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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).

2.3. Karyotype analyses

Males were netted in the field, and testes fixed either immediately or as soon as possible. Testes were preserved in Carnoy fixative (ethanol and glacial acetic acid, 3:1) for 2–6 months at 4 °C and then stained with 2% acetic orcein for 30 days at 20 °C. Cytogenetic analysis was conducted as previously described (Lukhtanov et al., 2005, 2006, 2008; Vershinina and Lukhtanov, 2010). In this study, we have counted the haploid chromosome numbers (n) in metaphase II of male meiosis and the number of bivalents in metaphase I of male meiosis. In total, preparations from 9 specimens and 5 taxa were analyzed (Table 2).

2.4. Phylogenetic and species tree inference

A molecular matrix was generated for each independent marker by editing and aligning using Geneious 4.8.3 (Biomatters Ltd., 2009). Phylogenetic resolution was evaluated by performing single-gene, mitochondrial and nuclear phylogenies (Fig. 1, Supplementary Figs. S1–S6) using the maximum likelihood criterion with the software Phyml 3.0 (Guindon et al., 2010). jModeltest ver. 0.118 (Posada, 2008) was executed to select the best-fitting DNA substitution models for each marker dataset according to the Akaike information criterion (AIC). As a result, the GTR + I + G model was used for *COI + tRNA-leu + COII*, GTR + G for *ITS2*, GTR for *28S*, HKY + I for *H3* and *Rpl5* and TN + I for *Wg* and *CAD*.

A Bayesian coalescent-based multilocus species tree approach was used to infer phylogenetic relationships among species. *BEAST 1.7.2 (Heled and Drummond, 2010) with a strict clock and a linear piecewise demographic model was set for a Markov chain Monte Carlo of 100 million generations sampled every 1000 iterations. Two independent runs were performed and convergence was checked using Tracer v1.5. Since fossil taxa are not available for age calibration in this group, we employed a molecular clock approach in dating the phylogeny. A substitution rate of 1.5% uncorrected pairwise distance per million years estimated using a variety of invertebrates (Quek et al., 2004) for *COI* was applied to the mitochondrial partition. Specimens were attributed to 13 described species (*L. albicans*, *L. caelestissima*, *L. hispana*, *L. coridon*, *L. bellargus*, *L. punctifera*, *L. melamarina*, *L. arzanovi*, *L. corydonius*, *L. sheikh*, *L. ossmar*, *L. dezina* and *L. syriaca*), and combinations of several taxa were also explored due to uncertainty about their taxonomic status within the group. The taxa *gennargenti*, *nufrellensis* and *philippi* were defined as *L. coridon* specimens according to their position in the ML phylogenies. Population sizes were extracted from *BEAST species trees inference using the Python package Biopy (http://code.google.com/p/biopy/). Since a piecewise linear model was used in *BEAST, posterior population sizes were variable along branches, resulting in values for the beginning and the end of each lineage. Net diversification rates were estimated for *Lysandra*, *Agrodiaetus* and the entire Polyommatina using Magallon and Sanderson's (2000) method with the function "bd.ms" in the R *geiger* package. Divergence times and species numbers were extracted from Talavera et al. (2013), except for *Lysandra*, which were based on estimates derived from this work.

2.5. Testing for hybridization events

Although *BEAST incorporates the uncertainty of the coalescent process in the phylogeny estimate, it assumes that no gene flow occurred after the initial split. To quantify the potential impact of horizontal gene flow, causes of discordance were investigated using coalescent approaches to test hybridization as an alternative explanation to incomplete lineage sorting. The method of Joly et al. (2009) was used as implemented in the software JML (Joly, 2012). The program calculates the minimum distance between sequences of two species and tests whether it is smaller than expected under a simulated scenario that includes incomplete lineage sorting but does not account for hybridization. Therefore, JML was used to perform 10,000 simulations for each gene tree by using the Seq-Gen code (Rambaut and Grassly, 1997). JML was run independently for each marker and parameters were extracted from the Phyml output for gene genealogies, including nucleotide frequencies, proportion of invariant sites and the gamma shape parameter when selected. The relative mutation rate mean of the species tree posterior distribution was used for each locus. A cutoff of 0.05 was applied and *BEAST inference repeated after removing sequences of detected cases of hybridization from the dataset to obtain the most accurate species tree possible. To detect residual hybridization, a cutoff of 0.15 was also explored.

3. Results

3.1. Phylogenetic analyses

The resolution of the phylogenies inferred by standard phylogenetic methods was poor, suggesting non-treelike evolutionary relationships among *Lysandra* species (Fig. 1, Supplementary Figs. S1–S6). Parsimony informative sites for each gene are shown in Supplementary Table S4. Maximum likelihood estimates for the individual nuclear genes generally show no tree structure, most likely because of incompletely sorted alleles. Gene phylogenies also showed generalized discordances among them, suggesting that the species share sequences to a high degree. Given such discordances among nuclear loci, phylogenetic inference of the species tree based on their concatenation would be prone to errors (Edwards, 2009). Thus we used the multi-species/multi-locus coalescent method implemented in *BEAST (Heled and Drummond, 2010) to estimate species trees from the distribution of single gene trees, co-estimating divergence times and the effective population sizes of tip and ancestral taxa. Initial results reported high levels of uncertainty as shown in Fig. 2A. Only two clades recovered strong statistical support, corresponding to the Iberian and the Middle East species-groups. Basal relationships were especially blurred, and no root could be recognized.

