saunders 2005, (3) Beaton et al. 2007, Ferrell & Beaton 2007, (4) Lynn & Strüder-Kypke 2006 and (5) Barth et al. 2006.

Taxon (#species)	Fragment length (bp)	Intra/Interspecific	Source
Diatoms (20)	420	0–3/15–20%	1
Red macro-algae (37)	664	0–0.3/4.4–14%	2
Marine Dinoflagellates (17)	Not shown	0–1/0–11%	3
Tetrahymena (8)	980	<1/1–12.4%	4
Paramecium (2)	880	7–9.5/~20%	5

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