

SUPPLEMENTARY MATERIAL

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

Gerard Talavera^{a,b,c}, Vladimir A. Lukhtanov^{b,d}, Lukas Rieppel^{c,e}, Naomi E. Pierce^e and Roger Vila^{a,*}

^aInstitut de Biologia Evolutiva (CSIC-UPF), Passeig Marítim de la Barceloneta, 37, 08003 Barcelona, Spain

^bFaculty of Biology & Soil Science, St Petersburg State University, Universitetskaya nab. 7/9, 199034 St Petersburg, Russia

^cDepartment of Organismic and Evolutionary Biology and Museum of Comparative Zoology, Harvard University, 26 Oxford Street, Cambridge, Massachusetts 02138, USA

^dDepartment of Karyosystematics, Zoological Institute of Russian Academy of Science, Universitetskaya nab. 1, 199034 St Petersburg, Russia

^eDepartment of History, Brown University, 79 Brown Street, Providence, RI 02192, USA

*Corresponding author: roger.vila@csic.es

SUPPLEMENTARY DISCUSSION

Phylogenetic relationships

We recover three well-differentiated clades plus three species with apparently no close relative (*L. syriaca*, *L. dezina* and *L. ossmar*). One of the strongly supported clades is formed by *L. punctifera* and *L. bellargus*, a grouping that corresponds very well to morphology. In fact *L. punctifera* and *L. bellargus* are so similar in their wing patterns that the taxon *punctifera*, first described by Oberthür in 1876 was initially considered a subspecies of *L. bellargus*. Much later, de Lesse (1959) assigned *punctifera* species status based on differences in chromosome number. These two taxa split ca. 0.74 Mya, possibly because of dispersal across the West Mediterranean (perhaps through the Gibraltar Strait), since *L. bellargus* is widespread in the Iberian Peninsula and across Europe into Western Asia, while *L. punctifera* is confined to the Southwestern Mediterranean shore (Morocco, N. Algeria and NW. Tunisia). The removal of the conflicting signal created by the mitochondrial sequence of *L. bellargus* JC96Q001 substantially increases the posterior probability of this clade (*pp* from 0.82 to 0.98). This specimen was collected in Germany, where *L. bellargus* flies syntopically and synchronically with *L. coridon*. Indeed, the introgressed mitochondrial haplotype has already been shown to be very widespread in Romania, where nine out of ten sequenced Romanian *L. bellargus* specimens carried the introgression (Dincă et al., 2011).

Another supported clade includes all Iberian taxa (*L. albicans*, *L. coelestissima*, and *L. hispana*) + *coridon* sensu stricto group (including the taxa *gennargenti*, *nufrellensis*, and *philippi*) (Figure 2A-2B). Two differentiated clades are recovered in the mitochondrial tree (Figure 1). One, which we will call the eastern clade, is well supported and comprises only *L. coridon*, mainly from central and eastern Europe. The

other, which we will call the western clade, is not well supported, and contains the remainder of the *L. coridon* samples from the Iberian Peninsula and Southern France, which cluster together with the other three Iberian taxa. The internal relationships for this group display a high degree of phylogenetic uncertainty. Notably, the four Iberian taxa and *L. coridon* are not recovered as monophyletic. Introgression is likely at play within this western clade, although JML could not differentiate it from the uncertainty created by incomplete lineage sorting. As shown in Figure 2C, the *L. coridon* specimens from the western clade were usually collected in sympatry or proximity to some of the other three Iberian taxa.

A macropopulation structure dividing *L. coridon* into an eastern and a western European form has been previously suggested (Schmitt and Seitz, 2001; Schmitt et al., 2002; Schmitt and Zimmermann, 2011). While our results generally agree with the proposed distribution of these two forms, remarkable additions to the known phylogeography of the species are worth discussing. First of all, a *L. coridon* specimen from Monte Pollino (Calabria, Southern Italy) displays a highly diverged haplotype with unresolved position in the tree, and it could represent a relict lineage that has survived in this rather isolated locality. Similarly, the Sardinian taxon *gennargenti*, while apparently related to the eastern clade, is also substantially diverged, which suggests that it has remained isolated for a substantial amount of time. Rather surprisingly, the geographically close taxon *nufrellensis* from Corsica belongs instead to the eastern clade, and its mitochondrial sequence is close to that of one French *L. coridon narbonensis* specimen studied, among others. Thus, *nufrellensis* is apparently the outcome of a more recent colonization of Corsica from the mainland, despite having some phenotypic

differentiation that could be either the result of a founder effect, drift or adaptation to insular conditions.

The other insular population studied is from the UK, which also belongs to the eastern clade and is almost identical to the French *L. coridon narbonensis* specimen. Thus, we can conclude that *L. coridon* may have colonized Great Britain quite recently from the mainland.

Our results show that the clear-cut division proposed for the two *L. coridon* forms, with a contact zone in north-eastern Germany, along the mountain ranges of the German–Czech border and throughout the eastern Alps (Schmitt and Zimmermann, 2012), is much more complex than originally proposed, at least for mitochondrial markers. Indeed, at least two specimens from the eastern clade were collected in surprisingly western locations: one was *L. coridon narbonensis* MAT99Q932 from Mende (Languedoc region, France). The other specimen is *L. coridon asturiensis* RV07C272 collected in the extreme north-western Iberian Peninsula (Figure 2C). Worth noting, this novel population, which represents the westernmost locality for *L. coridon*, occurs in an isolated small cape situated outside the area of influence of any other Iberian *Lysandra* species.

