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GENETICALLY DETERMINED COLOUR POLYMORPHISM IN LARVAE OF *CERIAGRION CHAOI* (INSECTA: ODONATA: COENAGRIONIDAE)

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ABSTRACT. — Although genetically determined colour polymorphism is quite common in adult odonates, there is no report on this phenomenon in the larvae of any odonate species up to now. This paper reports, for the first time, the occurrence of two colour morphs (dark and brown) in both the male and female larvae of the damselfly *Ceriagrion chaoi* Schmidt. The species identity of these colour morphs was confirmed by the partial sequences of 16S rRNA gene as well as observation on emergence. Only a single invariant haplotype was observed, which differed from a congeneric species *Ceriagrion cerinorubellum* (Brauer) by 39 base pairs. The partial sequences of 16S rRNA gene constitute the first report for these damselflies. Available data indicate that environment/habitat does not seem to play a role in the determination of the colouration in the larvae of *C. chaoi*. The inheritance and significance of the colour polymorphism however remain to be verified.

KEY WORDS. — colour polymorphism, odonate larva, damselfly, *Ceriagrion chaoi*, *Ceriagrion cerinorubellum*, 16S rDNA sequences

INTRODUCTION

Female-limited colour polymorphism is quite common in adult odonates. It has been documented for more than hundred species, comprising the suborders Anisoptera and Zygoptera (Fincke et al., 2005). Two examples are the trimorphic females in the blue-tailed damselfly *Ischnura elegans* (Vander Linden) in Europe (Sánchez-Guillén et al., 2005) and the common bluetail *Ischnura senegalensis* (Rambur) in Asia and Africa (Orr, 2005; Tan et al., 2010). By contrast, male-limited polymorphisms are not so common (van Gossum et al., 2008).

Generally odonate larvae are green or brown, resembling or blending with their backgrounds (Corbet, 1999). A particular case of polymorphism in odonate larvae is the strip pattern of first instars of Aeshnidae, which seems to serve as a disruptive colouration (Rowe, 1991). Some larvae, when they moult, may be able to take on the predominant colour of their backgrounds (Silsby, 2001). An example is the dragonfly *Epiophlebia superstes* Selys (Eda, 2007; Tabaru, 1984). As far as known, there is no published record or encounter of the occurrence of genetically determined colour polymorphism in odonate larvae (Matti Hämäläinen, pers. comm., 2008).

Ceriagrion chaoi is a member of the zygopteran family Coenagrionidae (Silsby, 2001). It occurs in Peninsular Malaysia, Singapore, Thailand and Myanmar. In Peninsular Malaysia it is rare and local in open habitats (Orr, 2005;

Yong & Hämäläinen, 1994), in Singapore recorded only in Bishan Park (Tan et al., 2010), and in Thailand with scattered records (Hämäläinen & Pinratana, 1999).

This paper reports the novel finding of two colour morphs (dark and brown) in male and female larvae of the fiery coraltail *Ceriagrion chaoi* Schmidt, and the fist record of the partial sequences of 16S rRNA gene for *C. chaoi* and *C. cerinorubellum*.

MATERIAL AND METHODS

Field observations of *C. chaoi* were carried out at the botanical garden of University of Malaya, Kuala Lumpur, Peninsular Malaysia on many occasions over the years. This population of damselfly was first recorded in 1994 (Yong & Hämäläinen, 1994). Intensive observations on the emergence of larvae were carried out throughout the months of Oct. and Nov.2008, but discontinued due to disturbance of the habitats (tanks containing aquatic plants at different density in the open as well as net house exposed to natural sunlight). The number of brown and dark (black) larvae or the exuviae (Fig. 1) for the day were recorded. Representative male and female adults, tenerals and larvae as well as exuviae were collected, preserved in absolute alcohol and stored in deep freezer for DNA study. Identification of the adult damselfly was based on Orr (2005), Tan et al. (2010), and Yong & Hämäläinen (1994).