When testing for hybridization, 11 distances between mitochondrial alleles were smaller than the 5th quantile for the posterior predictive distributions of JML (Table 1) and 16 more if a permissive P-value <0.15 was considered. No case of introgression involving nuclear genes was detected, excluding those between specimens that lacked specific sequences or contained a considerable amount of missing data, which may produce artefactual predictions in JML. The *L. bellargus* specimen JC96Q001 was found to be the most conflicting because its mitochondrial sequence was unexpectedly similar to that of *L. coridon* and, with a smaller P-value, to that of other species. To a lesser extent, *L. ossmar* (RV07F170) also reflected small genetic distances from apparently distant taxa (*L. coridon* and *L. bellargus*). Although such distances were not statistically significant at the 5% level (i.e. incomplete lineage sorting cannot be significantly rejected), they were recurrent and small enough to cautiously consider this specimen as conflicted. Indeed, this specimen, similar to the *L. bellargus* specimen JC96Q001, did not cluster with the rest of its conspecifics in the mitochondrial tree (Fig. 1). Hence both mitochondrial sequences were removed from the final dataset.

In addition to the two specimens suggested by JML, we also excluded the mitochondrial sequence of *L. corydonius* specimen VL01L120. This sequence is almost identical to those of typical *L. ossmar* specimens, and clusters with two *L. ossmar* specimens with high support in the mitochondrial tree (Fig. 1). However, the nuclear sequences of this specimen are identical to those of other

