

## Phylogeny of *Agrodiaetus* Hübner 1822 (Lepidoptera: Lycaenidae) Inferred from mtDNA Sequences of *COI* and *COII* and Nuclear Sequences of *EF1- $\alpha$* : Karyotype Diversification and Species Radiation

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**Abstract.**— Butterflies in the large Palearctic genus *Agrodiaetus* (Lepidoptera: Lycaenidae) are extremely uniform and exhibit few distinguishing morphological characters. However, these insects are distinctive in one respect: as a group they possess among the greatest interspecific karyotype diversity in the animal kingdom, with chromosome numbers (*n*) ranging from 10 to 125. The monophyly of *Agrodiaetus* and its systematic position relative to other groups within the section *Polyommatus* have been controversial. Characters from the mitochondrial genes for *cytochrome oxidases* I and II and from the nuclear gene for *elongation factor 1 $\alpha$*  were used to reconstruct the phylogeny of *Agrodiaetus* using maximum parsimony and Bayesian phylogenetic methods. Ninety-one individuals, encompassing most of the taxonomic diversity of *Agrodiaetus*, and representatives of 14 related genera were included in this analysis. Our data indicate that *Agrodiaetus* is monophyletic. Representatives of the genus *Polyommatus* (*sensu stricto*) are the closest relatives. The sequences of the *Agrodiaetus* taxa in this analysis are tentatively arranged into 12 clades, only 1 of which corresponds to a species group traditionally recognized in *Agrodiaetus*. Heterogeneous substitution rates across a recovered topology were homogenized with a nonparametric rate-smoothing algorithm before the application of a molecular clock. Two published estimates of substitution rates dated the origin of *Agrodiaetus* between 2.51 and 3.85 million years ago. During this time, there was heterogeneity in the rate and direction of karyotype evolution among lineages within the genus. Karyotype instability has evolved independently three times in the section *Polyommatus*, within the lineages *Agrodiaetus*, *Lysandra*, and *Plebicula*. Rapid karyotype diversification may have played a significant role in the radiation of the genus *Agrodiaetus*. [*Agrodiaetus*; *cytochrome oxidase I*; *cytochrome oxidase II*; *elongation factor 1 $\alpha$* ; karyotype diversification; phylogeny; *Polyommatus*; speciation.]

According to the biological species concept, “species are groups of interbreeding natural populations that are reproductively isolated from other such groups” (Mayr, 1970:12). In other words, species are closed genetic systems with unique gene pools. The appearance of new species is accompanied by the establishment of reproductive barriers that are typically viewed as the consequence of geographic isolation of nascent species (Mayr, 1970; White, 1973). Hybrids between species with different karyotypes may have reduced fertility due to problems in meiotic segregation (White, 1973; Gropp et al., 1982; John et al., 1983; King, 1993). Thus, fixed chromosome differences between species may contribute to postzygotic barriers to gene flow. The role of chromosome rearrangements in maintaining postzygotic isolation between well-established species is not controversial, but whether such rearrangements also play a causal role in the initial stages of speciation has excited vigorous scientific debate. The spectrum of views on this problem is broad, from the general denial of their causal role in animals, accepting only polyploidy and special cases such as monobrachial centric fusion (Coyne and Orr, 1998; Spirito, 1998) to the claim that isolation by chromosomal rearrangements could be a major mode of speciation (White, 1973; King, 1993). Theories of chromosomal speciation have been revisited recently, but the emphasis has been on the barriers that rearrangements create to recombination between coadapted gene complexes rather than on the reduction in hybrid fertility (Rieseberg,

2001; Rieseberg and Burke, 2001; Navarro and Barton, 2003).

The degree of hybrid sterility is a function of both karyotypic (White, 1957, 1973; Gropp et al., 1982; King, 1993; Noor et al., 2001; Piálek et al., 2001) and genetic (“genic” of Dobzhansky, 1933, 1937; Coyne, 1984) divergence between populations. It is hard to separate the effects of these two components and determine *a posteriori* which component has been principally responsible for establishing barriers to gene flow between species. In this study, we used genetic divergence to deduce the phylogeny of *Agrodiaetus*, a species-rich genus of butterflies that shows extreme karyotypic diversity. A reliable phylogeny is a necessary prerequisite to evaluation of the relative roles of genetic and karyotypic divergences for speciation within the genus.

