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Genetic evidence of phenotypic polymorphism in the aeolid nudibranch *Flabellina verrucosa* (M. Sars, 1829) (Opisthobranchia: Nudibranchia)

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Abstract

The conspecificity of two forms of *Flabellina verrucosa* (M. Sars, 1829), one form with short and one with long cerata, was tested by sequencing the mitochondrial COI and the nuclear 5.8S-ITS2 genes. We could not establish any genetic differences between the two forms and conclude that they belong to the same species. Thus, *Flabellina verrucosa* is polymorphic in ceratum length.

Keywords: Molecular systematics; COI; 5.8S-ITS2; *Flabellina verrucosa*
See also **Electronic Supplement** at: http://www.senckenberg.de/odes/06-02.htm

Introduction

The nudibranch *Flabellina verrucosa* exists in two sympatrical forms (Figs. 1, 2; for color versions see an Organisms Diversity and Evolution Electronic Supplement at http://www.senckenberg.de/odes/06-02.htm) in the northern boreal-arctic region (e.g. Odhner 1907; Løyning 1922; Morse 1969; Thompson and Brown 1976; Kuzirian 1979). The short-cerata form was originally described as *Eolidia verrucosa* Sars, 1829, the long-cerata form as *Eolis rufibranchialis* Johnston, 1832. Hereafter, we will refer to these forms as the *verrucosa*- (short cerata) and *rufibranchialis* (long cerata) forms, respectively. Several authors have compared these two forms without finding any morphological differences other than in cerata shape and arrangement (Løyning 1922; Odhner 1922, 1926, 1939; Kuzirian 1979). This has led to the uniting of the two forms under the name *F. verrucosa* (Sars) (e.g. Løyning 1922; Lemche 1941). One argument for this was the existence of an intermediate stage, which according to Løyning (1922) and Odhner (1939) has longer cerata than typical specimens of the *verrucosa* form, but shorter cerata than typical specimens of the *rufibranchialis* form. Intermediate stages have been considered an indicator of interbreeding populations (Raahauge and Kristensen 2000; Diekmann et al. 2001).

In the present study we investigate whether the ceratal differences (cf. Figs. 1, 2) are concordant with genetic differences, by comparing nucleotide sequences from both forms. The genes investigated are the mitochondrial protein-coding cytochrome *c* oxidase subunit I (COI), and the nuclear non-coding second internal transcribed spacer together with the contiguous 5.8S gene (5.8S-ITS2). These genes have proven useful for investigations of close relationships in several taxa, including opisthobranchs (King et al. 1999; Medina and

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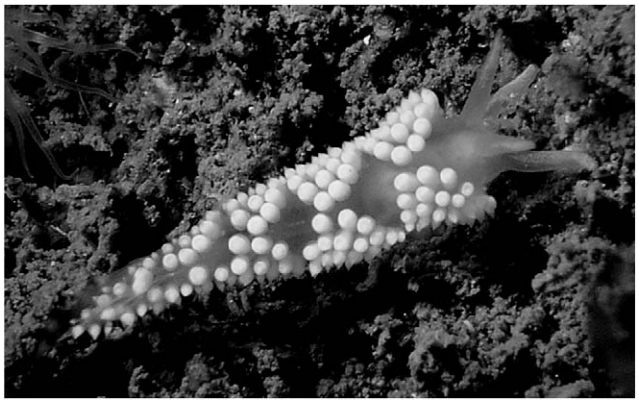

Fig. 1. *Flabellina verrucosa* with short cerata, i.e. the *verrucosa* form; body length approx. 15 mm; photographed in situ at Yttre Vattenholmen by R. Eriksson.

Fig. 2. *Flabellina verrucosa* with long cerata, i.e. the *rufibranchialis* form; body length approx. 25 mm; photographed in situ at Jordfall by R. Eriksson.

Walsh 2000; Raahauge and Kristensen 2000; Kojima et al. 2001; Hertel et al. 2002).