The two *L. coridon* clades may be the outcome of two different glacial refugia (the western clade in the Iberian Peninsula and the eastern clade in the Italian or Balkan Peninsulas, with postglacial dispersal creating the current distribution (Schmitt et al., 2002). However, we propose that isolation during the last glaciation has not generated most of the intraspecific divergence detected in *L. coridon*, but that the four taxa in the

Iberian refugium shared mitochondrial sequences in a high degree. The hypothesis of introgression between *L. albicans*, *L. coelestissima*, *L. hispana* and western *L. coridon* is further supported by the species tree treating separately the *L. coridon* eastern and western clades, which recovers them as paraphyletic lineages because the western clade is sister to the other three Iberian taxa (Figure 2D). While introgression within the Iberian Peninsula could not be unambiguously demonstrated by JML, this hypothesis is more likely than solely incomplete lineage sorting being the cause. Indeed, the presence of the eastern form in the extreme northwest Iberian Peninsula can hardly be explained by recent long-range dispersal. This isolated and ecologically unique population, located outside the area of influence of the other Iberian taxa and of other *L. coridon* populations (Figure 2C), shares a chromosome number with its geographical neighbours, and is most probably a relict of “pure” Iberian *L. coridon* that was not introgressed, which would mean that most of the western clade genetic differentiation is actually derived from events of introgression.

Within the *coridon* group, we consider the somewhat morphologically differentiated taxa *gennargenti* and *nufrellensis* as *L. coridon* subspecies because their genetic divergences fall within that of *L. coridon* sensu stricto. The taxon *gennargenti* is morphologically different from other forms of the *L. coridon* complex and due to the blue color of wing upperside in both males and females it was considered sometimes as a species distinct from *L. coridon* (Jutzeler et al., 2003). However, recent hybridization experiments (Schurian et al., 2011) demonstrated an absence of reproductive isolation between the taxa *gennargenti* and *nufrellensis*, concluding conspecificity of both taxa with *L. coridon*. No information is available on their chromosome numbers, and they are allopatric. The taxon *philippi*, which flies in Northern Greece, is genetically

identical to *L. coridon graeca* from Central Greece. Indeed, the taxon *philippi* was described as a separate species due an erroneous chromosome number count: Brown and Coutsis (1978) determined its chromosome number to be $n=20-26$, but no figures were provided. Later on, Coutsis et al. (2001) examined the karyotype of *philippi* again and found $n=88-90$, with one large chromosome. As its chromosome number does not differ from nearby *L. coridon*, its species status no longer seems warranted.

The generally parapatric, but sometimes locally sympatric, Iberian taxa require more complex assessment. Even if they frequently hybridize, they seem to present stable differences in chromosome number, and we tentatively consider *albicans*, *hispana* and *coelestissima* as three recent species.

Lastly, we recover the *corydonius* group as monophyletic. This includes four taxa from the Caucasus (*corydonius*, *arzanovi*, *melamarina* and *sheikh*) that are often considered subspecies of a single species, *corydonius* (Vodolazhsky and Stradomsky, 2008). Their divergence is minimal and they are estimated to have diverged ca. 0.25 Mya. They all seem to be allopatric, and differ in certain morphological characteristics and voltinism. The taxon *melamarina* is bivoltine, and differs from other representatives of the *coridon* subgroup by a very light, whitish color of the underside wing. The taxon *sheikh*, however, is monovoltine, and close to other representatives of the *coridon* subgroup, although displaying more bluish upperside wings in males and larger marginal black spots. The taxon *arzanovi* was described on the basis of putative chromosomal differences, as well as tiny differences in male genitalia (fine structure of gnathos) and male wing color (wing underside is grey whereas it is whitish in *melamarina* and brown in *corydonius* and *sheikh*).

However, since the chromosomal data we provide (n=84 for *L. melamarina*, *L. sheikh* and *L. corydonius*) cast doubt on previously reported chromosomal differences within this complex, we propose provisionally treating these taxa as conspecific until further evidence is obtained. Indeed, no predominant fixed barriers seem to exist between them, and the fact that they appear to be isolated, parapatric populations with some degree of phenotypic differentiation encourages us to treat them as subspecies *sensu* Braby et al. (2012).

The case of *L. ossmar*, a taxon that is usually considered to be closely related to the parapatric *L. corydonius* (Schurian, 1989; Hesselbarth et al., 1995), is especially interesting. While Schurian (1989, p. 158) conducted a morphological and ecological analysis that recovered *L. ossmar* as sister to the *L. corydonius* clade, in our dataset this is shown to be an effect produced by detected cases of mitochondrial introgression between these two taxa. Indeed, when removing the two potentially introgressed sequences, *L. ossmar* is recovered as an independent lineage with no close relative and unresolved position, similarly to the result for the middle-eastern taxa *L. dezina* and *L. syriaca*.

References:

Braby, M.F., Eastwood, R., Murray, N., 2012. The subspecies concept in butterflies: has its application in taxonomy and conservation biology outlived its usefulness? Biol. J. Linn. Soc. 106, 699–716.

de Lesse H., 1959. Sur la valeur spécifique de deux sous-espèces d'*Agrodiaetus* (Lep. Lycaenidae) récemment décrites. Bull. mens. Soc. Linn. Lyon. 28, 312–315.

Jutzeler, D., Casula, P., Gascoigne-Pees, M., Grill, A., Leigheb, G. 2003. Confirmation du statut spécifique de *Polyommatus gennargenti* (LEIGHEB, 1987) de Sardaigne comparé à *Polyommatus coridon* (PODA, 1761) de la région de Schaffhouse (CH) par élevage parallèle (Lepidoptera: Lycaenidae) 1ère partie. Linneana Belgica 19(3), 109-118.

Oberthür, C., 1876. Faunes entomologiques; descriptions d'insectes nouveaux ou peu connus. Imprimerie Oberthür. Rennes.

Schmitt, T., Seitz, A., 2001. Allozyme variation in *Polyommatus coridon* (Lepidoptera: Lycaenidae): identification of ice-age refugia and reconstruction of post-glacial expansion. J. Biogeogr. 28, 1129–1136.

Schmitt, T., Gießl, A., Seitz, A., 2002. Postglacial colonisation of western Central Europe by *Polyommatus coridon* (Poda 1761) (Lepidoptera: Lycaenidae): evidence from population genetics. Heredity 88, 26–34.

Schmitt, T., Zimmermann, M., 2012. To hybridize or not to hybridize: what separates two genetic lineages of the Chalk-hill Blue *Polyommatus coridon* (Lycaenidae, Lepidoptera) along their secondary contact zone throughout eastern Central Europe? J. Zoo. Syst. Evol. Res. 50, 106–115.