Newly rearranged chromosomes usually occur in heterozygotes and are often associated with heterozygote disadvantage. Therefore, their spread to fixation within a species is considered unlikely. Not surprisingly, many taxa do not undergo extensive karyotypic changes during speciation and tend to be karyotypically conservative at the genus or family level. For example, most species in the order Lepidoptera (Insecta) have karyotypes with 31 pairs of chromosomes (Robinson, 1971; Stekolnikov et al., 2000). This chromosome number arose in the Mesozoic and has remained unchanged in most groups for more than 70 million years (Suomalainen, 1969; Lukhtanov, 2000). Some lepidopteran taxa have distinct modal chromosome numbers, however. Most lycaenid butterflies,

for example, have a haploid complement of either 23 or 24 chromosomes (Lesse, 1960b; Robinson, 1971; Lorkovic, 1990; Stekolnikov et al., 2000). Almost every lycaenid with a haploid chromosome number other than 23 or 24 belongs to one of three closely related genera: *Agrodiaetus* (n = 10–125), *Lysandra* (n = 24–92), or *Plebicula* (n = 134–225).

The genus *Agrodiaetus* Hübner, 1822 is distributed through the western Palearctic, predominantly in the Caucasus, Iran, Turkey, and Central Asia. In the most recent review of the genus, Häuser and Eckweiler (1997) recognized 56 species, 113 subspecies, and 28 well-differentiated taxa of unclear taxonomic status, either species or subspecies. The genus currently includes about 90 valid species when new species described since 1997 are added (Olivier et al., 1999; Carbonell, 2000, 2001; Dantchenko, 2000; Hagen and Eckweiler, 2001; Schurian and Hagen, 2001, 2003; Skala, 2001; Lukhtanov and Dantchenko, 2002a).

Species within *Agrodiaetus* are extremely uniform and exhibit few differences in characters traditionally used in classification, such as wing pattern and/or aspects of the male and female genitalia. However, these species vary greatly in their karyotypes, ranging from n = 10 (*A. posthumus*; Lesse, 1959a) to n = 125 (*A. dolus*; Lesse, 1962b; for karyotype images, see Lukhtanov and Dantchenko, 2002a, 2002b). Karyotypes may provide the only diagnostic characters for some *Agrodiaetus* species (Lesse, 1960a, 1961; Lukhtanov, 1989; Kandul, 1997; Kandul and Lukhtanov, 1997), and a description of the karyotype has become a necessary requirement for describing new *Agrodiaetus* species (Lesse, 1957, 1959a, 1959c, 1960a; Lukhtanov et al., 1997; Olivier et al., 1999; Lukhtanov and Dantchenko, 2002a).

Eliot (1973) included the genus *Agrodiaetus* within the section *Polyommatus*. Some taxonomists have preferred to consider *Agrodiaetus* as a subgenus of the large polytypic genus *Polyommatus* Latreille, 1804 (Dantchenko and Lukhtanov, 1993, 1994; Hesselbarth et al., 1995; Eckweiler and Häuser, 1997; Häuser and Eckweiler, 1997; Lukhtanov et al., 1997, 1998; Koçak and Seven, 1998; Dantchenko, 2000). Different groups within *Polyommatus* (*sensu lato*) do not show clear phenotypic differences. Therefore, placements of species within *Polyommatus* (*sensu lato*) have changed repeatedly since the comprehensive revisions by Forster (1936, 1938) and Stempffer (1937) and are still disputed (Hesselbarth et al., 1995). This confusion prompted Bálint and Johnson (1997) to abrogate the taxon *Agrodiaetus* entirely and to consider *Polyommatus* as a polytypic genus containing many species groups, with the species of *Agrodiaetus* parceled out into different species groups. Although the monophyly of different groups within *Polyommatus* (*sensu lato*) and their relationships are questionable, for clarity we have followed Eliot (1973) rather than Bálint and Johnson (1997) and refer to these groups as genera within the *Polyommatus* section.

*Agrodiaetus* species are small (wing span 1.8–3 cm) brown or blue butterflies. Female butterflies are always warm brown, whereas males can have either

TABLE 1. Recognized *Agrodiaetus* species groups and their relationships.

