

Large genetic distances within a population of *Amphipholis squamata* (Echinodermata; Ophiuroidea) do not support colour varieties as sibling species

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ABSTRACT: The brittle star *Amphipholis squamata* is paradoxical in that it lacks an obvious dispersive phase yet has a world-wide distribution. Although individuals from distant populations are morphologically similar, a recent phylogenetic analysis found multiple clades separated by large genetic divergences. These clades were not phylogeographically structured and genetic divergences within populations were typically as high as those amongst populations. The recent suggestion that the sympatric colour varieties 'orange', 'dark brown', 'beige', 'black' and 'grey' represent sibling species, led us to test whether colour variety and phylogeny were congruent. Genetic distances among sequences of the mitochondrial gene 16S rRNA from the colour varieties were surprisingly high (up to 13% uncorrected distance) and phylogenetic analyses using maximum parsimony, maximum likelihood and neighbour joining gave well supported, congruent phylogenies. However, the clades were not consistent with colour variety. When clades were constrained to make colour varieties monophyletic, tree scores were always significantly worse. We conclude from the results of this study that colour varieties do not represent distinct phylogenetic lineages. We discuss the implications of our results in the light of the possibility of clonality or self-fertilization in this species.

KEY WORDS: Cryptic species · Phylogeny · 16S mtDNA · Bioluminescence · Speciation · Colour morphs

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INTRODUCTION

The small cosmopolitan ophiuroid *Amphipholis squamata* (Delle Chiaje, 1828) (disc diameter of 3 mm) is a simultaneous hermaphrodite that broods its offspring. Although there is an apparent lack of a dispersive phase in this species, the outer skeletal morphology of individuals is reported to be similar irrespective of geographical origin (Hyman 1955, pers. obs.), and hence *A. squamata* is currently considered to be a single species world-wide (see Clark 1987). It has been

shown, however, that DNA sequence divergence of specimens, both within and between populations, is often high, attaining genetic distances of up to 20% (Kimura 2-parameter distance) for the mtDNA (Sponer et al. 1999). The basis of this genetic variation is unclear and does not appear correlated with geographic distance or habitat (Sponer et al., unpubl. data).

Amphipholis squamata is a polychromatic species (Tortonese 1932), with individuals having different colour patterns of the disc and the arms (Binaux & Bocquet 1971, Deheyn et al. 1997). The species is also bioluminescent. Bioluminescence is produced only in the arms and is attributed to specific photocytes under ganglionic control (Deheyn et al. 2000a). It has been shown that each colour variety produces light of characteristic intensity, irrespective of the geographic origin

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of specimens (Deheyn et al. 1997, Deheyn 1998). Populations are often polymorphic for a number of colour varieties as well as bioluminescence intensity and kinetics. In breeding experiments over 3 generations of the 'beige' and 'black' colour varieties (which differ in light intensity by 2 orders of magnitude), Deheyn & Jangoux (1999) showed that colour pattern and bioluminescence intensity are associated and heritable traits. When the 2 varieties were kept together in tanks (allowing for cross-fertilisation) offspring were always of the same variety as the brooding parent, without intermediate forms occurring. From this observation, Deheyn & Jangoux (1999) suggested that colour varieties are reproductively isolated sibling species. Although not explicitly stated by these authors, this conclusion is valid only under the biological species concept (BSC) and related concepts (e.g. recognition concept), which assume reproductive isolation in outcrossing species. The sibling species hypothesis based upon colour has gathered some support in recent years (Deheyn et al. 2000b, Feral in press). Genetic studies on colour morphs in other invertebrates suggest that colour polymorphism can be indicative of cryptic species status, as found in the sea anemone *Actinia equina* (Allcock et al. 1998) and the nemertean *Lineus torquatus* (Manchenko & Kulikova 1996). However, no genetic differentiation was found between 2 colour morphs of the flea beetle *Phyllotreta tetrastigma* (Verdyck et al. 1998).

