



Phylogenetic relationships and the evolution of mimicry in the *Chauliognathus* yellow-black species complex (Coleoptera: Cantharidae) inferred from mitochondrial COI sequences

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Abstract

The phylogenetic relationships of twelve species of *Chauliognathus* were investigated by studying the mitochondrial cytochrome oxidase I gene. A 678 bp fragment of the COI gene was sequenced to test the hypothesis that the Müllerian mimicry species of the “yellow-black” complex make up a monophyletic clade, separated from species with other colour patterns. The data set was analysed by neighbour-joining, maximum parsimony and maximum likelihood procedures.

The results support a single origin of the yellow-black colour pattern during the evolution of the genus, with one main clade formed by *Chauliognathus lineatus*, *C. tetrapunctatus*, *C. riograndensis*, *C. flavipes*, *C. octomaculatus*, *C. fallax*, and another one formed by two species, *C. expansus* and *C. sp 1*, plus an orange-black-coloured species. The nucleotide divergences found between *C. sp 3* (black) and the other species studied fall within the level expected for species from different genera. The similarity of colour patterns of the yellow-black species has been considered an example of Müllerian mimicry by conservation of the ancestral state with some minor modifications.

Key words: Cantharidae, *Chauliognathus*, COI, Coleoptera, Müllerian mimicry, phylogeny, yellow-black complex.

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Introduction

The genus *Chauliognathus* is distributed over the Americas and Australia, with more than 250 described species (Delkeskamp, 1939), but, despite its wide geographical distribution, only few studies have addressed its assortative mating features (references in McLain, 1982, 1985, and Bernstein and Bernstein, 1998), allozyme variability (Howard and Shields, 1990), colour polymorphism and mating systems (Machado and Araújo, 1995, 1998, 1999, 2001), and cytogenetics (Machado *et al.*, 2001). Taxonomy at the species level has been poorly studied, since a systematic revision was carried out only in U.S. species (Fender, 1964), whereas the fauna of Mexico and the rest of the Neotropical region remains to be revised.

The present work is part of a long-term study of colour polymorphism in *Chauliognathus*, along with its morphology, cytogenetics, and molecular variability. Our study was focused on the species of the “yellow-black” complex. The similarity in colour pattern in these species, as well as that shown by morphometric analysis, together with ecological data suggest that they form a Müllerian-type mimicry ring. The existence of colour polymorphism and the factors which maintain this polymorphism in Müllerian mimetic organisms have recently generated much debate, renewing the interest in mimicry (Turner *et al.*, 1984; Speed, 1993; MacDougall and Dawkins, 1998; Joron and Mallet, 1998; Mallet and Joron, 1999; Edmunds and Golding, 1999). In *Chauliognathus*, this polymorphism could have evolved as a response to visually oriented predators and, on theoretical grounds, it could be a case of classic Müllerian mimicry, made up by species of different intrageneric lineages (Machado *et al.*, 2001). Alternatively, the morpho-

logical similarity shown by species of the “yellow-black” complex could be explained by common ancestry.

Molecular phylogenetic studies have been used for generating and testing evolutionary hypotheses in beetles, such as the reconstruction of the history of host plant use by *Ophraella* (Futuyma and McCafferty, 1990; Futuyma *et al.*, 1993, 1994), the origin of defensive strategies in *Cicindela* (Vogler and Kelley, 1998), colonisation processes (Juan *et al.*, 1995, 1996, 1998; Emerson *et al.*, 1999, 2000; Rees, 2001), and chromosome evolution (Galián *et al.*, 2002).

This study includes 12 species of *Chauliognathus* and is the first attempt to generate a phylogenetic hypothesis for the evolution of beetles belonging to the family Cantharidae. The aim of the work was to test the hypothesis that the species of the “yellow-black” species complex make up a monophyletic clade, separated from species with other colour patterns. Therefore, it could be inferred that this colour pattern is due to common ancestry. According to the classical Müllerian mimicry theory, the pattern should be the result of parallel or convergent evolution.