Schurian, K.G., Westenberger, A., Diring, Y., Wiemers, M. 2011. Contribution to the biology, ecology and taxonomy of *Polyommatus (Lysandra) coridon nufrellensis* (Schurian, 1977) (Lepidoptera: Lycaenidae), Part III: an experimental hybridisation of *P. (L.) c. gennargenti* x *P. (L.) c. nufrellensis*. Nachrichten des Entomologischen Vereins Apollo 31(4), 177-186.

Vodolazhsky, D.I., Stradomsky, B.V., 2008. A study of blues butterflies of the group of *Lysandra corydonius* (Herrich-Schäffer, 1804) (Lepidoptera: Lycaenidae) with the use of mtDNA markers. Caucas. Entomol. Bull. 4, 353–355.

SUPPLEMENTARY TABLES AND FIGURES

Table S1. Samples used in this study: taxon name, sample accession number at MCZ and sample collection locality.

Genus	Species & ssp.	Sample code	Locality
<i>Lysandra</i>	<i>albicans albicans</i>	RV03H582	Puebla de Don Fadrique, 1295 m, Granada, Spain
<i>Lysandra</i>	<i>albicans arragonensis</i>	MAT99Q969	Una, Cuenca, 970m, Spain
<i>Lysandra</i>	<i>arzanovi</i>	SH02H019	Aibga-1 Pass. 1850m, Krasnaya Polyana, Aibga Mts., Sochi, Krasnodar Region, Russia
<i>Lysandra</i>	<i>arzanovi</i>	SH02H020	Aibga-1 Pass. 1850m, Krasnaya Polyana, Aibga Mts., Sochi, Krasnodar Region, Russia
<i>Lysandra</i>	<i>bellargus</i>	AD00P129	Aragatz Mt., Amberd Valley, 2300m, Transcaucasus, Armenia
<i>Lysandra</i>	<i>bellargus</i>	JC96Q001	Gambach, Bavaria, Germany
<i>Lysandra</i>	<i>bellargus</i>	MAT99Q882	Rúbies, Catalonia, Spain
<i>Lysandra</i>	<i>bellargus</i>	RV04G399	Saimbeyli Valley, 1445m (Adana), Turkey
<i>Lysandra</i>	<i>bellargus</i>	VL02X510	Masuleh, 1900-2100m, Gilan, Iran
<i>Lysandra</i>	<i>caelestissima</i>	MAT99Q959	Ciudad Encantada, 1440m, Uña, Cuenca, Spain
<i>Lysandra</i>	<i>caelestissima</i>	MAT99Q966	Uña, Cuenca, 970m, Spain
<i>Lysandra</i>	<i>coridon apennina</i>	MB05G416	Mt. Pollino, Calabria, Italy
<i>Lysandra</i>	<i>coridon asturiensis</i>	JR04G493	Albelda, 900m, La Rioja, Spain
<i>Lysandra</i>	<i>coridon asturiensis</i>	RV07C272	Cedeira, Capelada, Galicia, Spain
<i>Lysandra</i>	<i>coridon borussia</i>	AD00P192	Tula region, Tatinki, 120 m., W. Russia
<i>Lysandra</i>	<i>coridon cataluniae</i>	RV03H454	El Brull, Catalonia, Spain

<i>Lysandra</i>	<i>coridon coridon</i>	VD02T008	Romania
<i>Lysandra</i>	<i>coridon gennargenti</i>	KS05I874	Orgosolo, 1250m, vic. Monte Novo S. Giovanni, Sardinia Is.
<i>Lysandra</i>	<i>coridon gennargenti</i>	KS05I875	Orgosolo, 1250m, vic. Monte Novo S. Giovanni, Sardinia Is.
<i>Lysandra</i>	<i>coridon insulana</i>	RE04C165	Therfield Heath, Royston, UK
<i>Lysandra</i>	<i>coridon narbonensis</i>	MAT99Q932	Mende, 780m, Languedoc region, France
<i>Lysandra</i>	<i>coridon</i>	AD00P045	Volgograd region, Kamyshinsky v., 200 m., Low Volga, South Russia
<i>Lysandra</i>	<i>coridon</i>	RE07G279	NE Bezandun-sur-Bine, 735 m, Drome, France
<i>Lysandra</i>	<i>coridon</i>	RV06A183	Sorteny, Andorra
<i>Lysandra</i>	<i>coridon</i>	RV07E302	Baile Herculane, Pecinisca, 220-320m, Caras-Severin, Romania
<i>Lysandra</i>	<i>coridon graeca</i>	JXC02G002	Mt. Timfristos (=Mt. Veluhi), 1300-1500m, Sterea Ellas, Greece
<i>Lysandra</i>	<i>corydonius caucasica</i>	VL01L120	Hasköy, 12 km SW Gümüşhane, Gümüşhane Prov., Turkey
<i>Lysandra</i>	<i>corydonius caucasica</i>	AD00P435	Aiodzor Mts., Gnishyk 1800 m., Transcaucasus, Armenia
<i>Lysandra</i>	<i>corydonius corydonius</i>	VL03F932	Talysh Mts, SE Azerbaijan
<i>Lysandra</i>	<i>corydonius corydonius</i>	VL05N131	Iran Azerbaijan-e Sharqi, pass 25 km NW Varzaqan; 2050-2170 m
<i>Lysandra</i>	<i>dezina</i>	09X500	Kurdistan
<i>Lysandra</i>	<i>hispana hispana</i>	MAT99T993	Coll d'Estenalles, 870m, Parc Natural de Sant Llorenç del Munt, Spain
<i>Lysandra</i>	<i>hispana hispana</i>	RV07F312	El Mont, Albanyà, Alt Empordà, Girona, Spain, 860m
<i>Lysandra</i>	<i>hispana semperi</i>	RV02N590	Ares del Maestre, 1150m, Castello, Spain
<i>Lysandra</i>	<i>melamarina</i>	SH02H007	Gelendjik, Betta Mts., 150m, Krasnodar Region, Russia
<i>Lysandra</i>	<i>melamarina</i>	SH02H010	Gelendjik, Betta Mts., 150m, Krasnodar Region, Russia
<i>Lysandra</i>	<i>nufrellensis</i>	KS05I821	Corsica , 1300m
<i>Lysandra</i>	<i>nufrellensis</i>	KS05I822	Corsica , 1300m
<i>Lysandra</i>	<i>ossmar ankara</i>	RV04G136	Kargasekmez Geçidi, Kizilcahamam, 1150m (Ankara) Turkey
<i>Lysandra</i>	<i>ossmar ossmar</i>	RV04G356	3Km NW Urgüp, 1140m (Kapadokya) Turkey
<i>Lysandra</i>	<i>ossmar ossmar</i>	RV07F170	Yelatan, 15 km S. of Çamardi, Nidge, Turkey , 1330m
<i>Lysandra</i>	<i>philippi</i>	SI03K025	Mt. Phalakro, 1600 m, District (Nomos) Drama, Greece
<i>Lysandra</i>	<i>philippi</i>	SI03K037	Mt. Phalakro, 600 m, District (Nomos) Drama, Greece
<i>Lysandra</i>	<i>punctifera</i>	NK02A026	Ait-b-Yahya, 1900m, Rich, Morocco
<i>Lysandra</i>	<i>punctifera</i>	NK02A027	Col Taghzoum, 1900m, High Atlas Range, Morocco
<i>Lysandra</i>	<i>sheikh</i>	VL03F998	Altyagach, 1300m, Azerbaijan near the border with Dagestan, Russia
<i>Lysandra</i>	<i>sheikh</i>	VL03H615	Altyagach, 1300m, Azerbaijan near the border with Dagestan, Russia
<i>Lysandra</i>	<i>syriaca burak</i>	RV07F139	13 km N. of Saimbeily, 1505m (Adana) Turkey
<i>Polyommatus</i>	<i>amandus amurensis</i>	AD02W109	Primorski Krai, S. Ussuri, Khanka Lake, Poganichnoye, Russia
<i>Neolysandra</i>	<i>diana</i>	AD00P081	Gegamsky Mts., 1800m, Gegadyr, Armenia
<i>Polyommatus</i>	<i>myrrha cinyraea</i>	AD00P389	Zangezur Mts., Akhtchi, Armenia