Table 1. Sequenced individuals: individual number, collecting site, collecting depth, and GenBank accession numbers for the sequenced COI and 5.8S-ITS2 genes

No.	Site	Depth (m)	Accession numbers	
			COI	5.8S-ITS2
rufi1	Jordfall	3–5	AB180812	AB180831
rufi2			AB180813	AB180832
rufi3			AB180812	
rufi4			AB180812	
rufi5			AB180814	
rufi6			AB180812	AB180833
rufi7			AB180812	
rufi8			AB180812	
rufi9			AB180815	
rufi10			AB180812	
rufi11			AB180812	
rufi12			AB180816	
rufi13			AB180817	
rufi14			AB180818	
rufi15			AB180812	
rufi16			AB180819	
verr1	Yttre Vattenholmen	20–25		AB180834
verr2			AB180817	AB180835
verr3			AB180816	AB180835
verr4			AB180820	
verr5			AB180821	
verr6			AB180822	
verr7			AB180823	
verr8			AB180824	
verr9			AB180825	
verr10	Lunneviken	20–25	AB180826	AB180836
verr11			AB180827	
verr12			AB180812	AB180837
verr13			AB180828	
verr14			AB180829	
verr15			AB180830	

Note that identical sequences are deposited under one accession number.
Abbreviations: rufi = *rufibranchialis*, verr = *verrucosa*.

Materials and methods

The animals were collected by SCUBA diving in the Gullmar fjord (at Jordfall: all *rufibranchialis* individuals) and in Strömstad archipelago (at Yttre Vattenholmen: *verrucosa* individuals nos. 1–9; and at Lunneviken: *verrucosa* individuals nos. 10–15) in December 2003. Unique sequences were deposited at GenBank (see Table 1 for accession numbers). In total, 31 individuals were sequenced: 15 of the *verrucosa*-, and 16 of the *rufibranchialis* forms. Nudibranch tissue was stored in 70% EtOH and/or in RNAlater™. DNA was extracted with the DNeasy Tissue Kit (Qiagen), according to the manufacturer's protocols. We used primers LCO1490 and HCO2198 (Folmer et al. 1994) for COI, and ITS1d and ITS4r (Oliverio and Mariottini 2001) for 5.8S-ITS2, in both amplification and sequence reactions. Amplification of the PCR product was made with TaKaRa LA Taq (Takara Bio Inc.) according to the supplied protocol, using the following temperature profile: 96 °C/120 s–(94 °C/30 s–47 °C/30 s–72 °C/60 s) × 40–72 °C/480 s for COI, and 96 °C/120 s–(93 °C/30s–*a* °C/30 s–72 °C/60 s) × 45–72 °C/420 s for 5.8S-ITS2 (where $a = 48–60$). PCR products were purified with the QIAquick PCR purification kit (Qiagen), and DNA concentrations were measured with a Hoefer Dyna Quant 200 fluorometer. Each sequence mixture contained 1 µl primer (5 mM), 4 µl DTCS Quick Start Mix, purified amplification

Table 2. COI sequence distance matrix; distances expressed as percent difference between two sequences

