Molecular Phylogenetics and Evolution 49 (2008) 227–239

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Molecular phylogenetics of tsetse flies (Diptera: Glossinidae) based on mitochondrial (*COI*, *16S*, *ND2*) and nuclear ribosomal DNA sequences, with an emphasis on the *palpalis* group

N.A. Dyer [a,*], S.P. Lawton [a,d,e], S. Ravel [c], K.S. Choi [a], M.J. Lehane [a], A.S. Robinson [b], L.M. Okedi [g], M.J.R. Hall [e], P. Solano [f], M.J. Donnelly [a]

[a] *Vector Group, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, Merseyside, L3 5QA, UK*
[b] *FAO/IAEA Agriculture and Biotechnology Laboratory, A-2444 Seibersdorf, Austria*
[c] *UMR177, IRD-CIRAD, TA A17/G, Campus International de Baillarguet, 34398 Montpelllier Cedex 5, France*
[d] *Institute of Biological Sciences, University of Wales Aberystwyth, UK*
[e] *The Natural History Museum, London, UK*
[f] *IRD/CIRDES, 01 BP 454, Bobo-Dioulasso 01, Burkina Faso*
[g] *Livestock Health Research Institute, P.O. Box 96, Tororo, Uganda*

ARTICLE INFO

Article history:
Received 25 March 2008
Revised 8 July 2008
Accepted 13 July 2008
Available online 22 July 2008

Keywords:
Tsetse
Glossina
Diagnostic PCR
Palpalis
Cryptic species

ABSTRACT

Relationships of 13 species of the genus *Glossina* (tsetse flies) were inferred from mitochondrial (*cytochrome oxidase 1*, *NADH dehydrogenase 2* and *16S*) and nuclear (*internal transcribed spacer 1* of rDNA) sequences. The resulting phylogeny confirms the monophyly of the morphologically defined *fusca*, *morsitans* and *palpalis* subgenera. Genetic distances between *palpalis* and *morsitans* subspecies suggest that their status needs revision. In particular, *cytochrome oxidase 1* sequences showed large geographical differences within *G. palpalis palpalis*, suggesting the existence of cryptic species within this subspecies. The morphology of *palpalis* group female genital plates was examined, and individuals were found varying outside the ranges specified by the standard identification keys, making definitive morphological classification impossible. A diagnostic PCR to distinguish *G. palpalis palpalis*, *G. tachinoides* and *G. palpalis gambiensis* based on length differences of *internal transcribed spacer 1* sequences is presented.

1. Introduction

Tsetse flies (*Glossina*) are the major vectors of trypanosomiasis throughout sub-Saharan Africa, causing extensive morbidity and mortality in humans and live stock (Leak, 1999). Morphological characters such as genitalia, wing size and shape and abdominal colouration have been used to resolve the phylogenetic relationships of tsetse flies. However, the division of species into the three groups, (*morsitans*, formally and synonymous with subgenus *Glossina*; *palpalis*, formally and synonymous with subgenus *Nemorhina*, and *fusca*, formally and synonymous with subgenus *Austenina*) is primarily based on the differences in structural complexity of the genitalia and is supported by patterns of body hairs and habitat choice (Gooding and Krafsur, 2005; Gooding et al., 1991). Subgenus *fusca*, comprised of 12 species, forms a sister group to the *palpalis* and *morsitans* subgenera, and probably inhabits the ancestral habitat of tsetse flies (Leak, 1999). With the exception of *G. longipennis*, *fusca* group flies inhabit forests or dense thickets providing heavy shade (Leak, 1999). *palpalis* group flies inhabit vegetation close to water, including forests, small island forests, gallery forests, "sacred" woods, banks of lakes, "niayes" and mangroves. Some species colonise cocoa, coffee or mango plantations (Solano et al., 2008). In contrast, *morsitans* group flies inhabit savannah and are generally more tolerant of desiccating conditions (Bursell, 1958). Savannah vegetation appeared in sub-Saharan Africa around the Miocene to Pliocene boundary, approximately 7–8 million years ago (Cerling et al., 1997). It is postulated that the *morsitans* group may have evolved from within the *fusca* group to adapt to this new savanna habitat (Bursell, 1958). Bursell did not address the evolution of *palpalis* group flies, but Machado discussed speciation within the *palpalis* group in an earlier publication (Machado, 1954). He suggested that the *fuscipes* and *palpalis* species, within the *palpalis* group, underwent allopatric speciation prior to the mid Quaternary. During this period the Congo River had no outflow into the Atlantic, and formed a barrier between West Africa and the Congo Basin. The three allopatric *fuscipes* subspecies *fuscipes*, *quanzensis* and *martinii* are thought to have evolved within the Congo basin due to retractions and separations of their forest habitats during dry periods in the Pliocene (Machado, 1954).