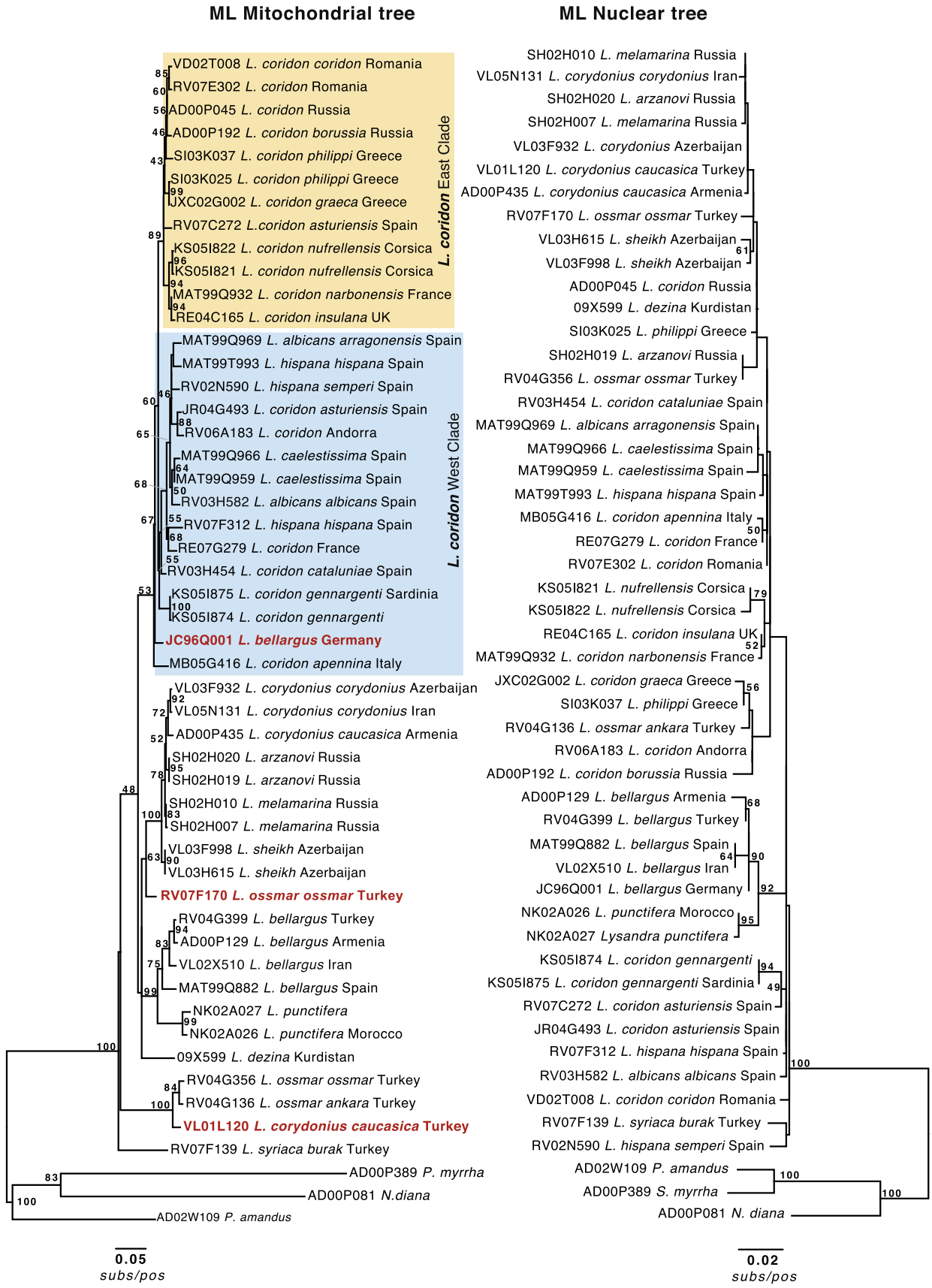

Fig. 1. Maximum likelihood phylogenetic trees inferred from mitochondrial and nuclear data independently. Mitochondrial sequences from specimens highlighted in red were removed to avoid introgression noise in species tree inference. Boostrap support greater than 40% is shown at nodes. Scale bars represent substitutions/position.

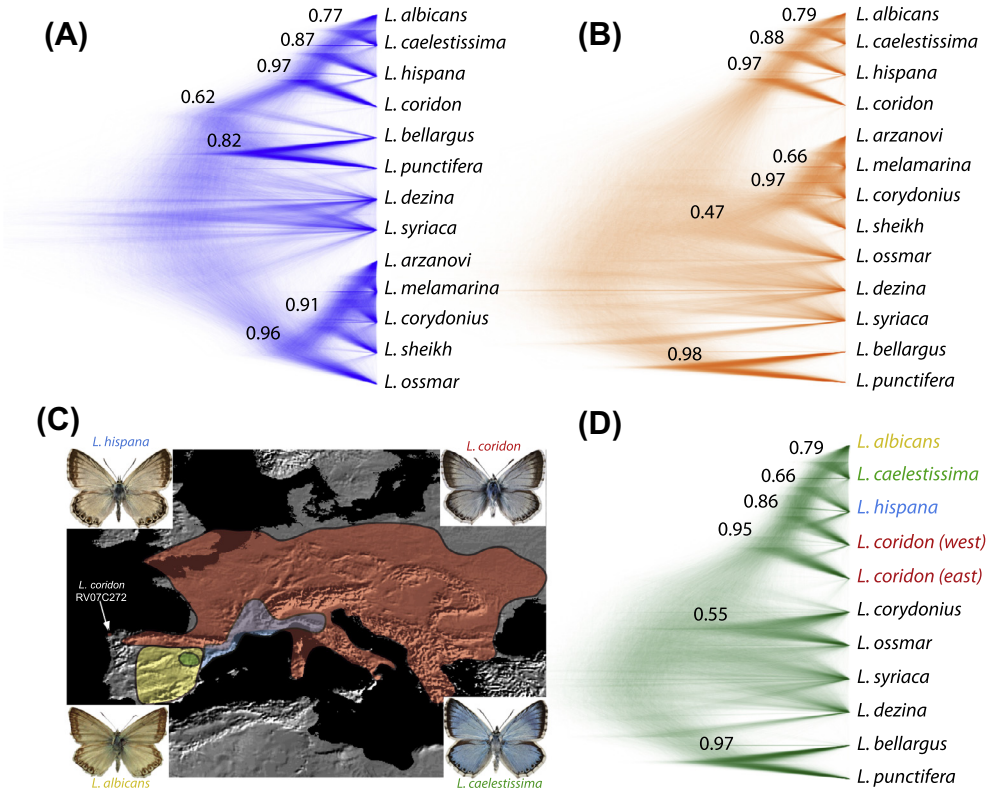

Fig. 2. *BEAST species trees for *Lysandra* based on seven independent genetic markers, (A) without accounting for hybridization, (B) after removing apparently introgressed mitochondrial sequences, (C) map of distributions for the Iberian taxa of the *coridon* group, where *L. coridon* specimen RV07C272 is shown in the periphery of the distribution of all other Iberian *Lysandra* taxa and (D) species tree after removing apparently introgressed mitochondrial sequences, considering two *L. coridon* groups, and considering the taxa of the *corydonius* group as conspecific. Trees are figured with DensiTree (Bouckaert, 2010), displaying a subsample of the Markov chain Monte Carlo of 10,000 trees. Higher levels of certainty are represented by higher densities, and posterior probabilities for nodes >0.50 are indicated.

representatives of *L. corydonius*. *Lysandra ossmar* and *L. corydonius* are parapatric in Eastern Turkey and are known to hybridize locally (Hesselbarth et al., 1995). We carefully examined wing morphology and did not detect any traces of hybridization. *Lysandra ossmar* and *L. corydonius* are clearly different in male wing colors: *L. ossmar* has a whitish-violet wing upperside whereas in *L. corydonius* the wing upperside is blue (Hesselbarth et al. 1995). The specimen VL01L120 of *L. corydonius* has this blue color, which is indistinguishable from those found in other specimens of *L. corydonius*. Thus, no intermediate color with *L. ossmar* was found in this specimen as expected from hybrids, and it is highly suggestive of old genetic introgression events, although JML could not significantly discard the possibility of incomplete lineage sorting. Indeed, JML often has difficulty detecting hybridization when dealing with recently diverged species (Joly, 2012). Similarly, introgression may have occurred within the *coridon*-clade but might be too recent for JML to pinpoint unambiguously.