	rufi1	rufi2	rufi3	rufi4	rufi5	rufi6	rufi7	rufi8	rufi9	rufi10	rufi11	rufi12	rufi13	rufi14	rufi15	rufi16	verr2	verr3	verr4	verr5	verr6	verr7	verr8	verr9	verr10	verr11	verr12	verr13	verr14	verr15	AJ223254
rufi1	—																														
rufi2	1.0	—																													
rufi3	0.0	1.0	—																												
rufi4	0.0	1.0	0.0	—																											
rufi5	1.3	1.0	1.3	1.3	—																										
rufi6	0.0	1.0	0.0	0.0	1.3	—																									
rufi7	0.0	1.0	0.0	0.0	1.3	0.0	—																								
rufi8	0.0	1.0	0.0	0.0	1.3	0.0	0.0	—																							
rufi9	0.5	1.5	0.5	0.5	1.9	0.5	0.5	0.5	—																						
rufi10	0.0	1.0	0.0	0.0	1.3	0.0	0.0	0.0	0.5	—																					
rufi11	0.0	1.0	0.0	0.0	1.3	0.0	0.0	0.0	0.5	0.0	—																				
rufi12	0.5	0.8	0.5	0.5	1.2	0.5	0.5	0.5	1.0	0.5	0.5	—																			
rufi13	0.8	0.5	0.8	0.8	0.5	0.8	0.8	0.8	1.4	0.8	0.8	0.7	—																		
rufi14	0.2	1.2	0.2	0.2	1.5	0.2	0.2	0.2	0.7	0.2	0.2	0.7	1.0	—																	
rufi15	0.0	1.0	0.0	0.0	1.3	0.0	0.0	0.0	0.5	0.0	0.0	0.5	0.8	0.2	—																
rufi16	1.0	0.7	1.0	1.0	0.7	1.0	1.0	1.0	1.5	1.0	1.0	0.8	0.2	1.2	1.0	—															
verr2	0.8	0.5	0.8	0.8	0.5	0.8	0.8	0.8	1.4	0.8	0.8	0.7	0.0	1.0	0.8	0.2	—														
verr3	0.5	0.8	0.5	0.5	1.2	0.5	0.5	0.5	1.0	0.5	0.5	0.0	0.7	0.7	0.5	0.8	0.7	—													
verr4	0.3	0.7	0.3	0.3	1.0	0.3	0.3	0.3	0.8	0.3	0.3	0.2	0.5	0.5	0.3	0.7	0.5	0.2	—												
verr5	0.7	0.3	0.7	0.7	0.7	0.7	0.7	0.7	1.2	0.7	0.7	0.5	0.2	0.8	0.7	0.3	0.2	0.5	0.3	—											
verr6	1.0	0.7	1.0	1.0	0.7	1.0	1.0	1.0	1.5	1.0	1.0	0.8	0.2	0.8	1.0	0.3	0.2	0.8	0.7	0.3	—										
verr7	1.0	0.7	1.0	1.0	0.7	1.0	1.0	1.0	1.5	1.0	1.0	0.8	0.2	1.2	1.0	0.3	0.2	0.8	0.7	0.3	0.3	—									
verr8	0.3	0.7	0.3	0.3	1.0	0.3	0.3	0.3	0.8	0.3	0.3	0.2	0.5	0.5	0.3	0.7	0.5	0.2	0.0	0.3	0.7	0.7	—								
verr9	0.8	1.2	0.8	0.8	1.5	0.8	0.8	0.8	1.4	0.8	0.8	0.7	1.0	1.0	0.8	1.2	1.0	0.7	0.5	0.8	1.2	1.2	0.5	—							
verr10	0.7	1.0	0.7	0.7	1.4	0.7	0.7	0.7	1.2	0.7	0.7	0.2	0.8	0.8	0.7	1.0	0.8	0.2	0.3	0.7	1.0	1.0	0.3	0.8	—						
verr11	0.8	1.2	0.8	0.8	1.5	0.8	0.8	0.8	1.4	0.8	0.8	0.7	1.0	1.0	0.8	1.2	1.0	0.7	0.5	0.8	1.2	1.2	0.5	1.0	0.8	—					
verr12	0.0	1.0	0.0	0.0	1.3	0.0	0.0	0.0	0.5	0.0	0.0	0.5	0.8	0.2	0.0	1.0	0.8	0.5	0.3	0.7	1.0	1.0	0.3	0.8	0.7	0.8	—				
verr13	0.8	1.2	0.8	0.8	1.5	0.8	0.8	0.8	1.4	0.8	0.8	0.7	1.0	1.0	0.8	1.2	1.0	0.7	0.5	0.8	1.2	1.2	0.5	1.0	0.8	1.0	0.8	—			
verr14	0.7	1.7	0.7	0.7	2.0	0.7	0.7	0.7	1.2	0.7	0.7	1.2	1.5	0.8	0.7	1.7	1.5	1.2	1.0	1.4	1.7	1.7	1.0	1.5	1.4	1.5	0.7	1.5	—		
verr15	0.7	1.0	0.7	0.7	1.4	0.7	0.7	0.7	1.2	0.7	0.7	0.5	0.8	0.8	0.7	1.0	0.8	0.5	0.3	0.7	1.0	1.0	0.3	0.5	0.7	0.8	0.7	0.8	0.8	—	
AJ223254	18.4	18.5	18.4	18.4	18.7	18.4	18.4	18.4	18.5	18.4	18.4	18.4	18.2	18.2	18.4	18.4	18.2	18.4	18.2	18.4	17.9	18.4	18.3	18.6	18.4	17.7	18.4	18.5	18.5	18.3	—

