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Recognition of a Novel Melanotic Mutant in a Field Population of *Culex pipiens quinquefasciatus* in Southern Zambia

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Abstract

Genetic mutations controlling eye color, fat body color, structural abnormalities, and insecticide resistance are common in mosquitoes. We have identified a novel color variant of *Culex pipiens quinquefasciatus* characterized by a heavily pigmented integument in adult specimens circulating in field populations of this species in southern Zambia. Mosquitoes were collected monthly by pyrethrum spray catch between November and May 2004–05 and 2005–06, with between 25% and 80% of the total *Cx. p. quinquefasciatus* collections comprising this pigmented variety. The identity of pigmented specimens was morphologically confirmed as *Cx. p. quinquefasciatus* by examination of orientation of the dorsal and ventral arms of the male genitalia of F1 male progeny reared in the laboratory, and molecularly verified by diagnostic polymerase chain reaction and nicotinamide adenine dinucleotide phosphate dehydrogenase subunit 4 nucleotide sequence homology. Preliminary laboratory rearings indicated that the pigmentation was heritable and not influenced by larval habitat and environmental conditions. Further investigation into the mechanism of the mutation, inheritance patterns, and potential linkage to additional markers is pending.

Keywords

Culex pipiens quinquefasciatus; melanotic mutant; Zambia; Macha

Genetic mutations controlling eye color, fat body color, structural abnormalities, and insecticide resistance are common in mosquitoes. A darkened or melanized integument is another notable phenotype that can arise from genetic mutation. Seawright and Anthony (1972) and Seawright et al. (1985) described lethal color mutants in *Anopheles quadrimaculatus* Say and *An. albimanus* Wiedemann, respectively. The mutant larvae of these species were typified by black or dark coloration of either the head capsule or entire body and progressed normally through larval development but died during pupation. Many such genetic mutations including those that affect integument color have been described in the *Culex pipiens* complex (e.g., VandeHey 1964, 1969; Barr 1975; Subbarao and Adak 1978; Sakai et al. 1980; Shetty and Chowdaiah 1983; Suguna 1985, 1986a, 1986b; Das et al. 1990; Rasgon and Scott 2004). “Black head capsule” (*Bl*) is a dominant, spontaneous mutation on chromosome 1 associated with slow development in *Cx. pipiens* larvae

(VandeHey 1967, Barr 1975). “Melanotic” (*mel*) is a recessive, spontaneous, lethal mutation on chromosome 3 associated with dark pigmentation in the epidermis (Kitzmiller 1953, Barr 1975), and “Dunkel” (*du*) is a recessive, spontaneous, mutation also mapped to chromosome 3 and associated with dark coloration in larvae (Dennhöfer 1973, Barr 1975). Suguna (1986b) described a novel, non-lethal melanotic brown mutant isolated from natural populations of *Cx. p. quinquefasciatus* Say larvae in southern India that was similar in phenotype and occurred in the same linkage group as “Dunkel.” The colors of the adult stages of these mutant strains were either not described or were not observable because of the lethality of the mutation during the larval stages or pupation. Sakai et al. (1972) reported a nonlethal melanotic mutation on chromosome 2, *ebony*, in *Cx. tritaeniorhynchus* Giles that was expressed in larval, pupal, and adult stages. Alternatively, pigmentation in insects can be regulated by the production of various hormones in response to environmental conditions (Fuzeau-Breash 1972, Raabe 1983). The production of pigments in various species of mosquitoes in response to the color of the larval habitat has also been described (Benedict and Seawright 1987).

Culex p. quinquefasciatus is 1 of 31 mosquito species that has been identified to date at the Johns Hopkins Malaria Research Institute field station in Macha, located in the Southern Province of Zambia (Kent 2006). Although this species is universally recognized as a pale mosquito, during the beginning of the 2004–05 rainy season we collected what appeared to be a black color variant of this species characterizing a large proportion of the total adult *Cx. p. quinquefasciatus* capture in Chidakwa village. This dark form was morphologically identical to the traditionally recognized pale form except for very dark pigmentation of the integument (Fig. 1). These dark *Cx. p. quinquefasciatus* were collected from the same village and houses as the pale forms. Our objectives were to confirm the identity of these dark mosquitoes as *Cx. p. quinquefasciatus*, and characterize the relative prevalence of this dark color variant in natural populations. Further study involving genetic crosses between dark and pale forms and the inheritance patterns of the pigmentation are pending.