Lesse (1960a)	Hesselbarth et al. (1995)	Eckweiler and Häuser (1997)
Complex 1: Monomorphic species, well-developed androconial scale tuft	<i>admetus</i> group	<i>admetus</i> group
Complex 2: Dimorphic and monomorphic species, well-developed androconial scale tufts	<i>dolus</i> group	<i>dolus</i> group
Complex 3: Dimorphic species, no well-developed androconial scale tufts	<i>damon</i> group <i>actis</i> group <i>transcaspicus</i> group <i>damone</i> group <i>carmon</i> group <i>poseidon</i> <sup>a</sup> group Not considered	<i>damon</i> group <i>dama</i> group <i>iphigenides</i> group (Central Asia) <i>dagmara</i> group (Pamir region) <i>erschoffii</i> group (Eastern Iran and Central Asia)
Dimorphic and monomorphic species, no androconial scale tufts	Not considered	Not considered

<sup>a</sup>Eckweiler and Häuser (1997) split the *poseidon* group of Hesselbarth et al. (1995), with some species transferred to an expanded *damon* group and the remainder forming the new *dama* group. The *dama* and *dagmara* groups were not sampled in our analysis.

blue or brown wings. In the latter case, they resemble females. Thus, a species can be classified as either dimorphic or monomorphic depending on the color of males. Lesse (1960a) divided the genus into three species complexes based on male coloration and the presence of well-developed tufts formed by androconial scales (Table 1). Forster (1956, 1960, 1961) divided the genus into numerous polytypic species based on geographic distribution and classic morphological characters (wing color patterns and genital structure). The karyotype studies of Lesse (1957, 1959a, 1959c, 1960a, 1960b, 1962b), which appeared concurrently with Forster's revision, revealed that *Agrodiaetus* species had extremely diverse karyotypes. Furthermore, some of the species in Forster's system appeared to be complexes of sympatric sibling species that could be identified by their sharply differing karyotypes (Lesse, 1957, 1959a, 1959b, 1960a, 1962a). New *Agrodiaetus* species were described exclusively on the basis of sympatric and temporal cooccurrence of karyotypically distinct "races" (Lesse, 1960a).

Subsequent analyses of the scarce morphological characters resulted in partitioning of *Agrodiaetus* into species groups named after their oldest members (Table 1). In the classification of Hesselbarth et al. (1995), *Agrodiaetus* was divided into eight species groups: *actis*, *admetus*, *carmon*, *damon*, *damone*, *dolus*, *poseidon*, and *transcaspicus*. Eckweiler and Häuser (1997) recognized the *admetus* and *dolus* groups but argued that available evidence was too weak to support the remaining groups. Instead, they erected a more inclusive *damon* group that combined the membership of Hesselbarth et al.'s *actis*, *carmon*, *damon*,

*damone* and *transcaspicus* groups with some species from the *poseidon* group. The remainder of the *poseidon* group was renamed the *dama* group, and three additional species groups, the *dagmara*, *erschoffii* (= *Paragrodiaetus* Rose and Schurian, 1977) and *iphigenides* groups, were erected to accommodate species from eastern Iran and Central Asia that had not been considered by Hesselbarth et al. (1995).

Although *Agrodiaetus* has recently attracted interest from taxonomists (Carbonell, 1998, 2000, 2001; Koçak and Seven, 1998; Olivier et al., 1999; Lukhtanov and Dantchenko, 2002b; Rose, 2002), in only one study has the monophyly of *Agrodiaetus* been tested. Mensi et al. (1994) found *Agrodiaetus* to be monophyletic, but their study included representatives of only 3 of the 12 species groups and used only a single outgroup from within the section *Polyommatus*. Therefore, basic questions remain to be resolved regarding the monophyly of *Agrodiaetus* and evolutionary relationships among its species before the role of karyotype diversification in the radiation of *Agrodiaetus* can be analyzed.