Species trees retained high levels of uncertainty after removing potentially introgressed mitochondrial sequences as shown in Fig. 2B, but relevant differences were observed mainly involving two phylogenetic clusters. First, the posterior probability supporting the grouping of *L. bellargus* + *L. punctifera* increased considerably (from 0.82 to 0.98), and second, the posterior probability supporting the sister taxa relationship between *Lysandra ossmar* and the *corydonius* clade dramatically decreased from 0.96 to 0.47, with *L. ossmar* now recovered in an unresolved phylogenetic position. Three well-supported clades remained: (1) the *coridon* clade, (2) the *corydonius* clade and (3) *L. punctifera* + *L. bellargus* (Fig. 2B).

3.2. Karyotype analyses

The karyotypes of western European and North African species of *Lysandra*, as well as two Asian taxa (*L. corydonius caucasica* and *L. syriaca syriaca*), were previously studied by de Lesse (1960). However, karyotypes of the *Lysandra* taxa from South-East Turkey, Azerbaijan and South of Russia have only been examined in one study (Stradomsky and Shchurov, 2005) (but see our comments on this publication below). We were able to investigate the karyotypes of the following taxa: *L. syriaca burak*, *L. melamarina*, *L. sheikh* and *L. corydonius corydonius*. We also analyzed the karyotype of *L. bellargus* from the easternmost parts of its distribution range (Table 2 and Fig. 3).

For the rare taxon *L. syriaca burak*, we were able to collect and analyze only a single male specimen. In the first meiotic division, 30 chromosome units were observed in most cells (Fig. 3) and apparently all these units were bivalents. Thus we estimate the haploid chromosome number (n) of *L. syrica burak* as $n = 30$. The chromosomal units vary in size gradually, without the existence of discrete size types. We conclude that this taxon differs from *L. syriaca syriaca* ($n = 24$; de Lesse, 1960) by at least 6 fixed chromosomal fissions.

For the taxa *L. melamarina*, *L. sheikh and L. corydonius corydonius*, similar karyotypes with $n = 84$ were found (Table 2), including two large bivalents always observed in thecenter of the MI metaphase plates and numerous small bivalents. This result disagrees with previously assigned counts of $n = 24$–27 for *L. melamarina* (Stradomsky and Shchurov, 2005), which may be due to chromosome counts in atypical cell divisions that were not suitable for

Table 1
JML results. Distances with *P*-values <0.05 and $p < 0.15$ according to the posterior predictive distributions for mitochondrial sequences are listed. Distances including sequences with large amounts of missing data were not considered. Specimens in bold indicate those whose signal was considered in significant conflict with that of conspecifics and therefore removed from the original dataset.

Individual 1	Individual 2	Obs. distance	*P*-value
JML testing hybridization ($p < 0.05$)			
JC96Q001 *L. bellargus*	JXC02G002 *L. coridon*	0.00739713	0.0055
JC96Q001 *L. bellargus*	SI03K025 *L. coridon*	0.00739713	0.0055
JC96Q001 *L. bellargus*	KS05I874 *L. coridon*	0.00785945	0.0076
JC96Q001 *L. bellargus*	KS05I875 *L. coridon*	0.00785945	0.0076
JC96Q001 *L. bellargus*	RV07E302 *L. coridon*	0.00832178	0.0122
JC96Q001 *L. bellargus*	KS05I821 *L. coridon*	0.0087841	0.0186
JC96Q001 *L. bellargus*	RV07C272 *L. coridon*	0.00924642	0.0268
JC96Q001 *L. bellargus*	VD02T008 *L. coridon*	0.00924642	0.0268
JC96Q001 *L. bellargus*	AD00P045 *L. coridon*	0.00924642	0.0268
JC96Q001 *L. bellargus*	RE07G279 *L. coridon*	0.00970874	0.0384
JC96Q001 *L. bellargus*	MAT99Q959 *L. caelestissima*	0.0106334	0.0496
JML testing hybridization ($p < 0.15$)			
JC96Q001 *L. bellargus*	RE04C165 *L. coridon*	0.0106334	0.073
JC96Q001 *L. bellargus*	MAT99T993 *L. hispana*	0.011558	0.1008
JC96Q001 *L. bellargus*	SH02H019 *L. arzanovi*	0.0166436	0.1158
JC96Q001 *L. bellargus*	SH02H020 *L. arzanovi*	0.0166436	0.1158
JC96Q001 *L. bellargus*	VL03H615 *L. sheikh*	0.0166436	0.117
JC96Q001 *L. bellargus*	MAT99Q969 *L. albicans*	0.0120203	0.1186
RV07F170 *L. ossmar*	RV07E302 *L. coridon*	0.0157189	0.1242
RV07F170 *L. ossmar*	VL02X510 *L. bellargus*	0.0166436	0.1298
RV07F170 *L. ossmar*	RV04G399 *L. bellargus*	0.0166436	0.1298
JC96Q001 *L. bellargus*	SH02H010 *L. melamarina*	0.0171059	0.1337
RV07F170 *L. ossmar*	RV07C272 *L. coridon*	0.0161812	0.1416
RV07F170 *L. ossmar*	JXC02G002 *L. coridon*	0.0161812	0.1416
RV07F170 *L. ossmar*	SI03K025 *L. coridon*	0.0161812	0.1416
RV06A183 *L. coridon*	MAT99Q959 *L. caelestissima*	0.00554785	0.1418
RV07F170 *L. ossmar*	RV04G399 *L. bellargus*	0.0171059	0.1466
JC96Q001 *L. bellargus*	SH02H007 *L. melamarina*	0.0175682	0.1497