A 678 bp fragment of the mitochondrial cytochrome oxidase I (COI) gene was sequenced. This approach offers an independent phylogenetic framework, based on characters which are unrelated to colour polymorphism. This gene region has also been used for reconstructing phylogenies of low-ranked taxa, including some Coleopteran groups (Juan *et al.*, 1995, 1996; Galián *et al.*, 1999). Furthermore, this gene has been used to address the evolution of mimicry in butterflies of the genus *Heliconius* (Brower, 1996).

Material and Methods

Material

Twelve species of *Chauliognathus* were included in the study. Sampling localities are shown in Table 1. Eight of these species belong to the “yellow-black” complex (*C. flavipes*, *C. fallax*, *C. octomaculatus*, *C. lineatus*, *C. tetrapunctatus*, *C. riograndensis*, *C. expansus*, and *C. sp 1*).

Their main colour patterns are illustrated in Figure 1. The other four species show other colour patterns, namely, grey and black (*C. morios*), orange (*C. sp 2*), different striped combinations of brown and grey (*C. sp 4*), and black (*C. sp 3*). This material was identified by one of the authors (VM), based on characters of the external genitalia, following the criteria used by Fender (1964) for the North American species of the same genus. The new species will be described elsewhere; voucher genitalia preparations and remnants of investigated beetles are deposited in the Molecular Biology Laboratory of the Universidade do Vale do Rio dos Sinos, São Leopoldo, Brazil, and can be requested from VM. Sequence data were obtained from single individuals of each species, after an initial estimation of intraspecific sequence variation between 0.3 and 0.6 % in *C. flavipes*.

DNA amplification and sequencing

Total genomic DNA was isolated by grinding the head, thorax and legs of each specimen (one individual/species) in liquid nitrogen and resuspending the homogenate in buffer (20 mM Tris, 10 mM EDTA, 0.5% SDS) containing proteinase-K at a concentration of 50 mg/L. The mixture was incubated overnight at 56 °C, then the DNA was purified with phenol-chloroform and precipitated with ethanol (Sambrook *et al.*, 1989). The mtDNA template for sequencing was produced by polymerase chain reaction (PCR) with the primers 5'CAACATTTATTTTGATTTTTGG3' (at 715 bp upstream of the COI gene, corresponding to position 2183 of the mtDNA genome of *Drosophila yakuba*) and 5'TCAATTGCACTAATCTGCCATATT3' (corresponding to position 3014 of the mtDNA genome located in the tRNA^{Leu}). These primers produced a 831 bp fragment at the 3' end of the COI gene in all analysed species. Each PCR cycle was set to 94 °C for 30 s, 1 min at 50 °C, and 1 min at 72 °C. This cycle was repeated 34 times. PCR products were sequenced with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit using Ampli Taq DNA Polymerase, FS, in an ABI PRISM Tm 377 DNA Sequencer.

Table 1 - *Chauliognathus* species, locality of collection, and elytra colour pattern.

Species	Locality	Coordinates	Elytra colour
<i>C. flavipes</i>	Guaíba	30°05' S-51°24' W	Yellow-black
<i>C. fallax</i>	Guaíba		Yellow-black
<i>C. octomaculatus</i>	Guaíba		Yellow-black
<i>C. expansus</i>	Guaíba		Yellow-black
<i>C. lineatus</i>	Guaíba		Yellow-black
<i>C. tetrapunctatus</i>	Guaíba		Yellow-black
<i>C. riograndensis</i>	Santiago		Yellow-black
<i>C. sp 1</i>	Faxinal do Soturno	29°12' S-54°42' W	Yellow-black
<i>C. sp 2</i>	Faxinal do Soturno	29°35' S-53°26' W	Orange-black
<i>C. sp 3</i>	Pelotas		Black
<i>C. morios</i>	Santa Maria	31°46' S-52°20' W	Grey-black
<i>C. sp 4</i>	Coronel Barros	29°41' S-53°49' W	Striped
		28°23' S-53°55' W	