#### MATERIALS AND METHODS

##### *Agrodiaetus* Species Sampling and Choice of Outgroups

We sampled representatives from 10 of the 12 species groups of *Agrodiaetus* and from 14 related genera within the section *Polyommatus* (Appendix 1) in an effort to test *Agrodiaetus* monophyly. Two *Agrodiaetus* species groups were not included because we were unable to obtain specimens. In particular, species from the *dagmara* group occur in remote localities of the Pamir region of Tadjikistan and are extraordinarily difficult to collect. We were also unable to obtain specimens of the *dama* group. In choosing potential outgroups, we followed Forster (1938), who considered *Agriades*, *Albulina*, *Aricia*, *Cyaniris*, *Eumedonia*, *Lycalides*, *Lysandra*, *Meleageria*, *Polyommatus*, *Plebejus*, and *Vacciniina* to be closely related to *Agrodiaetus*. Since Forster's time, new genera have been delineated from among these taxa, including *Neolysandra*, *Plebicula*, *Rimisia*, and *Sublysandra*. We were able to obtain representatives of all these genera except *Albulina*. Of the taxa sampled in this study, only *Aricia*, *Agriades*, *Plebejus*, and *Polyommatus* were treated as distinct genera by Bálint and Johnson (1997). The rest of the taxa, including *Agrodiaetus*, *Cyaniris*, *Lysandra*, *Meleageria*, *Neolysandra*, *Plebicula*, and *Sublysandra*, were designated as synonyms of *Polyommatus*.

Four *Agrodiaetus* specimens originally were not identified to the species level. These specimens were assigned to species groups using morphological characters. We were unable to obtain karyotypes for three of these specimens. A karyotype was determined for the specimen VL01L342, but this did not help to identify it to the species level. The specimen was phenotypically close to *A. eriwanensis* ( $n = 34$ ) and *A. eriwanensis interjectus* ( $n = 29-32$ ; Lesse, 1960a), but its karyotype was different ( $n = 40-42$ ). In our analysis, *A. eriwanensis*

*eriwanensis* with  $n = 34$  was a sister species of this specimen. Hesselbarth et al. (1995) placed *A. interjectus* and *A. eriwanensis* in the *admetus* group. According to the recovered phylogeny, *A. eriwanensis* and the specimen VL01L342 clustered among species from the *dolus* group (Fig. 1). Unfortunately, *A. interjectus* was not represented in our analysis. Recently, the specimen VL01L342 was used as a paratype for the newly described *A. dantchenko* Lukhtanov and Weimers 2003 (Lukhtanov et al., 2003). The other three unidentified specimens have been given reference numbers.

##### DNA Extraction and Gene Sequencing

Many *Agrodiaetus* species are difficult to identify accurately without karyotype information. Therefore, testes were extracted from male specimens and fixed for karyotype analysis in freshly prepared Carnoy fixative (100% ethanol and 100% acetic acid, 3:1) before the specimen's body was put into 100% ethanol for DNA preservation. In most cases, the same *Agrodiaetus* specimen was used for karyotype and phylogenetic analyses. In some cases, we could not obtain karyotypes from collected specimens. In these cases, we used karyotypes previously obtained from the same population (if available) or from a different population of the same taxon. The specimens used in this study and the source of karyotype data are listed in Appendix 1. All specimens are deposited in the DNA and Tissues collection of the Museum of Comparative Zoology (Harvard University, Cambridge, MA).

Four abdominal segments were used for DNA extraction. The segments were homogenized in 2% SDS buffer and digested with proteinase K (20 mg/ml) for at least 3 hr at 60°C. DNA was purified through successive ethanol precipitations and stored in Tris-EDTA buffer (pH 8.0) at -20°C. Two mitochondrial genes, cytochrome oxidase subunit I (*COI*) and cytochrome oxidase subunit II (*COII*), and a nuclear gene, elongation factor 1- $\alpha$  (*EF1- $\alpha$* ), were used to reconstruct the phylogeny of *Agrodiaetus*. The primers used for the mitochondrial DNA (mtDNA) amplification have been described (Rand et al., 1999; Monteiro and Pierce, 2001). PCRs (50  $\mu$ l) were carried out in the DNA Engine thermal cycler (MJ Research) and typically contained 0.5  $\mu$ M of each primer, 0.8 mM dNTPs, Qiagen (Valencia, CA) PCR buffer with additional MgCl<sub>2</sub> to a final concentration of 2 mM, and 1.25 units Qiagen *Taq* DNA polymerase. The typical thermal profile was 37 cycles of 95°C for 60 sec, 47°C for 60 sec and 72°C for 90 sec. Touchdown PCR with a starting annealing temperature of 53°C and Qiagen Q solution was used to amplify three fragments of *EF1- $\alpha$*  with primers listed in Table 2. After amplification, the double-stranded DNA was purified using QIAquick PCR purification kits (Qiagen) prior to direct sequencing. Cycle sequencing reactions (10  $\mu$ l) were performed using ABI Prism Big Dye 2 terminator cycle sequencing kits (Applied Biosystems, CA). Both strands of the PCR product were sequenced in a 3100 Genetic Analyzer (Applied Biosystems/Hitachi).