Table S2. Primer sequences. mt: mitochondrial, n: nuclear. T = thymine, A = adenine, G = guanine, C = cytosine, K = G+T, W = A+T, M = A+C, Y = C+T, R = A+G, S = G+C, V = G+A+C, I = Inosine, N = A+C+G+T.

Primer location	Primer name	Direction	Sequence (5' to 3')
mt <i>COI</i>	LCO1490 ¹	forward	GGTCAACAAATCATAAAGATATTGG
mt <i>COI</i>	Ron ^{2,3}	forward	GGATCACCTGATATAGCATTCCC
mt <i>COI</i>	Nancy ³	reverse	CCCGGTAAAATTTAAATATAAACTTC
mt <i>COI</i>	Tonya ³	forward	GAAGTTTATATTTTAAATTTTACCGGG
mt <i>COI</i>	Hobbes ³	reverse	AAATGTTGNGGRAAAAAATGTTA
mt <i>COI</i>	TN2126 ⁴	forward	TTGAYCCTGCAGGTGGWGGAG
mt <i>COII</i>	George ^{3,5}	forward	ATACCTCGACGTTATTCAGA
mt <i>COII</i>	Phyllis ^{3,5}	reverse	GTAATAGCIGGTAARATAGTTCA
mt <i>COII</i>	Strom ^{3,5}	forward	TAATTTGAACTATYTTACCIGC
mt <i>COII</i>	Eva ^{3,5}	reverse	GAGACCATTACTTGCTTTCAGTCATCT
mt <i>COII</i>	JL3146 ⁴	forward	GAGTTTCACCTTTAATAGAACA
mt <i>COII</i>	B-tLys ²	reverse	GTTTAAGAGACCAGTACTTG
mt <i>COII</i>	JL2532 ⁴	forward	ACAGTAGGAGGATTAACAGGAG
n <i>CAD</i>	CAD787F ⁶	forward	GGDGTNACNACNGCNTGYTTYGARCC
n <i>CAD</i>	CADFa ⁷	forward	G DATGGTYGATGAAAATGTTAA
n <i>CAD</i>	CADRa ⁷	reverse	CTCATRTCCTAATCYGTRCT
n <i>H3</i>	H3F ⁸	forward	ATGGCTCGTACCAAGCAGACVGC
n <i>H3</i>	H3R ⁸	reverse	ATATCCTTRGGCATRATRGTGAC
n <i>ITS-2</i>	ITS-3 ⁹	forward	GCATCGATGAAGAACGCAGC
n <i>ITS-2</i>	ITS-4 ⁹	reverse	TCCTCCGCTTATTGATATGC
n <i>wg</i>	LepWg1 ¹⁰	forward	GARTGYAARTGYCAYGGYATGTCTGG
n <i>wg</i>	LepWg2E ⁷	reverse	ACNACGAACATGGTCTGCGT
n <i>wg</i>	Wg1n ¹¹	forward	CGGAGATGCGMCAGGARTGC
n <i>wg</i>	Wg2n ¹¹	reverse	CTTTTTCCGTSCGACACAGYTTGC
n <i>28S</i>	S3660 ¹²	forward	GAGAGTTMAASAGTACGTGAAAC
n <i>28S</i>	A335 ¹²	reverse	TCGGARGGAACCAGCTACTA
n <i>Rpl5</i>	F44 ¹³	forward	TCCGACTTTCAAACAAGGATG
n <i>Rpl5</i>	Lys3R ¹⁴	reverse	ACAGCTCTGGCGCAGCGAAG

¹ Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R.C. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Marine Biol. Biotech.* 3, 294-299.

² Simon, C., Frati, F., Beckebach, A., Crespi, B., Liu, H. & Flook, P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* 87(6), 651-701.

³ Monteiro, A. & Pierce, N.E. 2001. Phylogeny of *Bicyclus* (Lepidoptera: Nymphalidae) inferred from COI, COII, and EF-1alpha gene sequences. *Molecular Phylogenetics and Evolution* 18, 264-281.