Engorged mosquitoes were collected by pyrethrum spray catch and aspiration in Chidakwa village in the Macha region of the Southern Province of Zambia (16°42′30.2″S, 26°79′72.8″E). Pyrethrum spray catches were conducted with locally purchased DOOM Super® (Adcock Ingram Ltd., Bryanston, South Africa) combination synthetic pyrethroid (D-phenothrin 0.92 g/ kg, Prallethrin 0.4 g/kg, and Imiprothrin 0.25 g/ kg). Approximately 45 houses were sprayed per month between November and May during 2004– 05 and 2005– 06 as part of a larger study on the *Anopheles* mosquitoes and malaria transmission dynamics in this region. All specimens were individually placed in 1.5-ml tubes containing silica gel desiccant and cotton immediately following collection and stored at ambient temperature. Culicine mosquitoes were morphologically identified using the keys of Edwards (1941), Service (1990), and Jupp (1996).

Engorged *Cx. p. quinquefasciatus* of both dark and pale forms were also collected by aspiration and transferred immediately to the on-site insectary. Each female mosquito was placed individually in a half pint screened carton and supplied with an oviposition cup and a cotton ball soaked in 30% sucrose solution. Larval progeny from each female were reared separately in field water supplemented with Cerelac® baby food flakes. The insectary light

cycle was approximately 9L:15D. Insectary temperature and relative humidity fluctuated with ambient outdoor temperature, but was on average 25°C with 90% humidity during the period of larval rearings. Adult progeny from each female were sacrificed, transferred individually to silica tubes, and labeled to correspond to the appropriate female. To determine whether larval habitat influenced adult coloration, egg rafts of 2 dark and 2 pale females were split and each half reared separately in clear plastic pans placed on either a black or white surface. The orientation of the dorsal and ventral arms of the male genitalia (DV/D ratios), diagnostic for differentiating members of the *Culex pipiens* complex, was measured for 6 dark male progeny *Cx. p. quinquefasciatus* according to the methods of Sundararaman (1949).

Mosquito DNA was extracted using a modified salt procedure as described previously (Kent and Norris 2005). The relative quality of all DNA extractions was evaluated by polymerase chain reaction (PCR)-amplification of a fragment of the mitochondrial nicotinamide adenine dinucleotide phosphate dehydrogenase subunit 4 (ND4) using arthropod-specific primers (Simon et al. 1994, Kent and Norris 2005). Molecular confirmation for all pale and dark samples morphologically identified as *Cx. p. quinquefasciatus* was obtained using the Smith and Fonseca (2004) PCR diagnostic as described. Additionally, both pale and dark *Cx. p. quinquefasciatus* were screened for *Wolbachia* endosymbionts by targeted amplification of a portion of the *Wolbachia* surface protein (*wsp*) gene by PCR using primers 81F and 691R (Braig et al. 1998, Zhou et al. 1998). DNA amplifications were completed on a MJ Research PTC-200 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) and visualized after electrophoresis on ethidium bromide-stained 2% agarose gels. Electrophoresis was conducted with GeneRuler 100-basepair (bp) molecular mass marker (Fermentas Life Science, Hanover, MD). To identify nucleotide substitutions, insertions, and deletions in genetic markers within and between color phenotypes, ND4 and *wsp* PCR products from dark and pale *Cx. p. quinquefasciatus* were compared by single strand conformation polymorphism (SSCP) (Hiss et al. 1994, Black and DuTeau 1997, Kent et al. 2004) and sequencing.

The identity of the dark-pigmented mosquitoes was confirmed as *Cx. p. quinquefasciatus* morphologically by taxonomic keys and measurement of DV/D ratios, and molecularly by PCR (Fig. 2). The orientation of the dorsal and ventral arms of the male genitalia ratios for *Cx. p. quinquefasciatus* typically range between 0.5 and 1.4, as compared with *Cx. p. pipiens*, which is characterized by DV/D ratios between 0 and 0.5 (Sundararaman 1949, Sirivanakarn and White 1978). The DV/D ratios of progeny males reared from wild-collected dark females in Zambia ranged from 0.54 to 0.93 with a mean of 0.68, well within the range of expected values that characterize true *Cx. p. quinquefasciatus*.