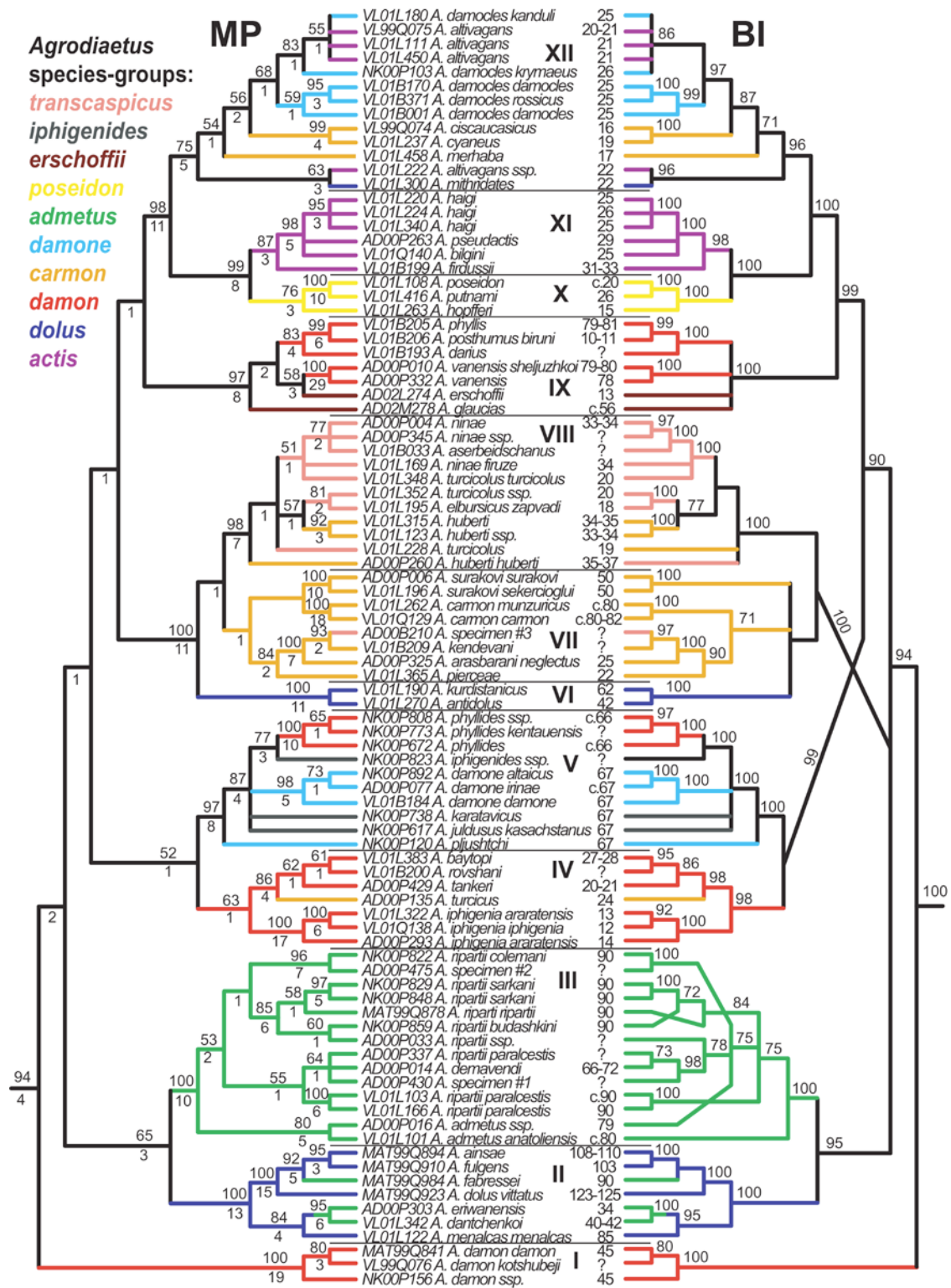


FIGURE 1. Maximum parsimony (MP) and Bayesian inference (BI) ingroup trees of *Agrodiaetus* inferred from 113 sequences of *COI* and *COII*. The strict consensus tree (MP) was constructed from 7,433 MP trees: Total length = 2,508; consistency index = 0.356; retention index = 0.719. Bootstrap values >50% and Bremer support are shown above and below recovered branches, respectively. The 70% majority consensus tree was recovered from Bayesian trees sampled during four independent Bayesian analyses under the GTR+I+Γ model for DNA substitution: mean negative log likelihood = 16147.25 ± 12.1. The posterior probability is shown above every branch on the BI tree. Recognized *Agrodiaetus* species groups are mapped on the inferred topologies. Recovered clades are numbered with successive Roman numerals. Clade VII is monophyletic on the MP tree but paraphyletic on the BI tree. Haploid chromosome numbers are shown to the right of the names of specimens (for details, see Appendix 1).

TABLE 2. Primers used for the amplification of *EF1-α*.

Primer <sup>a</sup>	Position	Sequence (5' → 3')	Max length of amplified product (bp)
ef135F	134	CAAATGYGGTGGTATYGACAAACG	555
ef684R	684	TCCTTRCGCTCCACSTGCCAYCC	
ef531F	530	TACAGYGAGCSCCGTTTTYGAGGA	445
ef929R	930	GCYTCYTGGAGAGCYTCGTGGTG	
ef51.9F	798	CARGACGTATACAAAATCGG	575
efrcM4R	1351	ACAGCVACKGYTGYCTCATRTC	

<sup>a</sup>F = Forward; R = reverse.

<sup>b</sup>Positions are given relative to the published *EF1-α* sequence of *Bombyx mori* L. (Kamiie et al., 1993).

### Phylogenetic Analysis

*Sequence alignments and characteristics.*—Mitochondrial and nuclear sequences were edited and aligned against the total mtDNA sequence (GenBank NC.002355; Lee et al., 1999) and the *EF1-α* sequence (GenBank, D13338; Kamiie et al., 1993) of *Bombyx mori* L. using Sequencher 3.1 (Genecodes Corporation, Ann Arbor, MI). Alignments were unambiguous for both mitochondrial and nuclear gene fragments. All fragments of translated genes were of equal length except the *COII* sequences of *Eumedonia persephatta minshelkensis* and *Vacciniina fergana*, which had a triplet insertion in the same position. This insertion was excluded from further phylogenetic analysis. Primer sequences were cropped, and missing data and ambiguities were designated by the letter "N." All sequences were submitted to GenBank (AY496709–AY496850).