We set out to test Deheyn & Jangoux's (1999) hypothesis of colour varieties as sibling species, using a molecular phylogenetic approach, from a wild population which exhibits several colour varieties. If their hypothesis is correct, under the BSC, we expected that colour varieties would be reciprocally monophyletic (all representatives of a particular colour variety are genealogically closest to one another).

The results from the present study do not support Deheyn & Jangoux's (1999) hypothesis; however, we believe that without full knowledge of the breeding system of *Amphipholis squamata*, questions of species status cannot easily be answered.

MATERIALS AND METHODS

Individuals of *Amphipholis squamata* were collected at low tide at Roscoff (Brittany, France) in September 1998. The collecting site (60 × 20 m, surface area) commonly called 'Le chenal de l'île verte' consists of pebbles scattered in beds of *Zostera marina* and sandy detritic areas. Adult individuals (disc diameter >1.8 mm) were collected randomly (regardless of their colour). The specimens were then sorted according to colour variety (as defined by Binaux & Boquet 1971) using a

compound microscope and preserved in absolute ethanol. Five specimens of each of the 5 colour varieties (with associated characteristic bioluminescence intensities; Deheyn et al. 1997, Deheyn 1998), 'orange', 'dark brown', 'beige', 'grey' and 'black', were used in this study.

DNA extraction. Two methods of DNA extraction were employed for this study, both using 2 to 3mm of an arm from each sample, broken into pieces or crushed in a 1.6 ml eppendorf tube. In most cases DNA was extracted using a Chelex procedure (Walsh et al. 1991): 250 µl of 5% Chelex solution was added to the tissue and heated to 65°C for 3 h, making sure the tissue was always submerged in the Chelex beads. Subsequently the solution was boiled at 100°C for 10 min and centrifuged at 13500g. Then, 5 µl of the supernatant of the cooled solution was used for PCR amplifications. Alternatively, the tissue was washed in TNE buffer (50 mM Tris pH 7.5, 400 mM NaCl, 100 mM EDTA) and subsequently incubated at 50°C in 250 µl 2× CTAB solution and 50 µl of 10 mg proteinase K ml⁻¹ for 2 h. After complete digestion, 75 µl of saturated NaCl solution was added, and the resultant precipitation centrifuged at 3000×g for 10 min. DNA was purified using ethanol precipitation (Sambrook et al. 1989).

PCR amplification, RFLP and sequencing. For this study we compared sequence data from the mitochondrial DNA (mtDNA) gene 16S rRNA (16S) and restriction fragment length polymorphism (RFLP) from the nuclear ribosomal internal transcribed spacer region (ITS). The 16S gene was chosen because initial trials using mtDNA cytochrome oxidase I (COI) gene revealed very large genetic divergences among specimens, which resulted in saturation of substitutions and thus homoplasy. In addition, several samples proved impossible to amplify for COI, presumably because of mismatches in the primer region. For this reason we proceeded with 16S which proved to have an evolutionary rate applicable to the comparison we intended to make among our samples and also consistently produced amplification product. ITS has been shown to provide good resolution at and below the species level in a wide range of organisms (e.g. Gardes et al. 1991, Bakker et al. 1992, Wesson et al. 1992, Baldwin et al. 1995) and has been used successfully in studies of hybridisation and introgression (Quijada et al. 1997, Van Oppen et al. 2000).

For 16S, the universal primers LR-j-12887 (alias 16Sbr) and LR-N-13398 (alias 16Sar) (Simon et al. 1994) were used for PCR and sequencing of 500 base pairs (bp) of the 16S gene. Amplification of double-stranded product was achieved using the following conditions: initial denaturation for 2 min at 94°C, 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 50 to 53°C, extension for 1 min at 72°C, and final

extension for 10 min at 72°C. PCR products were cleaned for sequencing using silica-gel-based purification columns. Cycle sequencing was accomplished using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer), using a temperature profile of initial denaturation of 2 min at 96°C, 25 cycles of denaturation for 15 s at 96°C, annealing for 7 s at 50°C, extension for 4 min at 60°C. Amplification products were run on an ABI 377 and analysed in the program SeqEd. Alignments were made using the clustal algorithm in 'Megalign', DNA star. In ingroup taxa, no indels are longer than 2 bp, all larger indels are due to sequence differences between the ingroup and outgroup. All sequences have been submitted to GenBank with accession numbers (AF331166 to AF331190).