DNA extraction, PCR amplification and sequencing. — Seven specimens of *C. chaoi* were used for DNA analyses: adult female CCHA1; female exuvia CCHA2; male brown larva CCHA4; male black larva CCHA6; dark larva CCHA13; brown larva CCHA15; adult male CCHA16. The total genomic DNAs were isolated from three legs of the larvae or the whole exuviae of the damselflies, using i-genomic CTB DNA Extraction Mini Kit (iNtRON Biotechnology, Inc, Korea). The partial sequences of 16S rDNA were amplified using the primer set of (16S-F) LR-J-13756 5'-TAGTTTTTTTAGAAATAAATTTAATTTA-3' and (16S-R) LR-N-13308 5'-GCCTTCAATTAAAAGACTAA-3' (Smith et al., 2003).

Fig. 1. Exuviae of brown (left) and dark (right) colour larvae of *Ceriagrion chaoi*.

Table 1. Number of brown and dark/black colour morphs of *Ceriagrion chaoi* larvae for Oct. and Nov.2008 at University Malaya campus.

Date	Brown	Dark	Total
Oct. 2008	138	36	174
Nov. 2008	49	7	56
Total	187	43	230

PCR amplification was carried out using MultiGene Gradient Thermal Cycler (Labnet, USA) using an i-Taq™ Plus DNA Polymerase Kit (iNtRON Biotechnology, Korea). The total volume for the PCR amplification was 50 µL consisting of 5.0 µL of 10x *i*-Taq™ *plus* buffer, 5.0 µL of dNTP mixture (2.5 mM each), 0.25 µM of each primer, 1.0 unit of *i*-Taq™ *plus* DNA polymerase, and 50 pg to 1.0 µg DNA. The parameters of PCR amplification were: 3 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min.

PCR products were assayed by electrophoresis on 1.0% agarose mini gel stained with SYBR®Safe DNA gel stain (Invitrogen, USA) and visualised under UV light. The target DNA fragments were isolated and purified by the LaboPass™ PCR purification kit (Cosmo Genetech, South Korea). The purified PCR products were sent to a commercial company for sequencing.

The partial 16S rDNA sequences were edited using ChromasPro V1.5 (Technelysium Pty Ltd), subsequently aligned using the CLUSTAL X programme (Thompson et al., 1997) and finally manually aligned. The partial 16S rDNA of adult male *C. cerinorubellum,* CCER1 collected from University of Malaya campus was used as comparison and aligned with the other seven sequences of *C. chaoi.*

RESULTS

Proportion of brown and dark larvae. — The number of brown and dark colour larvae for the months of Oct. and Nov.2008 is summarised in Table 1. For Oct.2008 there were about 3.8 times more brown larvae than dark larvae; for Nov.2008 there were 7.0 times more brown larvae than dark larvae. In total there were 4.4 times more brown larvae than dark larvae.

DNA sequences. — All the eight 16S rRNA sequences were deposited in GenBank with the following accession numbers CCHA1 (JQ890103); CCHA2 (JQ890097); CCHA4 (JQ890099); CCHA6 (JQ890100); CCHA13 (JQ890102); CCHA15 (JQ890098); CCHA16 (JQ890101) and CCER1 (JQ890104). The partial sequences of 16S rRNA gene revealed that the brown and dark colour morphs of the larvae were identical to the adult *C. chaoi* (Fig. 2).

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                10        20        30        40        50        60        70        80
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCHA1      GCCTTCAATTAAAAGACTAATGATTATGCTACCTTTGCACGGTCAATATACCGCGGCCGTTAAATTTAATTCACTGGGCA
CCHA2      ..................................................................................
CCHA4      ..................................................................................
CCHA6      ..................................................................................
CCHA13     ..................................................................................
CCHA15     ..................................................................................
CCHA16     ..................................................................................
CCER1      ..............................................................A...................