⁴ Canfield M.R., Greene E., Moreau C.S., Chen N., & Pierce N.E. 2008. Exploring phenotypic plasticity and biogeography in emerald moths: A phylogeny of the genus *Nemoria* (Lepidoptera: Geometridae). *Molecular Phylogenetics and Evolution* 49(2), 477-87.

⁵ Brower, A.V.Z. 1994. Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalidae). *Molecular Phylogenetics and Evolution* 3(2), 159-174.

⁶ Moulton, J.K. & Wiegmann, B.M. 2004. Evolution and phylogenetic utility of cad (rudimentary) among Mesozoic-aged eremoneuran Diptera (Insecta). *Molecular Phylogenetics and Evolution* 31, 363-378.

⁷ Vila, R., Bell, C.D., Macniven, R., Goldman-Huertas, B., Ree, R.H., Marshall, C.R., Bálint, Z., Johnson, K., Benyamini, D., & Pierce, N.E. 2011. Phylogeny and palaeoecology of *Polyommatus* blue butterflies show Beringia was a climate-regulated gateway to the New World. *Proceedings of the Royal Society B* 278(1719), 2737-2744.

⁸ Colgan, D.J., McLauchlan, A., Wilson, G.D.F., Livingston, S.P., Edgecombe, G.D., Macaranas, J., Cassis G., & Gray, M.R. 1998. Histone H3 and U2 snRNA DNA sequences and arthropod molecular evolution. *Australian Journal of Zoology* 46, 419-437.

- ⁹ White, T.J., Bruns, S., Lee, S., & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics in *PCR protocols: a guide to methods and applications*, edited by M.A. Innis, Gelfandm D.H., J.J. Snisky, & T. J. White. Academic Press, New York, pp. 315-322.
- ¹⁰ Brower, A.V.Z. & DeSalle, R. 1998. Patterns of mitochondrial versus nuclear DNA sequence divergence among nymphalid butterflies: the utility of wingless as a source of characters for phylogenetic inference. *Insect Molecular Biology* 7(1), 73-82.
- ¹¹ Designed by Ada Kalizewska (Harvard University, Cambridge, MA, USA).
- ¹² Sequeira, A.S., Normark, B.B., & Farrell, B. 2000. Evolutionary assembly of the conifer fauna: Distinguishing ancient from recent associations in bark beetles. *Proceedings of the Royal Entomological Society (London) B* 267, 2359-2366.
- ¹³ Mallarino R, Bermingham E, Willmott KR, Whinnett A. and CD Jiggins. 2005. Molecular systematics of the butterfly genus *Ithomia* (Lepidoptera: Ithomiinae): a composite phylogenetic hypothesis based on seven genes. *Molecular Phylogenetics and Evolution* 34, 625-644.
- ¹⁴ Designed in this study

Table S3. Genbank accession codes. GenBank codes used in this study.

Taxon	Specimen Code	COI + COII	Wg	CAD	ITS2	H3	28S	RpI5
<i>L. albicans albicans</i>	RV03H582	KF834395 (COI)	KF834556	KF834324	KF834475	KF834405	KF834305	KF834519
<i>L. albicans arragonensis</i>	MAT99Q969	KF834376	KF834541	KF834349		KF834441	KF834303	
<i>L. arzanovi</i>	SH02H019	KF834365	KF834566	KF834326		KF834412	KF834294	KF834492
<i>L. arzanovi</i>	SH02H020	KF834370	KF834559	KF834336	KF834462	KF834413	KF834295	KF834518
<i>L. bellargus</i>	AD00P129	JX093472	JX093299	JX093262	JX093380	JX093340	JX093225	KF834502
<i>L. bellargus</i>	JC96Q001	KF834378	KF834526	KF834338	KF834484	KF834422	KF834310	KF834513
<i>L. bellargus</i>	MAT99Q882	KF834397	KF834527	KF834352	KF834481		KF834317	KF834500
<i>L. bellargus</i>	RV04G399	KF834394	KF834528	KF834345	KF834482	KF834436	KF834312	KF834501
<i>L. bellargus</i>	VL02X510	KF834400	KF834529	KF834357	KF834485	KF834416	KF834313	
<i>L. caelestissima</i>	MAT99Q959	KF834398	KF834533	KF834356	KF834479	KF834442	KF834280	KF834489
<i>L. caelestissima</i>	MAT99Q966	KF834402	KF834537	KF834321	KF834471	KF834404	KF834281	KF834509
<i>L. coridon apennina</i>	MB05G416	KF834399	KF834561	KF834322	KF834487	KF834443	KF834283	KF834515
<i>L. coridon asturiensis</i>	JR04G493	KF834386	KF834532	KF834350	KF834474	KF834428	KF834316	KF834498
<i>L. coridon asturiensis</i>	RV07C272	KF834362	KF834534	KF834334	KF834457	KF834408	KF834288	KF834504
<i>L. coridon borussia</i>	AD00P192	JX093495			JX093377	JX093342	JX093227	KF834511
<i>L. coridon cataluniae</i>	RV03H454	KF834389			KF834478			KF834490
<i>L. coridon coridon</i>	VD02T008	KF834372	KF834553	KF834337	KF834449	KF834427	KF834302	KF834523
<i>L. coridon gennargenti</i>	KS05I874	KF834379	KF834564	KF834358	KF834455	KF834439	KF834278	KF834522
<i>L. coridon gennargenti</i>	KS05I875	KF834380	KF834565	KF834332	KF834456	KF834440	KF834279	
<i>L. coridon insulana</i>	RE04C165	KF834377	KF834531	KF834341	KF834472	KF834431	KF834284	KF834524
<i>L. coridon narbonensis</i>	MAT99Q932	KF834384	KF834530	KF834320	KF834477	KF834429	KF834315	KF834496
<i>L. coridon</i>	AD00P045	KF834373	KF834540	KF834331	KF834450	KF834421	KF834274	