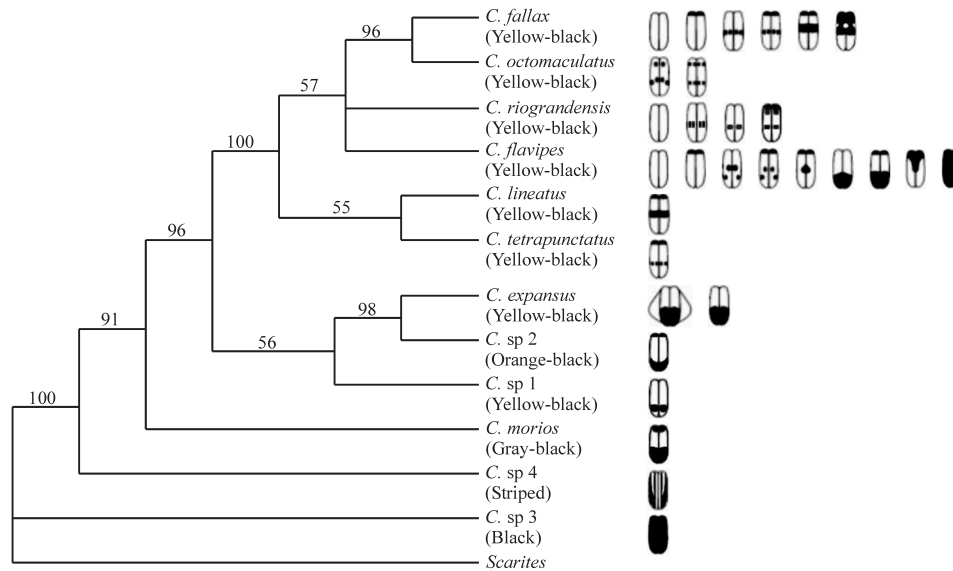


Figure 1 - Single most parsimonious tree obtained with heuristic search re-weighted by maximum value of re-scaled consistency index for the twelve species of *Chauliognathus*. Bootstrap values for 500 replicates are given at the nodes. Pattern of variation found in the elytra colour for the 12 species analysed of the genus *Chauliognathus* are figured to the right.

Phylogenetic analysis

Phylogenetic reconstruction was performed by applying the maximum parsimony (MP), maximum likelihood (ML) and neighbour-joining (NJ; Saitou and Nei, 1987) methods, using the PAUP*4.0b package (Swofford, 1998).

Maximum parsimony and maximum likelihood analyses were conducted using the branch swapping heuristic algorithm tree bisection-reconnection with random addition of taxa.

Phenograms based on sequence distances were constructed using the neighbour-joining analysis, with the distances corrected by Kimura's two-parameter model (Kimura, 1980) with gamma distribution of rates.

For the parsimony analysis, bootstrap tests (Felsenstein, 1985) based on 500 replications were used to assess support of the various phyletic groups. Bootstrap frequencies were interpreted as heuristic levels of support rather than statistical significance levels. Trees were rooted with the sequence of the ground beetle species of the genus *Scarites* (Carabidae), described by Galián *et al.* (1999), plus an additional fragment (Galián, unpubl. res).

Results

A fragment of 830 bp was amplified with the chosen primers in all species. Neither insertions nor deletions were found in this region. Within this gene segment, 338 positions were variable (including the outgroup species), and 170 of them were parsimony-informative. The third codon position was the most variable, while the second codon position was the most constant in this region. Across all sequences there was an excess of A (33.4%)

and T (36.7%) over G (15.0%) and C (14.9%) (Table 2). The proportion of A-T (70.7%) was high, falling between those found for *Drosophila yakuba* (78.6%, Clary and Wolstenholme, 1985) and for the genus *Pimelia* (64%, Juan *et al.*, 1995).