For analysis of ITS, we used the primers 18d and 28u (Hillis & Dixon 1991), which bind in the 18S rRNA and 28S rRNA, respectively, and can be used to amplify approximately 2000 bp of DNA, including ITS-1, 5.8S rRNA and ITS-2. PCR amplification was carried out as above. Ten restriction enzymes were tested for their ability to digest the fragment. The two 4-base cutters, *Rsa I* and *Alu I*, produced the clearest patterns and were therefore chosen for all subsequent RFLP analyses. Digests were run on 2% agarose gels and visualised with ethidium bromide staining.

Phylogenetic analyses. Aligned sequences were analysed using PAUP* (Swofford 1999) in order to infer phylogenetic relationships. Phylogenetic analyses were performed using maximum-parsimony (MP), maximum-likelihood (ML) and neighbour-joining (NJ) methods. Parsimony analysis was conducted using a 'heuristic' search algorithm with the following options: random taxon addition, TBR branch swapping, MULPARS, and branches with a maximum length of zero collapsed to yield polytomies. A total of 50 random taxon addition replicates were performed. Initial parsimony analysis weighted all characters equally; however, subsequent weighting took into account that transitions (ti) accumulate at a faster rate than transversions (tv) (Hillis & Moritz 1990, Collins et al. 1994). In addition, we tested the effect of treating insertions/deletions as (1) missing data or (2) characters (5th base). To circumvent a priori concerns over base composition variance across taxa (Collins et al. 1994, Lockhart et al. 1994), we compared the frequencies of all 4 bases in a matrix and assessed the distribution with a Chi-squared test. No significant differences in base composition were found across taxa ($p = 1.0$; Chi-square = 11.519674, $df = 72$), and subsequent phylogenetic inference was considered not to be influenced by compositional bias. The g_1 statistic of 1000 random trees found significant phylogenetic signal ($g_1 = -1.018428$, $p < 0.01$). The permutation-tailed probability (PTP) test also confirmed that the dataset

contained significant phylogenetic signal ($p = 0.02$; Faith & Cranston 1991). Kimura 2-parameter distances (Kimura 1980) with gamma approximation (Yang 1996) using a shape parameter of 0.22 (estimated empirically using ML methods) were used to construct phylogenetic trees using the NJ algorithm (Saitou & Nei 1987). Statistical support for internodes of phylogenetic trees was accomplished by running 500 bootstrap replications (Felsenstein 1985). Trees of different topology, namely monophyly of colour morphs, were compared statistically by ML methods using the Kishino & Hasegawa test (1989). As outgroups we used 2 specimens (1 from Hawaii and 1 from Sydney), the haplotypes of which are known to be basal to the ingroup (Sponer et al. unpubl. data).

RESULTS

Sequences of mitochondrial 16S rRNA

We obtained 16S sequence data of 501 bp length for a total of 23 specimens, including 5 specimens of each of the black, beige and orange varieties and for 4 specimens of each of the dark brown and grey varieties. The remaining 2 specimens (grey ID #3 and dark brown ID #2) did not give any PCR product, even after several attempts at low stringency conditions.

