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In the shadow of phylogenetic uncertainty: The recent diversification of *Lysandra* butterflies through chromosomal change

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ABSTRACT

The phylogeny of the butterfly genus *Lysandra* (Lycaenidae, Polyommatinae) has been intractable using both molecular and morphological characters, which could be a result of speciation due to karyotype instability. Here we reconstruct the phylogeny of the group using multi-locus coalescent-based methods on seven independent genetic markers. While the genus is ca. 4.9 Mya old, the diversification of the extant lineages was extremely recent (ca. 1.5 Mya) and involved multiple chromosomal rearrangements. We find that relationships are uncertain due to both incomplete lineage sorting and hybridization. Minimizing the impact of reticulation in inferring the species tree by testing for mitochondrial introgression events yields a partially resolved tree with three main supported clades: *L. punctifera* + *L. bellargus*, the *corydonius* taxa, and *L. coridon* + the Iberian taxa, plus three independent lineages without apparently close relatives (*L. ossmar*, *L. syriaca* and *L. dezina*). Based on these results and new karyotypic data, we propose a rearrangement recognizing ten species within the genus. Finally, we hypothesize that chromosomal instability may have played a crucial role in the *Lysandra* recent diversification. New chromosome rearrangements might be fixed in populations after severe bottlenecks, which at the same time might promote rapid sorting of neutral molecular markers. We argue that population bottlenecks might be a prerequisite for chromosomal speciation in this group.

1. Introduction

The main karyotypic features of organisms, particularly the number of chromosomes, tend to be stable within species (White, 1973; King, 1993). New chromosomal rearrangements usually originate as heterozygotes and are often – although not always – associated with heterozygote disadvantage. The spread of such rearrangements to fixation within a large population has low probability (King, 1993). Therefore, many organisms are characterized by chromosomal conservatism, a situation in which all closely related taxa demonstrate the same chromosome number. Lepidoptera (butterflies and moths) are a case in point: the modal haploid number of chromosomes (n) of $n = 31$ or $n = 30$ is preserved in the majority of lepidopteran families (Robinson, 1971; Stekolnikov et al., 2000). Within the butterfly family Lycaenidae (blues, coppers and hairstreaks), most species also have a conserved haploid chromosome number of either 23 or 24 (de Lesse, 1960; Lorković, 1990).

In contrast to chromosomal conservatism, chromosomal instability characterizes situations where multiple closely related taxa (populations, subspecies and/or species) belonging to a single phylogenetic lineage differ drastically from each other by major chromosomal rearrangements, sometimes resulting in high variability in chromosome number. Within the blue butterflies at least three clades of the subtribe Polyommatina (*Agrodiaetus*, *Plebicula* and *Lysandra*) represent intriguing exceptions to the general pattern of chromosomal conservatism, demonstrating a great range of derived chromosome numbers (Kandul et al., 2004).

Like the related *Agrodiaetus* and *Plebicula*, the genus *Lysandra* displays striking interspecific chromosome number variability, from $n = 24$ to $n = 93$ (de Lesse, 1969; Coutsis et al., 2001). *Lysandra* is exclusively Palaearctic, with two main centers of biodiversity in the Iberian Peninsula and the Middle East. The genus is sometimes cited as an example of difficult taxonomic resolution, and the exact number of species remains unknown due to poor morphological differentiation (De Bast 1985; Mensi et al., 1988; Schurian, 1989; Lelièvre, 1992; Wiemers, 2003; Descimon and Mallet, 2009). For example, the specific status of the taxa *caelestissima*, *gennargenti*, and *nufrellensis* within the *coridon* group, and the taxa *arzanovi*,

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sheikh and *melamarina* within the *corydonius* group is unclear. Current classifications rely primarily on chromosome number, number of annual generations and male wing color (Schurian, 1989) rather than formal phylogenetic investigation. Unlike *Agrodiaetus* and *Plebicula*, the karyotypes of some taxa within the genus have a chromosome number close to double that of other taxa, which has led some authors to hypothesize the occurrence of sequential polyploidy events in the group (Lorković 1941, 1949; Robinson, 1971). The species *Lysandra coridon* is also notable in having populations that exhibit intraspecific variability in chromosome number in a cline across Europe, with numbers apparently fixed in each population (de Lesse, 1969). Thus, *Lysandra* combines an array of characteristics (wide differences in chromosome number, potential for polyploidy, or alternatively for fusion/fission rearrangements, intra- and interspecific karyotype variability, and apparently recent speciation events) that render it an excellent model to study the role of chromosomal change on diversification.

Changes in ploidy as well as chromosome rearrangements such as fusion and fission events can result in reproductive isolation and promote speciation (King, 1993). These have been traditionally thought to cause meiotic problems in chromosomal heterozygotes that would translate into lower fitness (White, 1973). In this way, these phenomena could directly contribute to speciation, as well as prevent gene flow between existing species that might have originated by non-chromosomal mechanisms and differentiated secondarily in this respect. Such a process could contribute significantly to the generation of biodiversity evolution by preventing nascent species from fusing. Although this meiotic-suppression mechanism has been documented for only a few cases (Baker and Bickham, 1986), increasing recent evidence has supported the so-called recombination-suppression mechanism of chromosomal speciation (Faria and Navarro, 2010). According to this idea, chromosome rearrangements can contribute to speciation through suppression of recombination.