karyotype analysis. For the same reason, we are also uncertain about the karyotype estimation of $n = 19–20$ made by these authors for *L. arzanovi* (Stradomsky and Shchurov, 2005), which we conservatively consider to be unknown until further evidence is obtained.

Thus, the taxa *L. corydonius corydonius* (Azerbaijan), *L. melamarina* (*Russia*) and *L. sheikh* (Russia) (Fig. 3) are chromosomally undistinguishable from *L. corydonius caucasica* (East Turkey) (de Lesse, 1960), all displaying a karyotype with $n = 84$, including two large chromosome units. Although the chromosome number is identical, *L. ossmar* ($n = 84$) from Western and Central Turkey differs from the *corydonius*-group in having three large chromosome units (de Lesse, 1960) instead of two.

In *L. bellargus* from Azerbaijan and Iran, 45 chromosomes, including one large chromosome in the center of the metaphase plate, were observed (Fig. 3). These results support the published data for the karyotype of *L. bellargus* (Lorković, 1941; de Lesse, 1960).

Samples from the westernmost populations of *L. coridon* subspecies *asturiensis* from Spain (Pino-Pérez and Pino-Pérez, 2013) were also analyzed and found to contain 87 chromosomal units, thereby confirming previously assigned counts from eastern individuals of this subspecies (de Lesse, 1969).

4. Discussion

4.1. Phylogenetic relationships

Previous attempts to establish phylogenetic relationships or a taxonomic classification of the taxa within *Lysandra*, either based on morphological data, allozymes, or DNA sequences have all highlighted the difficulties that this genus entails (De Bast, 1985; Mensi et al., 1988; Schurian, 1989; Lelièvre, 1992; Wiemers, 2003). Based on morphology (wing color) and karyotype, we propose three hypothetical species-groups within the genus: (1) *syriaca* ($n = 24$;

Table 2
Karyotype results. Data and results of chromosomally studied material.

Taxon	Specimen code	Chromosome number (n)	Country	Locality
L. bellargus	VL508	$n = 45$	Iran	Gilan Prov. Masuleh (1900–2100 m)
L. bellargus	VL510	$n = ca45$	Iran	Gilan Prov. Masuleh (1900–2100 m)
L. bellargus	F938	$n = 45$	Azerbaijan	Talysh, Zuvand, Mistan (1700–1800 m)
L. bellargus	F941	$n = 45$	Azerbaijan	Talysh, Zuvand, Mistan (1700–1800 m)
L. corydonius corydonius	F932	$n = 84$	Azerbaijan	Talysh, Zuvand, Mistan (1700–1800 m)
L. corydonius melamarina	SH-2002-08	$n = 84$	Russia	Krasnodar Region, Gelendjik, Betta Mts (150 m)
L. corydonius sheikh	F998	$n = 84$	Azerbaijan	East Caucasus, Altyagach (1300 m)
L. corydonius sheikh	F999	$n = 84$	Azerbaijan	East Caucasus, Altyagach (1300 m)
L. syriaca burak	07F139	$n = 30$	Turkey	Adana, 13 km N. of Saimbeily
L. coridon asturiensis	07C272	$n \geqslant 86$	Spain	Cedeira, Capelada, Galícia
L. coridon asturiensis	07C273	$n = ca87$	Spain	Cedeira, Capelada, Galícia
L. coridon asturiensis	07F507	$n = 87$	Spain	Cedeira, Capelada, Galícia
L. coridon asturiensis	07C271	$n = 87$	Spain	Cedeira, Capelada, Galícia