The 113 continuous sequences of *COI*, *tRNA-leu*, and *COII* genes were aligned in a data set that was partitioned into respective genes using PAUP\* 4.0b10 (Swofford, 2000). The *tRNA-leu* gene was excluded from further phylogenetic analysis. Additional *EF1-α* sequences were obtained for a representative of each outgroup genus (Appendix 1) and each major *Agrodieta* clade recovered in our analysis (Fig. 1). *EF1-α* was also sequenced from *A. erschoffii* and *A. glaucias* because Rose and Schurian (1977) had segregated these species into a distinct genus,

*Paragrodiaetus*. Separate sequences of *COI*, *COII*, and *EF1-α* genes were concatenated into a single partitioned data set in MacClade (Maddison and Maddison, 1992). The character statistics were presented separately for total, ingroup, and outgroup sequences from each data set in Table 3. Base frequency homogeneity was tested separately for every data set with the  $\chi^2$ -test in PAUP\*. Because only informative sites can affect the phylogenetic analysis, uninformative sites were excluded from these tests.

We used a partition homogeneity test (incongruence length difference [ILD]; Farris et al., 1995) to acquire preliminary data on the homogeneity of our data sets. ILD tests were performed in PAUP\* using heuristic searches with tree bisection–reconnection (TBR) branch swapping and 100 random taxon addition replicates, saving no more than 100 equally parsimonious trees per replicate. Recent studies (Cunningham, 1997; Barker and Lutzoni, 2002; Darlu and Lecointre, 2002; Dolphin et al., 2002; Downton and Austin, 2002) have cast doubt on the utility of the ILD test for detecting incongruence between different data sets caused by differences in evolutionary constraints and/or tree topologies. Therefore, we used additional data to make a final decision whether to combine data sets or analyze them separately.