Pairwise distances for the Kimura two-parameter model corrected for rate heterogeneity with the gamma distribution were calculated between sequences, using the pairwise-deletion option of the MEGA computed distances menu (Table 3). The divergence observed between *Chauliognathus* sp 3 (black) and the other species of the genus was high, between 24% and 32%. For the other *Chauliognathus* species, the levels of divergence were between 8% and 21%. The smallest divergence value was observed between *C. octomaculatus* and *C. fallax* (8%).

A heuristic search with uniform weighting generated two MP trees of step length 680, with CI 0.613 and RI 0.395. Then, the characters were re-weighted by maximum value of re-scaled consistency indices, producing a single tree (Figure 1). That tree was similar to the neighbour-joining tree (not shown). These trees support monophyly for the "yellow-black" complex, with slight differences. An interesting aspect of these trees is that *Chauliognathus* sp 2

Table 2 - Proportion of each nucleotide by codon position in the region of COI gene analysed.

base	position			Total
	First	Second	Third	
A	33.8	19.5	46.8	33.4
T	28.7	43.9	37.3	36.7
G	24.3	16.5	4.3	15.0
C	13.2	20.0	11.5	14.9

Table 3 - Matrix of interspecific distance (Gamma distance for the Kimura two-parameter model) in the mitochondrial COI fragment among twelve *Chauliognathus* species.

OTUS	1	2	3	4	5	6	7	8	9	10	11	12	13
1		0.1209	0.1146	0.0855	0.1144	0.1334	0.1728	0.1536	0.1777	0.1640	0.2000	0.2953	0.3368
2			0.1220	0.1314	0.1289	0.1515	0.1848	0.1647	0.1818	0.1901	0.1988	0.3036	0.3273
3				0.1020	0.1081	0.1213	0.1531	0.1255	0.1519	0.1497	0.1929	0.2787	0.3149
4					0.1121	0.1163	0.1731	0.1533	0.1829	0.1690	0.1997	0.3036	0.3361
5						0.1046	0.1731	0.1434	0.2053	0.1715	0.1932	0.3092	0.3589
6							0.1452	0.1279	0.1712	0.1770	0.2066	0.2772	0.3246
7								0.1190	0.1724	0.1531	0.1645	0.2673	0.2969
8									0.1420	0.1467	0.1731	0.2417	0.2825
9										0.1614	0.1963	0.2574	0.2969
10											0.2184	0.2456	0.3267
11												0.3231	0.3677
12													0.2994
13													

OTUS: 1. *C. fallax*; 2. *C. riograndensis*; 3. *C. flavipes*; 4. *C. octomaculatus*; 5. *C. lineatus*; 6. *C. tetrapunctatus*; 7. *C. expansus*; 8. *C. sp 1*; 9. *C. morios*; 10. *C. sp 4*; 11. *C. sp 2*; 12. *C. sp 3*; 13. *Scarites*.

(orange pattern) is included in the yellow-black clade. On the other hand, *Chauliognathus* sp3 (black pattern) is situated in all analyses in a separated group, basal to the remaining species.

Heuristic maximum likelihood searches using the Hasegawa-Kishino-Yano (1985) model (not shown) did not agree with the previous analysis, since they showed some differences, especially in the position of *Chauliognathus* sp 4, that is clustered together within the “yellow-black” species.

In order to correct possible “evolutionary noise” caused by the more variable nucleotide positions, *i.e.* the first and third codon positions, a weighting of 2:5:1 to the first, second and third codon positions, respectively, was carried out. The branch and bound search produced two most parsimonious trees, similar to the heuristic search with a uniform weighting.