<i>L. coridon</i>	RE07G279	KF834360	KF834563	KF834333	KF834468	KF834425	KF834285	
<i>L. coridon</i>	RV06A183	KF834361	KF834558	KF834346	KF834476	KF834444	KF834287	
<i>L. coridon</i>	RV07E302	KF834363	KF834544	KF834343	KF834473	KF834437	KF834306	
<i>L. coridon graeca</i>	JXC02G002	KF834374	KF834555	KF834351	KF834454	KF834438	KF834275	KF834495
<i>L. coridon nufrellensis</i>	KS05I821	KF834382	KF834560	KF834339	KF834467	KF834423	KF834276	KF834505
<i>L. coridon nufrellensis</i>	KS05I822	KF834385	KF834567	KF834340	KF834466	KF834445	KF834277	KF834506
<i>L. coridon philippi</i>	SI03K025	KF834393	KF834535		KF834448	KF834414	KF834296	
<i>L. coridon philippi</i>	SI03K037	KF834387	KF834552	KF834348	KF834447	KF834435	KF834297	KF834497
<i>L. corydonius caucasica</i>	VL01L120	KF834392	KF834546	KF834327	KF834463	KF834415	KF834298	KF834494
<i>L. corydonius caucasica</i>	AD00P435	KF834388	KF834543	KF834319	KF834460	KF834406	KF834307	KF834493
<i>L. corydonius corydonius</i>	VL03F932	KF834383	KF834547	KF834328	KF834459	KF834417	KF834299	KF834491
<i>L. corydonius corydonius</i>	VL05N131	KF834367	KF834554	KF834330	KF834458	KF834420	KF834308	KF834521
<i>L. dezina</i>	O8X599	KF834403				KF834446	KF834318	
<i>L. hispana hispana</i>	MAT99T993	KF834375	KF834542	KF834353	KF834480	KF834430	KF834282	KF834514
<i>L. hispana hispana</i>	RV07F312	KF834369	KF834539	KF834347	KF834465	KF834426	KF834291	KF834499
<i>L. hispana semperi</i>	RV02N590	KF834401	KF834538	KF834323	KF834488	KF834432	KF834304	KF834510
<i>L. melamarina</i>	SH02H007	KF834364	KF834550	KF834335	KF834464	KF834410	KF834292	KF834508
<i>L. melamarina</i>	SH02H010	KF834366	KF834551		KF834461	KF834411	KF834293	KF834517
<i>L. ossmar ankara</i>	RV04G136	KF834390	KF834536	KF834355	KF834451	KF834433	KF834314	
<i>L. ossmar ossmar</i>	RV04G356	KF834391	KF834557	KF834342	KF834452	KF834407	KF834286	
<i>L. ossmar ossmar</i>	RV07F170	KF834359	KF834545	KF834344	KF834453	KF834409	KF834290	
<i>L. punctifera</i>	NK02A026	KF834396 (COI)	KF834525	KF834354	KF834483	KF834424	KF834311	KF834512
<i>L. punctifera</i>	NK02A027	JX093494	JX093411	JX093263	JX093391	JX093341	JX093226	KF834503
<i>L. sheikh</i>	VL03F998	KF834381	KF834548		KF834486	KF834418	KF834300	KF834507
<i>L. sheikh</i>	VL03H615	KF834368	KF834549	KF834329	KF834469	KF834419	KF834301	KF834520
<i>L. syriaca burak</i>	RV07F139	KF834371	KF834562	KF834325	KF834470	KF834434	KF834289	KF834516
<i>P. amandus amurensis</i>	AD02W109	JX093454	JX093419	JX093245	JX093366	JX093322	JX093215	
<i>N. diana</i>	AD00P081	JX093479	JX093416	JX093256	JX093370	JX093363	JX093203	
<i>P. myrrha cinyraea</i>	AD00P389	JX093473	JX093430	JX093243	JX093369	JX093326	JX093234	

Table S4. Parsimony informative sites and number of positions per each loci for the *Lysandra* species.

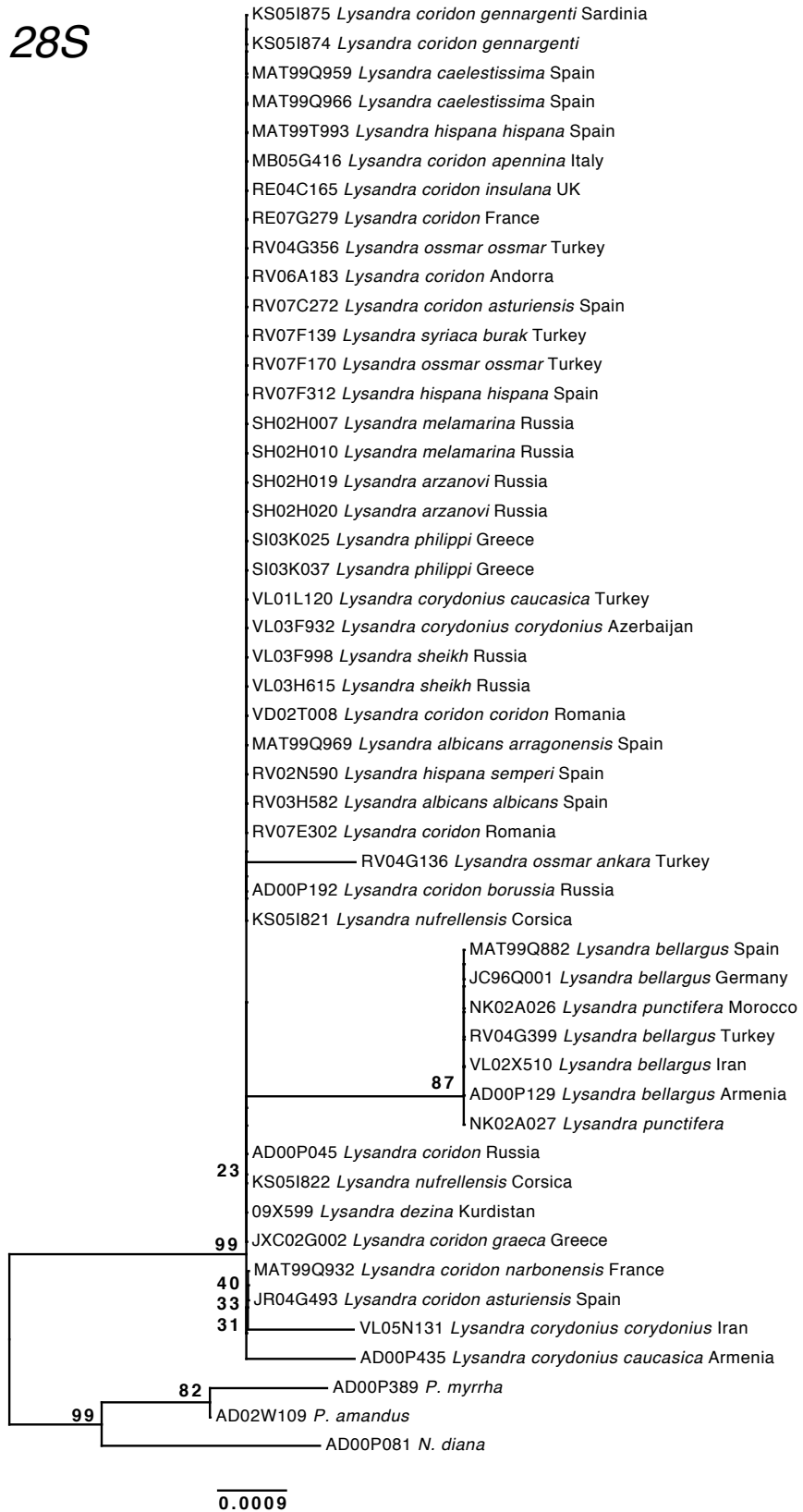
Gene	Parsimony informative sites	Number of positions
CO	153	2164
CAD	2	745
Wg	15	403
ITS2	10	635
28S	3	821
H3	4	329
Rpl5	21	873