The blue butterflies, like other Lepidoptera and some other insects, have holocentric chromosomes in which the centromere is not localized and centromeric activity is distributed along the length of the chromosome (Robinson, 1971; Wolf, 1996; Lukhtanov and Dantchenko, 2002; Lukhtanov and Kuznetsova, 2010). The bearers of holocentric chromosomes seem to have some evolutionary advantages when chromosomal fusions and fissions occur: the fused or fragmented chromosomes preserve normal kinetic activity during cell divisions and, therefore have a higher chance of being fixed. However, as in monocentric chromosomes, rearrangements of holocentric chromosomes can lead to meiotic problems and/or suppress recombination when they are in the heterozygous condition (Lukhtanov et al. 2011). To distinguish between polyploidy and fusion/fission events, to estimate the frequency of chromosome changes and to reveal the direction of chromosomal evolution and its relationship to species limits, the simultaneous study of karyotype structure and molecular markers to produce a solid phylogenetic framework are necessary.

Understanding the recent speciation history in *Lysandra* requires merging phylogenetic and population genetic approaches, taking into account both the persistence of ancestral polymorphisms and possible traces of hybridization events. Non-tree-like evolution is strongly related to the coalescent process, where gene discordance is common among closely related species. Hybridization between *Lysandra* species seems to be common in nature: potential hybrid specimens have been reported between *L. bellargus* and *L. coridon*, between *L. coridon* and other Iberian taxa, and between *L. corydonius* and *L. ossmar* (Schurian, 1989; Lelièvre, 1992; Hesselbarth et al., 1995; Gil-T, 2007; Descimon and Mallet, 2009). Establishing a link between gene genealogy and population or species divergence history requires the incorporation of the coalescence process, as well as the possibility of secondary exchanges after population splits. Distinguishing between these two major causes of conflicting signal across loci is of major importance, but notoriously difficult. Several methods to identify introgression events in a phylogenetic framework have been developed. While most of these methods either do not simultaneously account for the potential existence of incomplete lineage sorting (e.g. Bryant and Moulton, 2004; Jin et al., 2006; Gauthier and Lapointe, 2007), or do not distinguish the nature of the discordance (Ané et al., 2007), a few incorporate the coalescence of lineages while attempting to assess the possibility of gene introgression (Buckley et al., 2006; Joly et al., 2009; Kubatko, 2009). Although any genomic regions may be affected by introgression, most reports of reticulate evolution induced by introgression in animals involve mitochondrial DNA (mtDNA) (e.g. Ferris et al., 1983; Ruedi et al., 1997; Roca et al., 2005; Berthier et al., 2006; Melo-Ferreira et al., 2012), resulting in strong conflicting phylogenetic signals between nuclear and mtDNA markers (e.g. Buckley et al., 2006; Bossu and Near, 2009; Spinks and Shaffer, 2009).

Here we use multi-locus coalescent-based methods to reconstruct the *Lysandra* species tree based on data from seven genetic markers. We infer divergence times and demographic history. We observe low resolution in the selected markers, and generally discordant genealogies. Our results show that mitochondrial introgression within *Lysandra* is common and can lead to incorrect phylogenetic and taxonomic conclusions if not taken into account. By considering both introgression and incomplete lineage sorting, we obtain a partially resolved tree with three main supported clades. We also provide new knowledge on karyotypes for several taxa and discuss the role of chromosomal evolution in the *Lysandra* species radiation.

2. Material and methods

2.1. Taxon sampling

We used 48 representatives of the *Lysandra* species-group covering its entire distribution and including several specimens for each described species except the rare taxa *L. dezina* and *L. syriaca*, for which we were unable to obtain more than a single specimen each. The samples are stored in the DNA and Tissues Collection of the Museum of Comparative Zoology (Harvard University, Cambridge, MA, USA) and in the Butterfly Diversity and Evolution Lab (Institut de Biologia Evolutiva, Barcelona, Spain). Three outgroup taxa (*Polyommatus amandus*, *Polyommatus myrrha* and *Neolysandra diana*) were used for phylogenetic analyses, selected according to the general Polyommatina phylogeny of Talavera et al. (2013). All specimens used in this study are listed in the Supplementary Table S1.

2.2. Molecular data

Genomic DNA was extracted from a leg or from a piece of the abdomen of each specimen using DNeasy™ Tissue Kit (Qiagen Inc., Valencia, CA, USA) and following the manufacturer's protocols. Fragments from three mitochondrial genes (here treated as a single marker) – *cytochrome oxidase I (COI) + leu-tRNA + cytochrome oxidase II (COII)*; and from six nuclear markers – *28S ribosome unit (28S)*, *histone H3 (H3)*, *wingless (Wg)*, *carbamoyl-phosphate synthetase2/aspartate transcarbamylase/dihydroorotase (CAD)*, *internal transcribed spacer 2 (ITS2)* and *ribosomal protein L5 (Rpl5)* were amplified by polymerase chain reaction and sequenced as described in Vila et al. (2011). The primers employed are shown in Supplementary Table S2. The sequences obtained were submitted to GenBank (accession numbers in Supplementary Table S3).