Short communication

A molecular phylogeny of *Oxya* (Orthoptera: Acridoidea) in China inferred from partial cytochrome *b* gene sequences

Zhumei Ren, Enbo Ma*, Yaping Guo, Yang Zhong

* College of Life Science and Technology, Shanxi University, Taiyuan 030006, China

b Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, School of Life Sciences, Fudan University, Shanghai 200433, China

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Abstract

The grasshoppers of the genus *Oxya* are well known to damage rice, sugar cane, and other crops, yet their phylogenetic relationships have not been examined with molecular data. In this study, we obtained the 432bp DNA sequences of the mitochondrial cytochrome *b* gene from 91 individuals of nine *Oxya* species and two outgroups (*Gesonula punctifrons* and *Acrida cinerea*). Phylogenetic analyses for the molecular data set were then carried out using the maximum parsimony and neighbor-joining methods. The results showed that the nine *Oxya* species form four well-supported clades, which include (1) *O. intricata* and *O. flavefemura*; (2) *O. japonica* and *O. bicingula*; (3) *O. agavisa*; and (4) *O. chinensis*, *O. brachyptera*, *O. adentata*, and *O. hainanensis*, respectively. In particular, the monophyly of *O. hainanensis* and *O. agavisa* is strongly supported, respectively. However, *O. flavefemura* and *O. intricata*, *O. bicingula*, and *O. japonica* form paraphyletic groups, respectively, and *O. chinensis*, *O. adentata*, and *O. brachyptera* form a polyphyletic group, suggesting that they should be merged as few as three species.

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1. Introduction

The grasshoppers of the genus *Oxya* (Orthoptera: Acridoidea) are distributed across Pakistan, China, southeastern Russia, and Australia. Some species were also introduced to Hawaii in the 19th century (Hollis, 1971). This genus was established by J.G. Audinet-Serville in 1831 and revised by other authors for several times (Hollis, 1971, 1975; Uvarov, 1931; Willemese, 1925). In particular, D. Hollis published his revisions of *Oxya* in 1971 and 1975. He argued that the genus *Oxya* was an unnatural grouping of species; it could be retained as a taxonomic unit for purely practical positions but it was very difficult to determine its phylogenetic position. In Hollis's classification, 18 species were recognized based on eight morphological characters of the phallic complex, including eight species in China: *O. chinensis*, *O. agavisa*, *O. intricata*, *O. japonica*, *O. ningpoensis*, *O. tinkhami*, *O. adentata*, and *O. velox*. In recent decades, several new species of *Oxya* in China have been described, e.g., *O. hainanensis*, *O. yunnana*, and *O. anagavisa* (Bi, 1986); *O. brachyptera* and *O. flavefemura* (Zheng, 1993; Zheng and Huo, 1992); and *O. termac Ningpoensis* and *O. bicingula* (Ma, 1995; Ma et al., 1993). The taxonomic positions of these *Oxya* species were determined using morphological and cytological characters (Ma et al., 1994; Xu et al., 1997). However, the phylogenetic relationships among Chinese *Oxya* species are still poorly understood.

Mitochondrial DNA (mtDNA) has been widely used for elucidating the phylogenetic relationships of insects...
at both species and infraspecific levels (Aikhionbare and Mayo, 2000; Flook and Rowell, 1997; Huang et al., 2000), following amplification using the polymerase chain reaction (PCR). The insect mtDNA contains 13 protein coding genes, two ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes (Simon et al., 1994), in which the cytochrome b gene is one of the large protein-coding genes in the invertebrate mitochondrial genome. Due to its rapid evolutionary rate, the cytochrome b gene is suitable for the comparative studies of species within the same genus or the same family (Simmons and Weller, 2001). Currently, we sequenced the cytochrome b gene from the mtDNA of some Oxya species, such as O. agavisa and O. intricata, and then analyzed the sequence variations between and within these species (Ren et al., 2002a). In this study, we further investigate the phylogenetic relationships among nine Oxya species in China (88 individuals from 33 populations) based on their partial sequences of the cytochrome b gene.