*Maximum parsimony.*—Maximum parsimony (MP) analysis was conducted with PAUP\*. A heuristic search was performed with TBR branch swapping and 1,000 random taxon addition replicates, saving no more than 100 equally parsimonious trees per replicate. In addition, PAUPRat (Sikes and Lewis, 2001) was used for MP analysis of the large *COI* + *COII* data set because of its speed in searching large data sets (Nixon et al., 1998; Nixon, 1999). During 200 iterations in PAUPRat, 10%, 15%, and 20% of equally weighted characters were perturbed. Tree lengths reported in this study included parsimony-uninformative characters. To estimate branch support on the recovered topology, nonparametric bootstrap (bt) values (Felsenstein, 1985) and Bremer support (Br; Bremer, 1994; for the discussion on the interpretation of Bremer support, see DeBry, 2001) were assessed with

TABLE 3. Character statistics for different data sets used in the study.

No. sequences	No. (%) parsimony-informative sites	No. (%) 1st codon position sites	No. (%) 2nd codon position sites	No. (%) 3rd codon position sites	Frequency of A + T (mean ± SD)
<i>COI</i> (1,277 bp)					
Total: 113	330 (25.8)	57 (4.5)	12 (0.9)	261 (20.4)	0.7212 ± 0.007
Ingroup: 91	234 (18.3)	35 (2.7)	6 (0.5)	193 (15.1)	0.7193 ± 0.005
Outgroup: 22	205 (16)	29 (2.3)	5 (0.4)	171 (13.4)	0.7291 ± 0.007
<i>COII</i> (692 bp)					
Total: 113	183 (26.4)	32 (4.6)	12 (1.7)	139 (19.8)	0.7730 ± 0.006
Ingroup: 91	122 (17.6)	17 (2.5)	8 (1.2)	97 (14)	0.7724 ± 0.006
Outgroup: 22	102 (14.7)	17 (2.5)	5 (0.7)	80 (11.6)	0.7756 ± 0.008
<i>COI</i> + <i>COII</i> (1969 bp)					
Total: 29	327 (16.6)	46 (2.3)	13 (0.7)	268 (13.6)	0.7422 ± 0.008
Ingroup: 14	140 (7.1)	19 (0.9)	4 (0.2)	117 (5.9)	0.7375 ± 0.004
Outgroup: 15	234 (11.9)	31 (1.6)	8 (0.4)	195 (9.9)	0.7465 ± 0.008
<i>EF1-α</i> (1195 bp)					
Total: 29	88 (7.4)	6 (0.5)	0 (0)	82 (6.9)	0.3943 ± 0.005
Ingroup: 14	19 (1.6)	4 (0.3)	0 (0)	15 (1.2)	0.3923 ± 0.002
Outgroup: 15	54 (4.5)	2 (0.2)	0 (0)	52 (4.3)	0.3962 ± 0.006

PAUP\* and Autodecay 4.0.2' (Eriksson, 1998), respectively. One thousand bootstrap pseudoreplicates were analyzed under a heuristic search with TBR branch swapping and 100 random taxon addition replicates, saving no more than 100 equally parsimonious trees per replicate. The same settings for the heuristic search were used to estimate Bremer support.

**Substitution models.**—The general time reversible model with invariant sites and gamma distribution (GTR+I+ $\Gamma$ ) was the substitution model selected for the *COI* + *COII* data sets (113 and 29 sequences, respectively) by both hierarchical likelihood ratio tests (hLRTs; Huelsenbeck and Rannala, 1997) and the Akaike information criterion (AIC; Akaike, 1974) as implemented in Modeltest 3.06 (Posada and Crandall, 1998). The application of Modeltest 3.06 to the *EF1- $\alpha$*  data set resulted in two different substitution models. The Tamura and Nei (1993) model with invariable sites and gamma distribution (TrNef+I+ $\Gamma$ ) was chosen by hLRTs, and the GTR+I+ $\Gamma$  model was chosen by AIC. The GTR+I+ $\Gamma$  model was chosen for the combined *COI* + *COII* + *EF1- $\alpha$*  data set by both hLRTs and the AIC.