                90       100       110       120       130       140       150       160
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCHA1      GGCCAGACCTTTCAAAAAAAA-CGAAAGGACATGTTTTTGATAAACAGGCGAACATAATATTTGCCGAGTTCCTCATTTA
CCHA2      ....................-.............................................................
CCHA4      ....................-.............................................................
CCHA6      ....................-.............................................................
CCHA13     ....................-.............................................................
CCHA15     ....................-.............................................................
CCHA16     ....................-.............................................................
CCER1      ..........C.........A.....................................................AA..

               170       180       190       200       210       220       230       240
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCHA1      TATATCACGCAAAATAAATTGAAAACAAAATTATATACTAATTCAATCATAATTAATATAAATAAAAAATTAATTAATAT
CCHA2      ..................................................................................
CCHA4      ..................................................................................
CCHA6      ..................................................................................
CCHA13     ..................................................................................
CCHA15     ..................................................................................
CCHA16     ..................................................................................
CCER1      .....AGAAT.T......C.A......T..............T..............T....T..............A

               250       260       270       280       290       300       310       320
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCHA1      ATTTTAATAA-ATATCTAAATTAAAGAAAATCAAATATAATTGAATTATACTATAACGTAATAAAAAGATGGGTGATTTT
CCHA2      ..........-.......................................................................
CCHA4      ..........-.......................................................................
CCHA6      ..........-.......................................................................
CCHA13     ..........-.......................................................................
CCHA15     ..........-.......................................................................
CCHA16     ..........-.......................................................................
CCER1      ..........T.A........C.........TT.......A.....................T.....C..........

               330       340       350       360       370       380       390       400
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCHA1      ATTCCTACTACATTCAAAAAA--AATAAAATTATAGAAAAAAAAATAAAGCTTATCCCTTATCATTTTTAACTTGACAAAT
CCHA2      ....................--............................................................
CCHA4      ....................--............................................................
CCHA6      ....................--............................................................
CCHA13     ....................--............................................................
CCHA15     ....................--............................................................
CCHA16     ....................--............................................................
CCER1      ..........A..........TA..A................-..................T...........A......