2. Materials and methods

Dried samples representing nine Oxya species were collected from natural populations in China during 1989–1998, and some samples stored in absolute ethanol were also used in this study as experimental materials (Table 1). Gesomula punctifrons, which belongs to the same family and different genus to Oxya and they have close relationship (Zheng, 1993), and Acrida cinerea were selected as outgroup taxa based on current understanding of the phylogenetic relationships among the superfamilial Acridoidea (Ren et al., 2002b).

Total genomic DNA was extracted from a single leg of each individual of the Oxya species by the phenol–chloroform method. Mitochondrial DNA sequences from part of the cytochrome b gene were obtained by direct sequencing of PCR-amplified DNA (Ren et al., 2002a). Primer sequences were CB1 (5’-TAT GTA CTA CCA TGA GGA CAA ATA TC-3’) and CB2 (5’-ATT ACA CCT CCT AAT TTA TTA GGA AT-3’) (Simon et al., 1994).

DNA sequences of the cytochrome b gene were edited and aligned using DNASTAR package (SeqMen, EditSeq, and MegaLign) and checked manually. Phylogenetic analyses were performed using PAUP 4.0b4a (Swofford, 2000). Maximum parsimony (MP) and neighbor-joining (NJ) methods were used to construct the phylogenetic trees: most parsimonious trees (MPTs) were generated using heuristic search routines with 100 random-addition sequences and TBR branch swapping. The Kimura two-parameter model was used for correcting possible multiple hits of nucleotide substitutions (Kimura, 1980). Bootstrap analyses were carried out with 1000 replicates. All phylogenetic trees were rooted using G. punctifrons and A. cinerea as outgroups.

3. Results and discussion

Except for the cytochrome b sequences of four species, i.e., O. intricata, O. japonica, O. chinensis, and O. agavisa, that have been used in our pervious study (Ren et al., 2002a), a total of 52 individuals, including added individuals of the 4 Oxya species as well as the two outgroup species were sequenced in this study (Table 1). The 432bp fragment of the cytochrome b gene was obtained. Due to the fact that: (1) nuclear copies of mitochondrial sequences exist in a variety of organisms, including insects and other invertebrates, vertebrates, fungi, and plants (Zhang and Godfrey, 1996); and (2) that hundreds of mitochondrial pseudogenes appear to reside in the grasshopper nuclear genome (Bensasson et al., 2000), we considered the cytochrome b gene sequences reported to be solely mitochondrial in origin. In all cases, PCR amplification always produces one band of approximately 500bp (including primers). An alignment of the cytochrome b sequences obtained from the Oxya species and Locusta migratoria from GenBank (Accession No. NC001712), which belongs to the same superfamily, i.e., Acridoidea,
as *Oxya*, showed no nucleotide indels (insertion/deletion events) in the sequences. The sequences can also be fully translated using the *Drosophila* corresponding mitochondrial code without an intervening stop codon.

All of the sequences analyzed generate 41 different haplotypes, and the three individuals of the two outgroup species have different haplotypes (Table 1). Base compositional information for the sequences was also estimated from the aligned sequences. The 432bp sequences from the cytochrome *b* gene contain 99 variable sites (22.9%), which were not evenly distributed among the three codon positions, with 76 substitutions (76.8%) detected at the third codon position, 19 (19.2%) at the first codon position, and four (4.0%) at the second codon position. The distribution of variable sites reflects the majority of substitutions occurring at synonymous sites (codon third position and leucine codon first position). Eighty-one of the polymorphic sites are informative, among which 65 (80.2%) are in the third nucleotide site, 14 (17.3%) in the first site and two (2.5%) in the second site. As is typical for a protein-coding gene, variability is least in the second position, intermediate in the first position, and greatest in the third position (Simon, 1991). The nucleotide composition of this region was calculated from the different haplotypes; as a result, it varied slightly depending on different haplotypes in the same species and greatly on different species, but was commonly found to be 31.9% A; 39.1% T;

![Fig. 1. Phylogenetic trees of 9 Chinese *Oxya* species generated by means of the maximum parsimony (MP) (A) and the neighbor-joining (NJ) (B) methods. The numbers at each node represent bootstrap proportions based on 1000 replications. Aberrations of the samples corresponding to the haplotypes are shown in Table 1. *Gesonula punctifrons* and *Acrida cinerea* were used as outgroup species.](image)
16.6% C; and 12.4% G. Overall, the base composition shows extreme bias being 71.0% AT. The AT bias is strongest one at the third codon position (89.9%), and least at the first position (59.1%). It was reported that a high A+T bias may be a common phenomenon in insects and that an A+T nucleotide bias, when present, tends to accumulate in hypervariable sites (Simon, 1991).