Table S5. Demographic history from *BEAST species tree inference. Values are extracted from Biopy consensus tree summaries. The X axis represents divergence time and Y axis represents relative population sizes width. Demographic values (dmv) for coalescence beginnings and ending points in branches are shown according to piecewise linear model used in *BEAST. Node ages are summarized using TreeAnnotator.

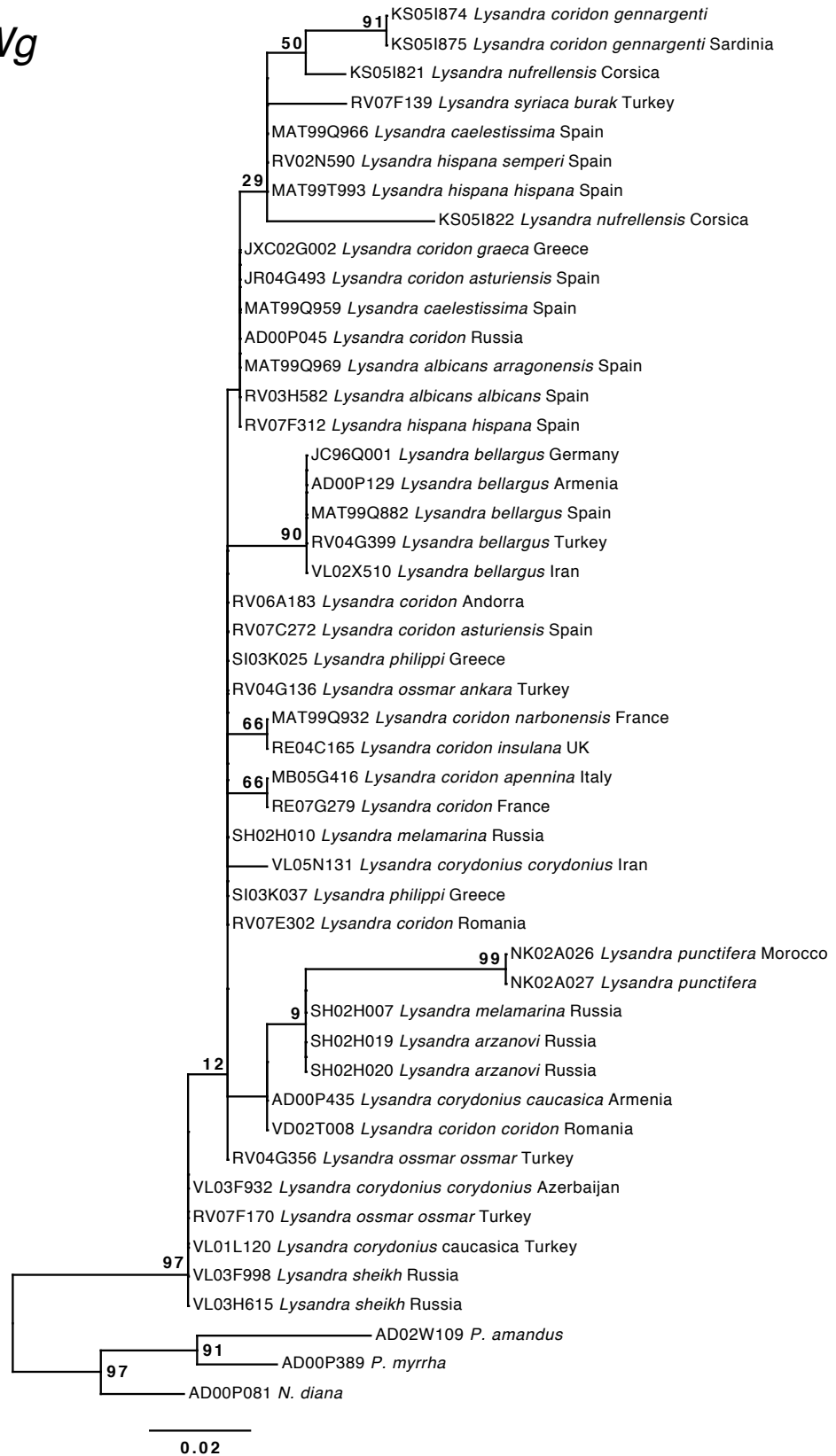
	dmv_b	dmv_e	dmv95_b	dmv95_e	Node age
<i>arz</i>	0.78	0.22	0.19	1.95	0.07
<i>mel</i>	0.90	0.25	0.23	2.12	0.07
<i>arz-mel</i>	0.47	0.26	0.18	1.75	0.15
<i>cory</i>	0.90	0.19	0.22	1.86	0.15
<i>cory-(arz-mel)</i>	0.45	0.13	0.12	1.18	0.25
<i>she</i>	0.76	0.16	0.16	1.80	0.25
<i>she-(cory-(arz-mel))</i>	0.29	0.26	0.14	0.83	0.60
<i>oss</i>	0.93	0.39	0.33	1.84	0.60
<i>oss-(she-(cory-(arz-mel)))</i>	0.65	0.61	0.35	1.67	0.89
<i>alb</i>	1.07	0.33	0.31	2.40	0.12
<i>cael</i>	0.94	0.23	0.24	1.99	0.12
<i>cael-alb</i>	0.57	0.40	0.24	1.88	0.25
<i>his</i>	1.18	0.39	0.38	2.22	0.25
<i>his-(cael-alb)</i>	0.78	0.28	0.25	1.49	0.38
<i>cor</i>	2.43	1.18	1.24	2.82	0.38
<i>cor-(his-(cael-alb))</i>	1.47	0.66	0.60	2.33	0.89
<i>(cor-(his-(cael-alb)))-(oss-(she-(cory-(arz-mel))))</i>	1.27	0.58	0.50	2.38	1.18
<i>dez</i>	1.02	0.35	0.30	2.38	1.18
<i>syr</i>	1.02	0.38	0.32	2.22	1.18
<i>dez-syr</i>	0.73	0.78	0.41	2.59	1.04
<i>(dez-syr)-(cor-(his-(cael-alb)))-(oss-(she-(cory-(arz-mel))))</i>	1.36	0.71	0.52	2.14	1.04
<i>bel</i>	1.00	0.18	0.26	1.26	0.72
<i>punc</i>	0.72	0.18	0.19	1.53	0.72
<i>bel-punc</i>	0.36	0.50	0.23	1.47	1.40
<i>(bel-punc)-rest</i>	1.21	1.21	0.77	2.45	1.40