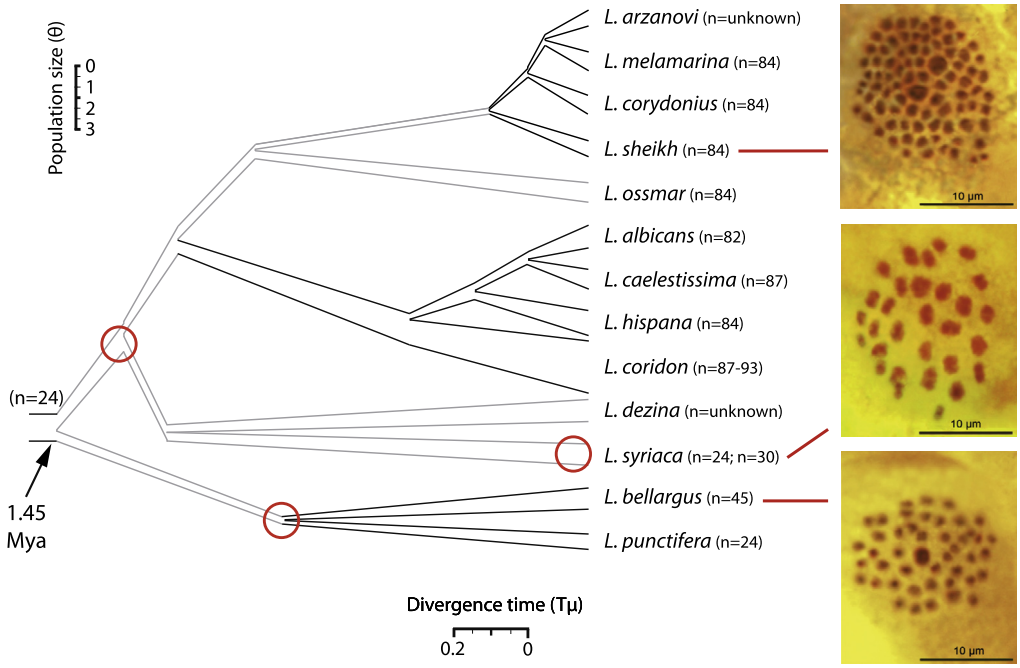

Fig. 3. Demographic history drawn on a consensus species tree. Variable population sizes along branches (piecewise linear model in *BEAST) are represented as width (in the *Y*-axis) according to the Biopy summary from *BEAST inference (after JML application). The *X*-axis represents divergence time in Mya according to a 1.5% evolutionary rate for insect mitochondrion. Red circles show the three estimated origins of chromosomal instability. Strong putative population bottlenecks along the phylogeny could promote rapid lineage sorting and fixation of chromosomal changes, leading finally to speciation. Meiotic karyotypes of *Lysandra* samples are shown in the right. The meiosis I metaphase plate of *L. corydonius sheikh* (specimen F999, Azerbaijan, Altyagach) displays 84 bivalents ($n = 84$). The meiosis I metaphase plate of *L. syriaca burak* (specimen 07F139, Turkey, Adana) displays 30 bivalents, a new chromosome number for the genus ($n = 30$). The meiosis II metaphase plate of *L. bellargus* (specimen F941, Azerbaijan, Talysh) displays 45 chromosomes ($n = 45$).

wing upperside in males with a wide black margin), (2) *bellargus* ($n = 45$, wing upperside in males with a very thin black margin), and (3)*coridon* ($n = 82–93$, wing upperside in males with a wide black margin). The attribution of the taxon *L. punctifera* to one of these groups is unclear under this hypothesis, since it is morphologically very close to *L. bellargus* and yet displays the same chromosomal number as *L. syriaca* ($n = 24$). The *coridon* group includes (a) the Iberian taxa (*albicans*, *hispana*, *calestissima*), (b) *coridon* sensu stricto and the taxa *gennargenti*, *nufrellensis* and *philippi*, (c) the *corydonius* subgroup (*corydonius*, *sheikh*, *melamarina*, *arzanovi*), (d) *ossmar*, and (e) *dezina*. These taxa might be also grouped because they share a high chromosome number ($n > 82$, although the chromosome number is still unknown in *L. dezina*). Our molecular results are unexpected in some respect and only partially support this hypothesis. We recover three well-differentiated clades after addressing artefacts created by potential cases of introgression: (1) *L. punctifera* + *L. bellargus*, (2) the Iberian taxa (*L. albicans*, *L. coelestissima*, and *L. hispana*) + *coridon* sensu stricto group (including the taxa *gennargenti*, *nufrellensis*, and *philippi*) (Fig. 2A and B) and (3) the *corydonius* group (*corydonius*, *arzanovi*, *melamarina* and *sheikh)*, plus three species with apparently no close relatives (*L. syriaca*, *L. dezina* and *L. ossmar*). These relationships are discussed in detail in the supplementary information.

We show that, at least in the case of the genus *Lysandra*, using a species tree approach that includes incomplete lineage sorting is not enough to obtain a phylogeny that faithfully reflects evolution, and that horizontal gene transfer in the form of introgression needs to be addressed to avoid incorrect taxonomic conclusions. Even by concatenating the JML test to the species tree approach, several internal relationships cannot be resolved, despite using characters from seven independent markers. *Lysandra* represents a major challenge even for the latest phylogenetic methods because it is an extremely recent radiation (ca. 1.45 [1.05,1.89] Mya), with incomplete lineage sorting for most clades, and widespread hybridization. We argue that in a deep coalescent scenario for the entire genus, the three supported clades have experienced rapid speciation events that fixed observable molecular plesiomorphic characters. However, our discovery of occasional introgression, combined with the regular difficulties of distinguishing some of the taxa morphologically, raise a question regarding the potential limits of species delimitation in this group. We suggest using the following criteria to define species in the genus *Lysandra*:

(1) Clusters of individuals that can be distinguished by morphological and/or molecular and/or chromosomal characters and can preserve their identity in sympatry or parapatry despite occasional hybridization.
(2) In the case of allopatric taxa, reciprocal monophyly, especially in combination with distinct differences in karyotype should be considered as evidence for different species (=non-conspecifity) (e.g. *L. punctifera* and *L. bellargus*: reciprocal monophyly and strong discontinuity in chromosome number ($n = 24$ and 45, respectively).
(3) Stability in nomenclature (if we see no clear evidence to change the generally accepted status of a taxon, we keep it, e.g. *L. dezina*).

Although chromosomal rearrangements restrict gene flow, whether chromosome number differences can create a complete postzygotic barrier in Lepidoptera remains an open question (Kandul et al., 2007; Vila et al., 2010; Lukhtanov et al., 2011). Mitochondrial introgression requires viable backcrosses, and this would be impossible if all F1 hybrids were completely infertile. However, in animals, most reported cases of hybridization resulting in viable offspring involve taxa with small differences in chromosome number (King, 1993), and, generally, a cumulative effect has been observed (i.e. fertility decreases proportionally with the level of

chromosomal differences) (King, 1993; but see Lyapunova et al., 2010). In this study, we report cases of introgression occurring between taxa with twice the number of chromosomes (Table 1). This scenario suggests two possibilities: either certain hybrids may retain some degree of fertility, or the hybridization occurred before the chromosomal reorganizations were fixed. The first option is most likely given evidence of current natural hybrids (King, 1993) and the small divergence of the introgressed sequences compared to those of the donor species, at least in the case of *L. bellargus* introgressed from *L. coridon* (see Dincă et al., 2011).

4.2. Karyotype evolution

The different chromosome numbers within *Lysandra* are roughly multiples of the minimum number within the genus, $n = 24$ (*L. syriaca* and *L. punctifera*), which is the usual number within the family *Lycaenidae* (Robinson, 1971; Stekolnikov et al., 2000). *L. bellargus* is $n = 45$, or roughly double the basal, and the rest of the species have chromosome numbers that are approximately four times the basal ($n = 82–93$). This chromosomal series might suggest a case of polyploidy. However, polyploidy, although a frequent mechanism of speciation in plants, is relatively unusual among bisexually reproducing animals (Mallet, 2007). No direct measures of DNA content in *Lysandra* have been published, but inspection of chromosome sets indicates that genome size is more or less the same in species with different chromosome numbers, because the higher the chromosome number, the smaller the chromosomes. Additionally, the meiotic products observed in putative natural hybrids between *L. coridon* and *L. bellargus* possess an intermediate number of chromosome elements of very different sizes, which is also more consistent with a fusion–fission hypothesis (de Lesse, 1960, pp. 162–164). This leads to the hypothesis that a general fission of chromosomes might be the most probable process accounting for karyotype evolution in *Lysandra* (De Bast, 1985).

Our results do not support the hypothesis of polyploidy. In the first place, the recovered phylogeny does not recover the taxa with $n = 24$ (*L. punctifera* and *L. syriaca*) as closely related. *Lysandra bellargus* ($n = 45$) is recovered as the sister of *L. punctifera* with high support, and thus it cannot represent the putative intermediate tetraploid leading to putative octoploids ($n = 88–93$). Moreover, we have found an intermediate between ancestral 24 and putative tetraploid number for the subspecies *L. syriaca burak* ($n = 30$), located in Eastern Turkey (Supplementary Table S1) that, together with the geographic cline in chromosome number in the species *L. coridon*, can be only explained as the result of fusion–fission processes.

Chromosomal fusion–fission events seem to be extremely common within *L. coridon*, but fixed within populations and approximating a longitudinal chromosome number cline across Europe, as de Lesse (1969) originally pointed out. Thus, *L. coridon* seems to be the only *Lysandra* species with widespread intraspecific variability in chromosome number, although our results for *L. syriaca burak* suggest that this species might represent a similar case. Neighboring *L. coridon* chromosomal races typically differ in chromosomal reorganization but with no apparent morphological or ecological differences. Thus there is no additional evidence pointing to speciation within *L. coridon*. A population study incorporating informative nuclear data would be required to fully understand the effect of chromosomal reorganizations on gene flow for *L. coridon* (cf. Rieppel, 2012 and in preparation).

Chromosomal instability seems to be a rule within the genus; however, how and when it originated is not clear. According to our results, the most likely explanation points to at least three independent origins of chromosomal instability (Fig. 3). One on the split of *L. bellargus* ($n = 45$) from the common ancestor with *L. punctifera* ($n = 24$), another within *L. syriaca* (supposedly at the split between the nominotypical subspecies ($n = 24$) and the subspecies *L. s. burak* ($n = 30$), and at least one more that produced the large numbers displayed by most species.