* Corresponding author. Fax: +44 (0) 151 705 3369.
E-mail addresses: Naomi.Dyer@liv.ac.uk, naomi223@googlemail.com (N.A. Dyer).

1055-7903/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2008.07.011

The genus *Glossina* is the only genus in the family *Glossinidae* (Brues et al., 1954; Pollock, 1971). The *Glossinidae* are placed in the superfamily *Hippoboscoidea*, previously proposed by Hennig as *Glossiniodea* (Hennig, 1971). The *Glossinidae* are in the dipteran group *Calyptratae* (Nirmala et al., 2001). Within the *Glossina*, provisional molecular analyses using isoenzyme electrophoresis (Gooding et al., 1991) and sequencing of an autosomal *ribosomal internal transcribed spacer region* (*ITS2*) provided good support for the monophyly of the three subgenera of *Glossina* but did not improve the resolution within each subgenus. These molecular analyses supported the contention that the forest dwelling *fusca* group forms a sister group to the *palpalis* and *morsitans* subgenera. Molecular analysis has also been used to investigate the position the *Glossinidae* within the *Hippoboscoidea*, and the *Hippoboicoidea* within the *Calyptratae*. Phylogenies of nuclear 18S and mitochondrial 16S rDNA, using *G. palpalis* and *G. morsitans* to represent *Glossinidae*, differ according to the method of phylogenetic inference (Dittmar et al., 2006; Nirmala et al., 2001). More recently, the phylogeny of the *Hippoboscoidea*, including seven *Glossina* species was estimated using two mitochondrial (*COI* and *16S*) and two nuclear (*CAD* and *28S*) markers (Petersen et al., 2007). This study confirmed the monophyly of the *Glossinidae*, and that the *fusca* group species *G. brevipalpis* was a sister group to all the other tsetse species sampled. Carlson et al. (1993) derived phenetic relationships between 26 species and subspecies using gas chromatographic analysis of cuticular alkenes. Again the three clades were broadly supported, but species considered to be within the *fusca* group such as *G. longipennis*, *G. medicorum* and *G. nigrofusca nigrofusca* were mixed within the *palpalis* clade. This apportioning of species to inappropriate clades is likely to be a result of convergence due to environmental adaptation as seen in other disease vectors (Maingon et al., 2003).

The taxonomic position of other species is also uncertain (Gooding and Krafsur, 2005). *Glossina austeni* was placed in the *morsitans* group based on classical taxonomy using characters of the male genitalia. However, female genital characters are shared with the *fusca* group and their ecology is very similar to that of *palpalis* group (Gooding and Krafsur, 2005). Enzyme analysis placed *G. austeni* as a sister group of *morsitans* and DNA sequence data indicate that *G. austeni* is more closely related to the subspecies of *G. morsitans* than to the species of the *palpalis* subgroup (Gooding et al., 1991). Although this indicates that *G. austeni* is more akin to the *morsitans* clade it does not imply that *G. austeni* should be placed within the *morsitans* subgenus. Chen et al. (1999) examined DNA sequence variation from the *internal transcribed spacer 2 ribosomal region* (*ITS2*) and concurred with the morphological classification of *G. austeni* and indeed the morphological phylogeny of the genus as a whole. However, Gooding and Krafsur (2005) highlighted that identification of the boundaries defining the subgenera cannot be inferred from current available genetic data. In Petersen et al. (2007), *G. austeni* partitioned with strong bootstrap support to the *morsitans* group, forming a sister clade with *G. pallidipes* to *G. morsitans* and *G. swynnertoni*. However, in the *palpalis* group, only *G. fuscipes* and *G. palpalis* were sampled, so a reliable phylogeny for the *palpalis* group is still lacking.