Figures S1-S6. Maximum Likelihood nuclear gene trees (*H3*, *28S*, *Wg*, *CAD*, *ITS2* and *Rpl5*). Highest values for bootstrap support are shown at nodes. Scale bar represents substitutions per position.

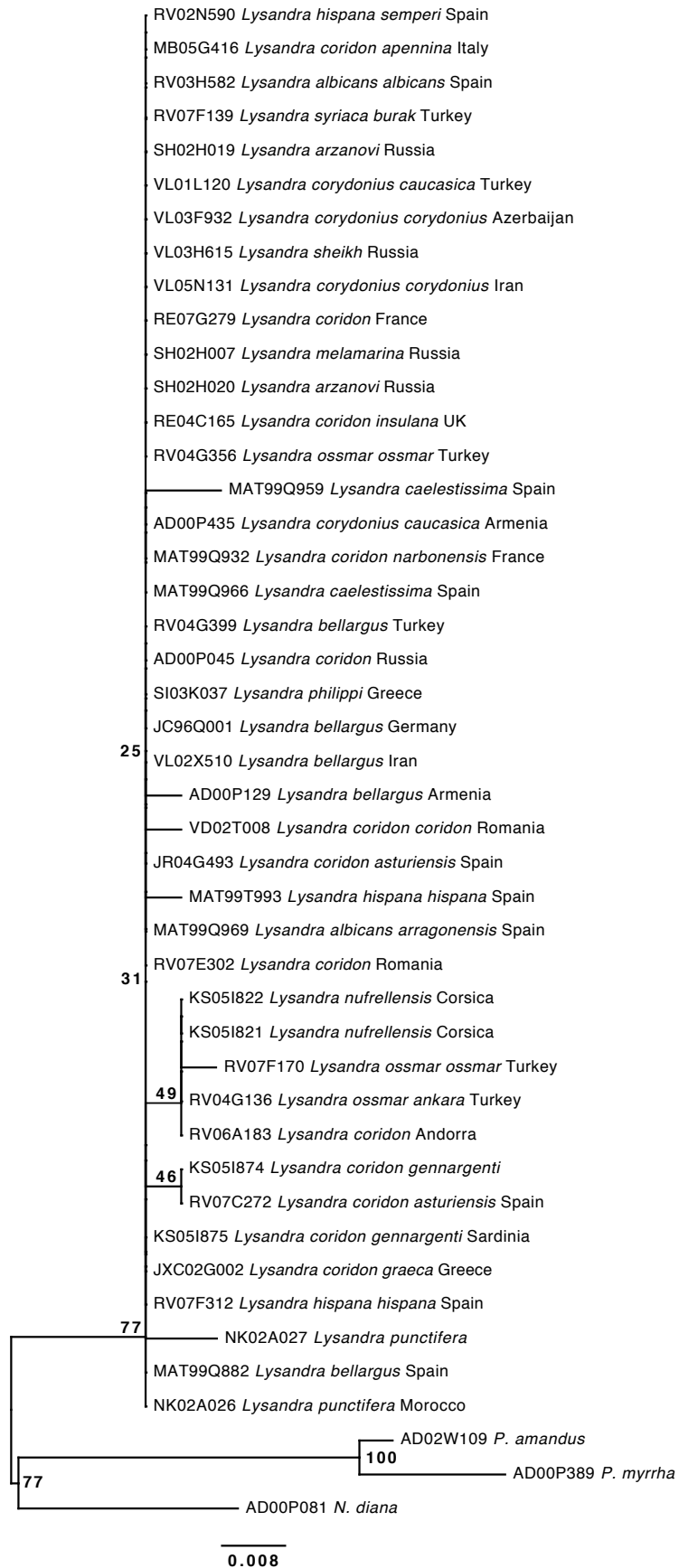




Wg



CAD



ITS2



0.008

Rpl5