The F1 progeny of pale *Cx. p. quinquefasciatus* from the same, split egg rafts reared on black or white surfaces were all pale as adults. Similarly, F1 progeny of dark *Cx. p. quinquefasciatus* reared on black and white surfaces were all dark as adults, indicating that in this case, the adult coloration is inherited and not influenced by larval habitat color or other environmental conditions. In contrast, Benedict and Seawright (1987) examined pigmentation responses of larval and adult mosquitoes reared on black, white, or green backgrounds and observed pronounced responses in larval *Anopheles* mosquitoes to adapt to

their background color. Moderate darkening of the fat body, setae, and heavily sclerotized structures was also noted for larval stages of *Cx. p. quinquefasciatus* and *Cx. salinarius* Coquillett reared on black substrates, but these changes were not carried over into the adult stage.

This dark color variant was PCR confirmed as *Cx. p. quinquefasciatus* based on sequence homology in the acetylcholinesterase 2 gene (Fig. 2). Additional molecular evidence for this identification was provided by SSCP analysis of mitochondrial ND4, and *Wolbachia* infection data. Dark and pale *Cx. p. quinquefasciatus* shared identical ND4 nucleotide sequences (EF030094, EF033661), and both forms were positive for *Wolbachia* endosymbionts that shared the same *wsp* genotype by SSCP. ND4 sequences for both pale and dark forms of Macha *Cx. p. quinquefasciatus* were identical to *Cx. p. quinquefasciatus* from Thailand (AY793692), the USA (AY793689), and South Africa (AY793691), and differed by 1 nucleotide from an additional ND4 haplotype reported from the USA, *Cx. p. quinquefasciatus* (AY793693). Lack of variation in ND4 is consistent with *Wolbachia* infection, driving the maternal inheritance of this gene to fixation (Rasgon et al. 2006). By comparison, there were 35 nucleotide substitutions in the ND4 amplicon between *Cx. p. quinquefasciatus* and *Cx. univittatus* Theobald (EF030093) from Macha.

In November 2004, 285 female *Cx. p. quinquefasciatus* were collected by pyrethrum spray catch from 46 houses in Chidakwa village. Of these, 167 were pale and 118 (41%) were of the dark, melanized form. In December 2004 and January 2005, the total collection of *Cx. p. quinquefasciatus* dropped to 44 and 36 mosquitoes, respectively, with the dark form comprising 65% of the collection in December and 25% of the total collection in January. In similar collections from Chidakwa village during the 2005–06 season, 8 of 10 (80%) of *Cx. p. quinquefasciatus* collected in November, 93 of 196 (47%) in December, and 0 of 66 in January 2006 were of the dark form. Individual mosquitoes with partial melanization, or patches of dark coloration on the pleural sclerites, were also noted during the 2005–06 season. Clearly, this dark variant is prevalent in wild populations of *Cx. p. quinquefasciatus* in this study region and is maintained from season to season. It is not known if any selective advantage is conferred to these melanized forms of *Cx. p. quinquefasciatus*. The dark variant appears to be similar to *ebony* (Sakai et al. 1972) in that melanotic expression occurs in the adult stage and the mutation is nonlethal. However, while *ebony* was observed in a *Cx. tritaeniorhynchus* laboratory colony, these color mutants were observed in field populations of adult *Cx. p. quinquefasciatus*. Studies on the mechanism and inheritance patterns of this mutation as well as potential linkage to other genetic markers are pending.

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Fig. 1.
Side by side images of field-collected dark and pale specimens of *Cx. p. quinquefasciatus* from Macha, Zambia.

Fig. 2.

Smith and Fonseca (2004) PCR diagnostic differentiating *Cx. p. pipiens* and *Cx. p. quinquefasciatus*. *Culex p. pipiens* is characterized by amplification of 610-bp fragment, whereas a 274-bp fragment is amplified from *Cx. p. quinquefasciatus*. Both fragments are amplified from species hybrids. Lanes 1–2, colony *Cx. p. pipiens*, Cornell, NY; Lane 3, *Cx. p. quinquefasciatus*, Bamako, Mali; Lane 4, *Cx. p. quinquefasciatus*, Kinmen, Taiwan; Lane 5, *Cx. p. quinquefasciatus*, South Africa; Lanes 6–7, pale *Cx. p. quinquefasciatus*, Macha, Zambia; Lanes 8–9, dark *Cx. p. quinquefasciatus*, Macha, Zambia; Lane 10, Slab colony *Cx. p. quinquefasciatus*, California, USA; Lane 11, negative control.