High similarity with published ophiuroid and asteroid 16S sequences (*Ophiothrix fragilis*, Baric & Sturmbauer 1999, GenBank accession number AJ002789; *Asterina pectinifera*, Jacobs et al. 1989, Genbank accession number D16387) confirmed the identity of the sequences amplified from *Amphipholis squamata* in this analysis, which corresponds to Base Pairs 7672 to 8271 of *Asterina pectinifera* mtDNA. When considering the entire dataset, the ti/tv ratio was found to average 1.6898 (averaged over all pairwise comparisons). Sequences contained 118 parsimony-informative sites (129 when treating insertions/deletions as 5th base). Pairwise sequence divergence (Kimura 2-parameter, with a γ correction of 0.221) for ingroup taxa ranged from 0% to 23.0% (uncorrected 'p' distance: 0% to 13%). Table 1 lists the range of genetic distances among specimens of the same colour variety. The average Kimura 2-parameter distance with γ correction between ingroup and outgroup taxa was 43.5% (uncorrected 'p' distance: 18.2%). Within colour varieties, identical haplotypes were found in 4 beige specimens, 2 dark brown specimens, 3 and 2 orange specimens, respectively, and 4 black specimens. In addition, 1 haplotype was shared between 2 dark brown and 4 beige specimens and another occurred in 1 grey and 4 black specimens. MP analysis of the 16S data yielded 5 most-parsimonious trees when the dataset was un-

weighted. All 5 trees divided the ingroup taxa into 2 major clades, 'A' and 'B' (Fig. 1). Clade A is divided further into 5 subclades. The assignment of individuals to clades and subclades was identical in all trees. The trees differed with respect to relationships among Clades 1 to 4 (Fig. 1). Weighting of transversions 2:1 or 10:1 to transitions resulted in 2 shortest trees. When insertions/deletions were treated as 5th base, only 1 shortest tree was obtained. Weighting and the use of indels as characters did not affect the composition of clades, and the topologies recovered with NJ and ML algorithms were also identical with respect to assignment of specimens to the major clades. All of the phylogenies we recovered with the various methods described above support the monophyly of the orange variety, but none support monophyly of any other colour varieties. Fig. 1 shows the MP bootstrap consensus tree. In order to test the original hypothesis that colour varieties are different species, we subsequently constrained the heuristic search to phylogenies in which colour varieties are monophyletic, using the constraint option in PAUP*. This was done to test whether monophyly of colour varieties results in significantly less parsimonious trees compared to the optimal unconstrained tree. The following constraints were tested: (1) reciprocal monophyly of all colour varieties and (2) monophyly of 1 colour variety (beige, black, dark brown and grey) at a time without constraining any of the others. All constrained trees were longer than the optimal unconstrained tree (Table 2). Analysis of the tree topologies with a Kishino-Hasegawa test revealed that tree lengths as well as ML scores of all constrained trees were significantly worse at the 0.001 confidence level (Table 2). In order to make colour varieties reciprocally monophyletic, 141 extra steps were required, for monophyly of any 1 of the colour varieties, 37 (for black) to 92 (for beige) extra steps were required (Table 2).

RFLP of nuclear ITS region

Only 2 distinct RFLP patterns were observed among all 23 individuals. The 2 patterns are concordant with the 2 main clades in the 16S phylogeny: Clade A and Clade B (Fig. 1). *Rsa I* produced 2 bands in Clade A and 3 bands in Clade B. One of the bands was shared between clades. *Alu I* produced 3 bands in both clades, 2 of which were shared.

DISCUSSION AND CONCLUSIONS

The motivation of this study was primarily to put bioluminescence variation into an evolutionary context,

Table 1. Genetic distances between members of the same colour variety. Kimura 2-parameter distances were corrected with a shape parameter of 0.221. This correction value was estimated empirically using maximum-likelihood methods in PAUP*

Colour morph	Uncorrected 'p' (%)	Kimura 2P with γ correction (%)
Dark brown	0 – 13.0	0 – 22.7
Grey	0 – 4.8	0 – 5.8
Black	0 – 5.0	0 – 6.2
Beige	0 – 11.8	0 – 19.5
Orange	0 – 0.6	0 – 0.7