Bootstrap analysis yielded strong support for certain groups, for example, all analyses separated the yellow-black species into two clades, one with six species (bootstrap value 100) and the other with two species plus the orange species *C. sp 2* (bootstrap value 84). These values were produced after re-weighting by maximum value of re-scaled consistency indices tree (Figure 1).

In all analyses, the species of the yellow-black complex make up two separated clades; one clade formed by six species of the “yellow-black” complex, *Chauliognathus lineatus*, *C. tetrapunctatus*, *C. riograndensis*, *C. flavipes*, *C. octomaculatus*, and *C. fallax* (bootstrap value 100) and the other clade formed by two species of the “yellow-black” complex, *C. expansus* and *C. sp 1*, plus *C. sp 2*, an orange-black species (bootstrap value 56).

Discussion

The results show that the region of the COI gene sequenced in *Chauliognathus* (corresponding to the 3' end) has the adequate level of divergence for assessing phylogenetic relationships among species of the same genus, as found in other animal groups (Lunt *et al.*, 1996). It has been postulated that the level of divergence for this gene ranges from 1% to 6% among populations within a species, and from 12.6% to 25% among species within a genus (Gallego and Galián, 2001; Simon *et al.*, 1994). The level of divergence calculated between *Chauliognathus* species falls, for most of them, within the range of distinct species, with the exception of *C. sp 3*. The lowest genetic distance value was found between *C. fallax* and *C. octomaculatus* (8%). However, this value of divergence was slightly higher than the one expected between populations within a species. Similar levels of divergence have been found in the Polyphagan beetle genera *Pimelia* (Juan *et al.*, 1995), *Hegeter* (Juan *et al.*, 1996), and *Ophraella* (Funk *et al.*, 1995), and in the Adephagan genus *Scarites* (Galián *et al.*, 1999). The level of divergence found between *Chauliognathus* sp 3 (black) and each of the other species falls into that expected for species of different genera.

According to the trees obtained after re-weighting by maximum value of re-scaled consistency indices, giving equal values to all codon positions, the “yellow-black” species have a common ancestor and form two clades. This grouping is consistent across different analytical methods (MP, NJ). In all analyses, *Chauliognathus* sp 3 is placed in a separated lineage, basal to all of the other species. The great genetic distance between this species and all the others suggests that a more comprehensive study, taking into

account the overall data (morphological and genetic), needs to be made in order to assess the taxonomic status of this species.

These results indicate that the colour pattern of the “yellow-black” complex is due to a common ancestry. The polymorphism in the elytral coloration in four out of the eight species of the complex (Diehl-Fleig and Araújo, 1991; Machado and Araújo, 1995; 1998; 1999) suggests that it (polymorphism) is also an ancestral feature of this genus. However, it is unclear whether the ancestor had a monomorphic yellow or black elytron, or a yellow elytron with black spots varying in size and location. Models for the evolution of colour patterns in heliconiine butterflies have hypothesised changes in regulatory genes (Gilbert, 2002; Jiggins and McMillan, 1997). This hypothesis may well be applied to the *Chauliognathus* “yellow-black” complex.

In any case, the colour pattern of the “yellow-black” complex is possibly a true case of Müllerian mimicry, as stated by Machado and Araújo (2001), with conservation of the ancestral state with some small modifications. This is in accordance with Brower *et al.* (1963), who stated that Müllerian relationships should not be excluded within closely related species, as the selective process that prevents divergence in colour pattern may be basically the same as those bringing about convergence in distantly related organisms. The study of the occurrence and maintenance of this variety of colour patterns in *Chauliognathus* offers a model to understand the mechanisms involved in the evolution of the polymorphism of Müllerian mimicry that is different from those applied to butterfly species. Furthermore, this species complex is a good study case for the species/population interface, through a phylogeographic approach. According to Templeton (1998), this approach is important for the study of the process involved in the origin of new species.

The nucleotide sequences of the 12 *Chauliognathus* taxa determined here were deposited in the GenBank Database under Accession Nos. from AY095214 to AY095225.

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