The occurrence of extensive karyotype diversity among species with little or no genetic and morphological divergence implies the possibility of chromosomal speciation (White, 1973; King, 1993). Within the genus *Lysandra*, chromosomal analyses of a few F1 hybrids between *L. coridon* ($n = 90$) and *L. bellargus* ($n = 45$) demonstrated an incomplete meiotic pairing of parental chromosomes resulting in intermediate number (between 45 and 90) of chromosome elements (multivalents + univalents) observed during first meiotic division (de Lesse 1960). The bearers of such meiotic cells are expected to have a significantly reduced fertility because the formation of balanced gametes has low probability. This seems to be the case because hybrids are relatively rare and seem to be evolutionary dead ends, although regularly appearing in different populations (de Lesse 1960; Schurian, 1989). But at the same time, mitochondrial introgression between different species (as detected between *L. coridon* and *L. bellargus*) could only be explained if the backcrosses of F1 hybrids with parental species resulted in viable offspring in some cases. The inferred cases of introgression in our phylogenetic tree argue against ancient hybridization as a potential hypothesis since they occur before the chromosome differences between these species appeared. In summary, we find evidence for the existence of limited gene flow across taxa with very different chromosome numbers (e.g. *L. coridon* and *L. bellargus*) that lead to genetic introgression that can be detected in some instances. But we also find evidence for the existence of hybrid depression: species do not fully merge, hybrids remain relatively uncommon in sympatry, and chromosomal races seem to be stable even within *L. coridon* despite geographical proximity.

The genus *Lysandra* could be seen as disproving the hypothesis relating karyotype instability with diversification: it is older but much less speciose (ca. 10 species) than the subgenus *Agrodiaetus*, which has produced ca. 120 species in only 2.3 [1.6,3.12] Mya (Talavera et al., 2013).However, our results offer an explanation for this discrepancy. Although the genus *Lysandra* split from the genus *Polyommatus* ca. 4.9 [3.43,6.37] Mya, a burst of diversification generating current species diversity did not apparently start until much more recently (1.45 [1.05,1.89] Mya). Since the common ancestor of extant species most likely had 24 chromosomes (as do almost all of the rest of the lycaenids), the hypothesis of karyotype diversification driving speciation in *Lysandra* is reinforced. In fact, if we assume that there are 10 species of *Lysandra* (as we conclude in this study), Magallon and Sanderson's net diversification rates give a value of 1.11 [1.53,0.85] lineages/My for *Lysandra* and 1.78 [2.56,1.31] lineages/My for *Agrodiaetus*, whereas a general rate accounting for all Polyommatina is significantly lower (0.40 [0.55,0.32] lineages/My).

Chromosomal changes within a lineage may promote the chance of speciation. If new chromosomal rearrangements are underdominant, their fixation (i.e. transition from the heterozygous condition to the homozygous state) may be promoted by population bottlenecks (King, 1993). At the same time, these bottlenecks may give rise to additional consequences, including rapid lineage sorting of neutral molecular markers. *Lysandra* may very well provide a case in point. First, the demographic history inferred from the species tree suggests that the lineages of this genus may have suffered substantial variation in their population sizes (Fig. 3, Supplementary Table S5), possibly in the form of bottlenecks. However, we also find strong phylogenetic support in three nodes, presumably because of the fixation of unique characters by rapid lineage sorting, a remarkable situation in the context of generalized incomplete lineage sorting. It is also relevant that *L. coridon*, a species displaying considerable chromosomal changes that

apparently have not yet resulted in speciation, also exhibits unusually large population sizes. Overall, this scenario suggests that deep coalescence correlates with karyotype stability and rapid lineage sorting with karyotype instability. Testing this hypothesis would require a much larger population genetic study that could unravel the role of demographic bottlenecks in the history of *Lysandra* diversification.

5. Conclusions

The genus *Lysandra* forms a clade displaying recent diversification events that started around 1.4 Mya. We obtain a partially resolved tree with support for three main clades: *L. punctifera* + *L. bellargus*, the *corydonius* taxa and *L. coridon* + the Iberian taxa, plus three taxa without close relatives recovered as lineages with unresolved position. Predominant incomplete lineage sorting seems to blur basal relationships. Our new karyotype findings within the *corydonius* group do not reveal differences among species, and given the low genetic divergences observed, we consider them as subspecific taxa. We show that mtDNA introgression in *Lysandra* is widespread and that not accounting for hybridization in species tree inference can lead to erroneous phylogenetic and taxonomic conclusions. *Lysandra coridon* displays two paraphyletic lineages roughly corresponding to eastern and western Europe, most likely because of massive introgression events between western specimens and the Iberian closely related taxa. However, we argue that the Iberian taxa correspond to true species, although extremely recent, given their apparently stable karyotype differences. Finally, we show that chromosomal instability has originated at least three independent times within the group. We also hypothesize that chromosomal instability may have played a crucial role in the *Lysandra* species radiation, where strong population bottlenecks may promote fixation of new chromosomal rearrangements at the same time that rapid lineage sorting of neutral molecular markers occurred, in a generalized scenario of incomplete lineage sorting.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.08.004.

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