The phylogenetic trees generated using the MP and NJ methods are shown in Figs. 1A and B, respectively. In both MP and NJ trees, the 9 *Oxya* species can be clearly classified into four clades as follows.

Clade I contains two *Oxya* species: *O. intricata* and *O. flavefemura*, in which *O. flavefemura* resembles *O. intricata* and was named as a new species mainly according to its color of hind femora (yellow), which is evidently different from other *Oxya* species (Zheng, 1993). In this study, however, *O. flavefemura* and *O. intricata* are shown to be paraphyletic.

Clade II contains two species, i.e., *O. bicingula* and *O. japonica*, among which *O. bicingula* was named as a new species according to the chromosome C-banding characters (Ma, 1995). However, recent morphological and cytological analyses indicated that this species should be merged into *O. japonica* (Ma, unpublished). This is supported by molecular data used in this study.

Clade III consists of only one species *O. agavisa*, whose distribution is restricted to areas of southern China. The monophyly of this species is well verified.

Clade IV contains four *Oxya* species, among which *O. hainanensis* is distinct from the other three species and this species should be recognized as a well-defined species. However, the other three species: *O. chinensis*, *O. brachyptera*, and *O. adentata* form a polyphyletic
group, thus they may be merged into one species. Hollis (1971) considered that O. adentata merely represent local and extreme variations of O. chinensis therefore it should be considered as the same species of O. chinensis. Ma and Zheng (1989) also found that O. adentata and O. chinensis have very similar patterns in the main marked chromosomes and few differences in the detailed C-banding structure. Our study supports the viewpoint of merging O. adentata and O. chinensis. In addition, O. brachyptera resembles O. adentata, but their major differences were in the following characters: shorter elytra, not reaching or reaching the knee of hind femora (Zheng and Huo, 1992). Based on the molecular data used in this study, O. brachyptera might be considered as an allopatric population of O. chinensis with sufficient identity.

In general, O. intricata, O. chinensis, O. agavisa, and O. japonica are distributed more widely in China than the other Oxya species, among which the latter three species are large in size and morphologically similar (Hollis, 1971; Zheng, 1993). Hollis (1971, 1975) argued that O. agavisa appears intermediate between O. chinensis and O. japonica, and that O. intricata is very widely distributed, suggesting that the hyla–intricata complex represents a superspecies, a sibling species-group, or a reticulum. In karyotypic and C-banding analyses, O. japonica and O. intricata were evidently different from O. chinensis (Ma and Zheng, 1989). According to the C-banding characters of the L2 chromosome, it is inferred on a preliminary basis that O. chinensis is relatively ancestral, and that O. japonica and O. agavisa may be evolved from O. chinensis (Ma et al., 2000). In addition, when combined with morphological and ecological characteristics, it is inferred that O. intricata is distinct from the other three species, i.e., O. chinensis, O. japonica, and O. agavisa (Ma et al., 1994). Xu et al. (1997) considered that O. chinensis is the most primitive species and that O. agavisa is the most evolved species according to their karyotypic characters. Our study does not completely support the results of previous studies.

Except for four major species i.e., O. chinensis, O. intricata, O. japonica, and O. agavisa, the other Oxya species are narrowly distributed and their damages to crop plants are relatively light. Although the analyses presented here are based on limited species and molecular markers, this study examines the preliminary relationships of nine Oxya species in China at the molecular level for the first time. Further examination of more Oxya species with additional molecular markers is needed for a more robust assessment of Oxya phylogeny.

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References