               410       420       430       440       450
           ....|....|....|....|....|....|....|....|....|....
CCHA1      AATTTATTAAAGAAGAAAAAAGTAAATACCAAGACTAAATTAAATTTATTTCTA
CCHA2      ......................................................
CCHA4      ......................................................
CCHA6      ......................................................
CCHA13     ......................................................
CCHA15     ......................................................
CCHA16     ......................................................
CCER1      ....C......A..............GT....GT....................
```

Fig. 2. Alignment of 454 bp partial sequences 16S rDNA of representative *Ceriagrion chaoi* and *Ceriagrion cerinorubellum*. CCHA1 = adult female of *C. chaoi*; CCHA2 = female exuvia of *C. chaoi*; CCHA4 = male brown larva of *C. chaoi*; CCHA6 = male black larva of *C. chaoi*; CCHA13 = dark larva* of *C. chaoi*; CCHA15 = brown larva* of *C. chaoi*; CCHA16 = brown larva of *C. chaoi*; CCER1 = *C. cerinorubellum*. *sex not determined.

The haplotype for all the *C. chaoi* specimens studied was invariant. It however differed from a congeneric species, *C. cerinorubellum* by 39 base pairs (Fig. 2).

DISCUSSION

Adult *C. chaoi* exhibits sexual dimorphism in body colour (Fig. 3). The mature male has reddish brown head and mouthparts, olive green thorax and red abdomen, while the female has an olive green thorax and dark olive head and abdomen. The emerging teneral male and female have similar colouration as the adults. Like other zygopterans, the adult damselflies have a slender and cylindrical built and a weak and fluttery flight. Their larvae are long and narrow with three caudal gills at the end of the abdomen.

In adult damselflies (Odonata: Zygoptera), female-limited polymorphism has been found to be genetically determined (for review, see Andrés & Cordero, 1999; Sánchez-Guillèn et al., 2005). In general, the polymorphism has a simple one-locus, two-allele inheritance. The colouration in adult odonates, in particular black patterning, has been hypothesized to be related to resistance against pathogens. However experimental evidence indicates that colour polymorphism in adult coenagrionid damselflies (as demonstrated in *Coenagrion puella*) is unlikely to be maintained by differences in immunity (Joop et al., 2006).

In adult *I. elegans* the female morphs differ in shape and development rate (Abbott & Svensson, 2008), as well as development time (Abbott & Svensson, 2008) and fecundity (Svensson & Abbott, 2005; Svensson et al., 2005). In the sister species *I. elegans* and *I. graellsii*, morph frequency dynamics indicates that hybridisation is likely to have important implications for the maintenance of multiple female morphs, in particular during the initial period of hybridisation (Sánchez-Guillén et al., 2011a). Both stochastic and selective forces play a role on population divergence of the colour polymorphism and the relative importance of these factors varies between geographical regions (Sánchez-Guillén et al., 2011b). To-date there are however no published record of genetically determined colour polymorphism in the larvae of any odonates.

The presence of two colour morphs in *C. chaoi* larvae can be reasonably attributed to the occurrence of polymorphism as they coexist in natural populations. There is no ambiguity in distinguishing the dark/black (Fig. 4) and the brown (Fig. 5) morphs, and no change in colouration upon emergence of the larvae. The emerging male (Fig. 6) and female (Fig. 5) could also be easily distinguished and identified. There

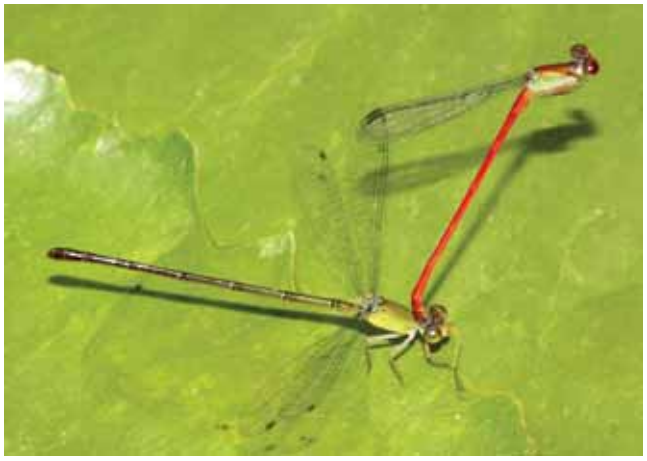

Fig. 3. Mating pair of *Ceriagrion chaoi*.

Fig. 5. Female *Ceriagrion chaoi* emerging from brown larva.

Fig. 4. Dark larva of *Ceriagrion chaoi*.

Fig. 6. *Ceriagrion chaoi* male emerged from dark larva.

was no doubt about the identity of the damselflies as *C. chaoi* is the only species of *Ceriagrion* present in the study area. Their identity was further confirmed by the partial sequences of 16S rRNA gene as well as direct observation of emergence. Only a single invariant haplotype was observed which differed from a congeneric species *C. cerinorubellum* by 39 base pairs (Fig. 2).

Both the dark and brown morphs emerged together at the same sites in two different but nearby locations (open and sheltered) in the University campus. It indicates that environment/habitat is unlikely to play a role in the maintenance of this colour polymorphism as both morphs occurred together in the same water body with the same plants and lighting condition. Furthermore, both colour morphs occur without disruption in time as evidenced by casual observations from time to time over the years since the initial discovery in 2008 until now. The density of aquatic plants, whether sparsely or completely covering the water surface, also does not seem to influence the colouration of the larvae.

The inheritance and significance of the colour polymorphism in *C. chaoi* larvae remain to be verified. Available data indicate that it is most likely to be under genetic control. It is hoped that the present finding will stimulate others to look for colour polymorphism in odonate larvae in other parts of the world.

In summary, this paper reports, for the first time the occurrence of two colour morphs (dark and brown) in both the male and female larvae of the damselfly *C. chaoi*. The partial sequences of 16S rRNA gene for *C. chaoi* and *C. cerinorubellum* constituted the first report for these damselflies.

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