Note that PAUP ignores nucleotide sequence ambiguities, thus ambiguities do not contribute to sequence differences. The matrix includes the outgroup sequence AJ223254. For abbreviations see Table 1.

product and ddH_20 to a total volume of 10 µl. The sequence reaction profile was as follows: (96 °C/30 s–*b* °C/30 s–60 °C/240 s) × 30 where $b = 50–54$ for COI, and $b = 50$ for 5.8S-ITS2. DNA sequences were obtained from a BeckmanCoulter™ CEQ8000 Genetic Analysis System, and were subsequently edited and aligned with Lasergene (DNASTAR) using the CLUSTAL-V algorithm (Higgins and Sharp 1989). Gap penalty and gap length penalty were both set to 10. GenBank sequences from *Acanthodoris pilosa* (Abildgaard in O.F. Müller, 1789) were used as outgroups, as this was the closest species from which both COI and 5.8S-ITS2 sequences were available (accession numbers AJ223254 and AY014155). Sequence distances were calculated with PAUP* 4.0b10 (Swofford 2003). Haplotype networks were constructed using TCS 1.13 (gaps treated as fifth state), implementing the methods of Clement et al. (2000).

Results

COI

Fourteen individuals of the *verrucosa*- and 16 of the *rufibranchialis* form were sequenced partially (593 bp) for COI. Thirteen characters are parsimony informative. Nucleotide distances range between 0.0% and 2.0% within the ingroup. Outgroup–ingroup differences range between 17.7% and 18.7% (Table 2). There are 18 haplotypes (excluding the outgroup), of which three are shared between the *verrucosa*- and the *rufibranchialis* forms (Fig. 3).

5.8S-ITS2

Five individuals of the *verrucosa*- and three of the *rufibranchialis* form were sequenced for the complete 5.8S-ITS2 (approx. 452 bp). Eight characters are parsimony informative, all confined to ITS2. Nucleotide distances range between 0.0% and 2.0% within the ingroup. Outgroup–ingroup differences range between 56.7% and 57.6% (Table 3). There are four haplotypes (excluding the outgroup); each individual of the *rufibranchialis* form represents its own separate haplotype, whereas all individuals of the *verrucosa* form belong to a single, fourth haplotype (Fig. 4).

Discussion

We could not find any consistent differences in sequences or haplotypes correlated to differences in morphotype, and thus conclude that the *verrucosa*- and the *rufibranchialis* forms are conspecific. In addition, COI haplotype sharing is evidence for interbreeding populations (e.g. Kojima et al. 2001), although we could not deduce whether this interbreeding is contemporary and/or historical. A comparison between COI (mitochondrial, coding) and ITS2 (nuclear, non-coding) results could, however, shed light on this, considering the different modes of inheritance and the coding/non-coding nature of these genes (Simon et al. 1994). King et al. (1999) suggested that lack of congruence between mitochondrial coding and nuclear non-coding genes was due to relatively recent reproductive-isolation events. In this comparison one expects the nuclear non-coding