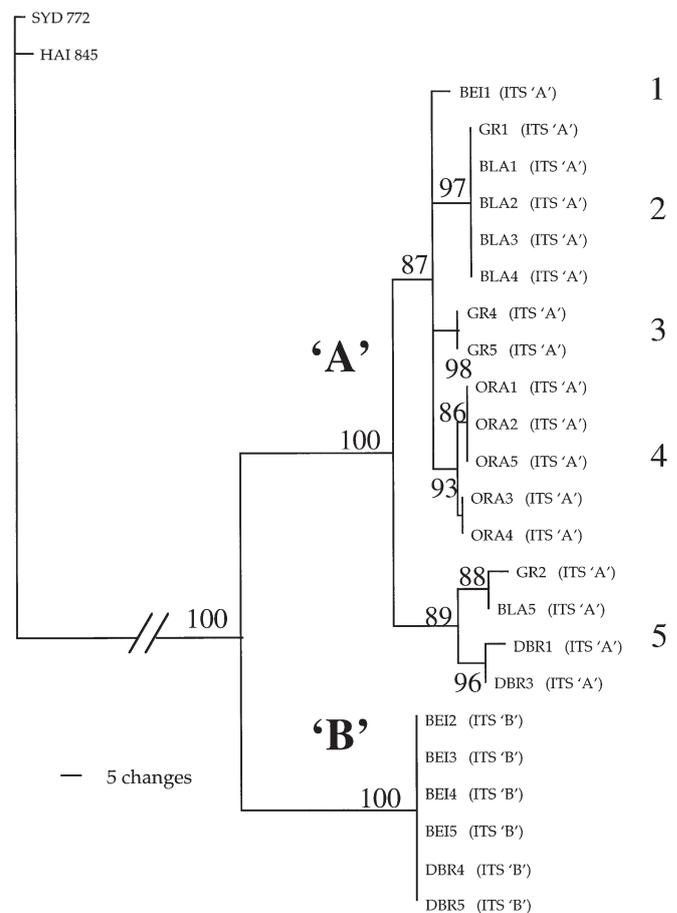


Fig. 1. Maximum-parsimony bootstrap consensus phylogeny based on 501 bp of 16S rDNA sequence. Indels were treated as 5th base and transversions weighted 2:1 over transitions. The 2 major clades referred to in the text as 'A' and 'B', subclades are numbered 1 to 5. Specimen ID numbers are followed by their ITS-RFLP type. Numbers next to nodes are percent bootstrap support values. Specimens from Hawaii (HAI 845) and Sydney (SYD 772) were used as outgroups. Optimal neighbour-joining and maximum-likelihood trees are identical with respect to major branching orders (tree length = 180, consistency index [CI] = 0.867, retention index [RI] = 0.953, rescaled consistency index [RC] = 0.826, homoplasy index [HI] = 0.133)

Table 2. Kishino-Hasegawa test assessing constrained and unconstrained MP trees. p: the probability of getting more extreme test statistics under the null hypothesis of no difference between the constrained trees (trees 2 to 6) and the unconstrained tree (Tree 1); 2 diff, difference in steps and 2 diff – ln L, respectively; constraints were: All, all colourmorphs are reciprocally monophyletic; Dbr, dark brown is monophyletic; Grey, grey is monophyletic; Black, black is monophyletic; Beige, beige is monophyletic

Maximum-likelihood scores				
Tree		–ln L	2 diff – ln L	p
Best	1	1560.45334	(best)	Unconstrained data
All	2	1928.11684	735.32700	<0.0001
Dbr	3	1756.53827	392.16986	<0.0001
Grey	4	1662.54133	204.17598	<0.0001
Black	5	1662.84791	204.78914	<0.0001
Beige	6	1767.49024	414.07380	<0.0001
Maximum-parsimony scores				
Tree		Length	2 diff	p
Best	1	300	(best)	Unconstrained data
All	2	441	282	<0.0001
Dbr	3	387	174	<0.0001
Grey	4	338	76	<0.0001
Black	5	337	74	<0.0001
Beige	6	392	184	<0.0001

since a number of comparative studies of bioluminescence in *Amphipholis squamata* had been conducted for both its ecological significance (e.g. Deheyn et al. 2000c, Dupont in press) and physiological control (e.g. De Bremaeker et al. 1999).