In addition to the uncertainties surrounding the validity of the subgeneric groupings within the *Glossina* genus there are a number of taxa of uncertain taxonomic status at the level of species/subspecies. Within the *palpalis* group there are five taxa originally accorded subspecific status by Machado (1954) and which have not been revised since this time. Even within these subspecies there is evidence for possible cryptic species, for example the hybrid sterility and differences in head morphology of *G .p. palpalis* in colonies originating in Bas Zaire (present day Democratic Republic of Congo) and Nigeria (Gooding et al., 2004).

Similarly within the *morsitans* subgroup there are three subspecific forms within the nominal taxon (Machado, 1970), although recently Krafsur and Endsley (2006) used microsatellite data to argue of the elevation of the three subspecies of *G. morsitans* (*G. morsitans morsitans*, *G. morsitans submorsitans* and *G. morsitans centralis*) to specific status.

In addition to the taxonomic importance of resolving the status and inter-relationships of the species within the genus *Glossina* there is also a compelling public-health rationale. The flies within the *morsitans* and *palpalis* groups are the major vectors of nagana or Animal African Trypanosomiasis (AAT) and Human African Trypanosomiasis (HAT) respectively. AAT renders much of sub-Saharan Africa unsuitable for livestock production resulting in restricted agricultural development which has a profound effect on the economy of much of the continent with estimated annual losses of 4.5 Billion US$. The World Health Organisation (WHO) conservatively estimates that 60 million people are at risk in 37 countries covering ~40% of Africa (11 M km^2). In 2004 the WHO reported 17,000 new HAT cases (WHO, 2006). After a devastating epidemic in the early 20th century when a million people died of HAT, the disease had almost disappeared by the 1960s. But another HAT epidemic occurred through the 1990s with a disease burden of 2.05 million disability adjusted life years. At present the limited amount of tsetse control conducted is reliant upon wide scale insecticide use involving cattle pour-ons, aerial spraying or targets (Allsopp, 2001). Sterile insect release programmes are also proposed for the later stages of control campaigns (Vreysen et al., 2000). These anti-vector measures are reliant upon accurate identification of vector species (Gooding and Krafsur, 2005).

In the present study, molecular phylogenies based on mitochondrial and nuclear gene sequences are used to investigate systematic and evolutionary relationship between the three tsetse groups, and particularly between species within the subgenera *morsitans* and *palpalis*. Sequence data from both mitochondrial (*cytochrome oxidase 1* (*COI*) *16S* ribosomal (*16S*), *NADH dehydrogenase subunit 2* (*ND2*)) and nuclear *internal transcribed spacer 1 of ribosomal DNA* (*ITS1*) loci are presented.

The aims of the study were to resolve interspecific relationships within the *palpalis* group of *Glossina*, to determine whether mitochondrial and additional nuclear markers support the elevation of the three subspecies of *G. morsitans* to specific status, to attempt to resolve the status of the putative subspecies within the *palpalis* group and to generate a diagnostic PCR to distinguish morphologically similar subspecies of *palpalis*.

2. Materials and methods

2.1. Taxon sampling

Thirteen of the 31 species/subspecies within the *Glossina* genus were obtained from a variety of sources, including both wild caught flies and a number of colonies that originated from material collected in sub-Saharan Africa (Table 1). Collection efforts focussed upon the *morsitans* and *palpalis* group given their public-health importance.

2.2. Molecular laboratory methods

The Ballinger-Crabtree method was employed to extract DNA from the flies (Ballinger-Crabtree et al., 1992). Only three legs from each individual were used for analysis in order to maintain the specimen for morphological classification and to ensure that no organisms from the gut or mouthparts would contaminate the fly sequences. DNA concentration was measured using the Quant-iT PicoGreen dsDNA reagent (Invitrogen) using the manufacturer's