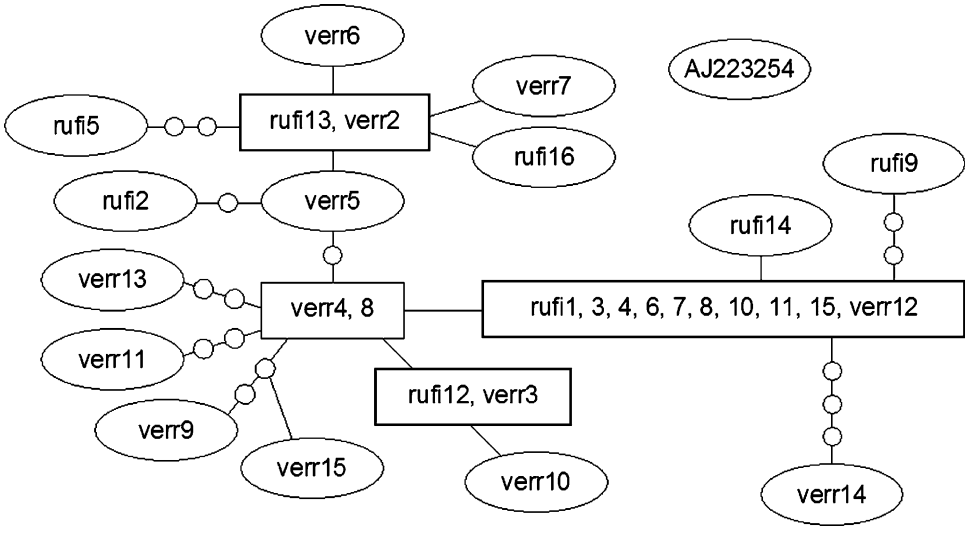

Fig. 3. TCS haplotype network for the COI dataset, including the outgroup AJ223254; for abbreviations see Table 1. Single and multiple sequences representing one haplotype are enclosed in ovals and boxes, respectively. Haplotypes shared between the *verrucosa*- and the *rufibranchialis* forms are enclosed in bold outline boxes. Missing haplotypes are indicated by small circles on the connecting lines.

Table 3. ITS2-5.8S sequence distance matrix; distances expressed as percent difference between two sequences

	rufi1	rufi2	rufi6	verr1	verr2	verr3	verr10	verr12	AY014155
rufi1	—								
rufi2	0.2	—							
rufi6	2.0	2.0	—						
verr1	1.6	1.6	0.2	—					
verr2	2.0	2.0	0.4	0.0	—				
verr3	2.0	2.0	0.4	0.0	0.0	—			
verr10	2.0	2.0	0.4	0.0	0.0	0.0	—		
verr12	1.8	1.8	0.2	0.0	0.2	0.2	0.2	—	
AY014155	57.4	57.6	56.7	56.7	56.8	56.8	56.8	57.0	—

Note that PAUP ignores nucleotide sequence ambiguities, thus ambiguities do not contribute to sequence differences. The matrix includes the outgroup sequence AY014155. For abbreviations see Table 1.

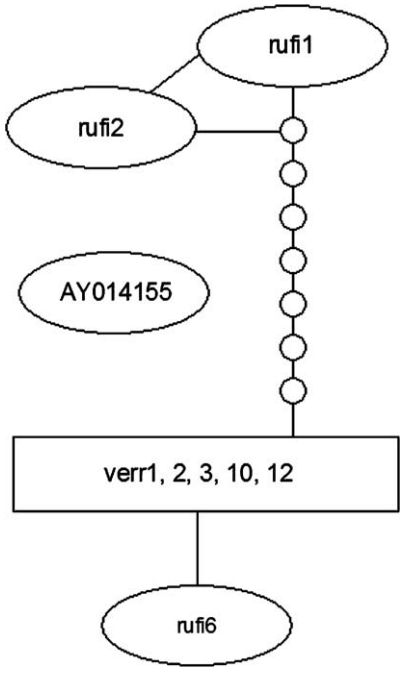

Fig. 4. TCS haplotype network for the 5.8S-ITS2 dataset, including the outgroup AY014155; for abbreviations see Table 1. Single and multiple sequences representing one haplotype are enclosed in ovals and boxes, respectively. Missing haplotypes are indicated by small circles on the connecting lines.

gene to evolve at a faster pace than the mitochondrial coding gene, thus to be able to produce a different grouping than the mitochondrial coding gene, i.e. to produce haplotype groups according to morphotype. In the present study, neither the mitochondrial coding COI nor the nuclear non-coding ITS2 (together with the contiguous 5.8S) yielded haplotype groups correlated to morphotype (see Figs. 3 and 4). This suggests that interbreeding between the *verrucosa*- and *rufibranchialis* forms is contemporary. The previous synonymization of the two corresponding species names (e.g. Løyning 1922; Odhner 1939; Lemche 1941) is supported and strengthened by the results presented herein.

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