Deheyn & Jangoux (1999) concluded from breeding experiments, in which offspring were always of the same colour variety as the brooding parent, that colour varieties are reproductively isolated sibling species. Their hypothesis was based on criteria set out in the biological species concept (Mayr 1982) for outcrossing species. Here, we tested this hypothesis on the colour varieties dark brown, beige, black, grey and orange of a French population of *Amphipholis squamata*. Under the 'sibling species hypothesis', we expected them to be phylogenetically distinct, and therefore to group into monophyletic clades (Cracraft 1983, 1989). However, all but the orange variety were paraphyletic or polyphyletic and so would not be considered true species under the phylogenetic species concept (Cracraft 1989). In addition, on 2 occasions, specimens of 2 different varieties shared identical haplotypes. Under the 'sibling species hypothesis' these results must be interpreted as evidence for hybridisation between the different colour varieties. The colour varieties used in this study occur in sympatry, in fact they can be found right next to each other (in syntopy), allowing ample opportunity for hybridisation to occur. Biological species

would be expected to have evolved effective reproductive barriers, especially when sympatric; however, the results presented here suggest that hybridisation is widespread, hence contravening the biological species concept (Mayr, 1982). Deheyn & Jangoux (1999) suggested that *A. squamata* may have undergone speciation recently and that colour varieties represent young sibling species. This argument would imply that parapatry of haplotypes could be due to a lack of lineage sorting (Avice 1994). This argument cannot be disproven; however, the concordance of 16S and ITS data with respect to the 2 main clades, 'A' and 'B', suggests that lineage sorting has occurred in these 2 clades. However, the phylogenetic split is not concordant with colour variety. Based on these data, we can reject Deheyn and Jangoux's (1999) hypothesis (under the BSC).

On the other hand, we believe that, without full knowledge of the breeding system of *Amphipholis*

squamata, questions of species status cannot be adequately resolved. Deheyn & Jangoux (1999) showed that *A. squamata* is capable of reproduction in isolation. It must therefore be assumed that *A. squamata* is a facultative (or obligate, although unlikely because this is very rare in the animal kingdom) self-fertilising or parthenogenetic organism. There is some evidence to suggest that self-fertilisation is likely: *A. squamata* is a simultaneous hermaphrodite (Oguro et al. 1982), and the motility of sperm is thought to be low since the flagellum is inserted at a large angle at the base of the head (Buckland-Nicks et al. 1984); therefore, the scope for cross-fertilisation may be restricted. Furthermore, preliminary genetic evidence suggests that self-fertilisation can occur in this species (Poulin et al. 1999). The occurrence of self-fertilisation or parthenogenesis could explain why, in Deheyn & Jangoux's (1999) breeding experiments, offspring were always of the same colour as their brooding parent. In the case of self-fertilisation, in order for 100% of offspring to be of the same colour as the parent, all colour-controlling genes would have to be in a homozygous state.

However, if the incidence of self-fertilisation and/or parthenogenesis is high, the biological species concept loses its meaning: in the extreme case of obligate clonality, each clone could be regarded as a different species.

In conclusion, in order to fully resolve questions of species status in *Amphipholis squamata*, it will be necessary to understand its breeding system. Based on the results from this study, it is clear, however, that whatever their taxonomic status, colour varieties are not reproductively isolated.

Acknowledgements. This work was funded by Otago University Research Grant ORG 98/747. R.S. was supported by a BMWF postgraduate scholarship from the Austrian Government. Thanks to Graham Wallis for helpful comments on earlier drafts.

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Editorial responsibility: Otto Kinne (Editor),
Oldendorf/Luhe, Germany

Submitted: September 18, 2000; Accepted: January 25, 2001
Proofs received from author(s): July 30, 2001