**Bayesian inference.**—Bayesian analysis was done in a likelihood framework as implemented by MrBayes 2.01 (Huelsenbeck, 2000; Huelsenbeck and Ronquist, 2001) with uninformative priors. Multiple Bayesian searches using Metropolis-coupled Markov chain Monte Carlo sampling were conducted. One cold and three heated Markov chains, applying MrBayes default heating values ( $t = 0.2$ ), were used in the analysis. Because the TrNef+I+ $\Gamma$  model is not implemented in MrBayes 2.01, the GTR+I+ $\Gamma$  model was used for every data set. Model parameter values were treated as unknown and were estimated in each analysis. To ensure that our Bayesian analyses were not trapped in local optima, each analysis was run four times, starting from different random trees, and average log-likelihood (lnL) values ( $\pm$ SD) at stationarity were calculated with Microsoft Excel and compared for convergence. Each Bayesian analysis was run for 1,000,000 generations, with trees sampled every 100 generations. The number of sampled trees to be discarded as representing the burn-in period was determined graphically. To estimate posterior probabilities (pP) of recovered branches (Larget and Simon, 1999; Huelsenbeck et al., 2001), 70% majority rule consensus trees were created from the remaining trees using PAUP\*. Phylograms were created as average-branch-length consensus trees with MrBayes.

**Dating phylogenetic events.**—To test the homogeneity of substitution rates (for the molecular clock hypothesis) across the average-branch-length consensus of Bayesian trees recovered for the larger *COI* + *COII* data set, we applied the LRT (Huelsenbeck and Rannala, 1997) with and without the enforcement of a molecular clock. Likelihood scores of the ingroup topology (Fig. 1) were assessed under the GTR+I+ $\Gamma$  model as implemented in PAUP\*. The LRT revealed a significant deviation from rate consistency ( $P < 0.001$ ) across different branches. Therefore, a nonparametric rate smoothing (NPRS) algorithm (Sanderson, 1997), implemented in TreeEdit 1.0

(Rambaut and Charleston, 2002), was used to homogenize evolutionary rates across the topology. Mean uncorrected pairwise distances, calculated in MEGA2 (Kumar et al., 2001), were used to calibrate the smoothed topology.

**Character-state reconstruction.**—MacClade 4.0 (Maddison and Maddison, 1992) was used to reconstruct the ancestral karyotype for the *Agrodiaetus* lineage. To decrease potential bias of certain chromosome numbers, only single individuals of every sampled *Agrodiaetus* species with known chromosome numbers was used. Thus, mitochondrial sequences of 71 distinct species were used to build a topology for the tracing of karyotype changes. The strict consensus was built from 56 recovered MP trees: total length = 2,144; consistency index (CI) = 0.392; retention index (RI) = 0.627. Haploid chromosome numbers (n) were arbitrarily coded as 12 ordered character states and mapped on the recovered phylogeny. The original PAUP\* and MacClade files are available from the authors.

## RESULTS

### *Analysis of the COI + COII Data Set*

The alignment of 113 *COI* + *COII* sequences recovered 513 parsimony-informative characters (26% of 1,969 sites; 25.8% from *COI* and 26.4% from *COII*; Table 3). The application of the  $\chi^2$ -test in PAUP\* did not reject the hypothesis of homogeneity of nucleotide frequencies for parsimony-informative characters in every pair of taxa ( $P = 0.99$ ). Estimates of substitution rates in *COI* and *COII* were similar for first and third codon positions, as reflected by the percentage of parsimony-informative characters. By contrast, the substitution rate for the second codon position in the *COII* gene (1.7%) was twice as high as that for the *COI* gene (0.9%, Table 3).

The frequency of adenine (A) and thymine (T) was slightly higher for outgroup sequences in every data set presented in Table 3. This higher frequency of A + T can be explained by the older age of the outgroup species and their concomitant higher saturation levels at the third codon position. The *COII* gene fragment had a higher saturation rate of A + T ( $0.7730 \pm 0.0006$ ) than did the *COI* gene fragment ( $0.7212 \pm 0.007$ , Table 3). High levels of A + T saturation in mitochondrial sequences (>75%) are well known for insects, especially at third codon positions (Crozier et al., 1989; Pedersen, 2002). The relative increase of A + T saturation between *COII* and *COI* was greater for first and second codon positions (6.6%) than for third codon positions only (2.6%). Comparing the sequenced genomes of *Apis mellifera* and *Drosophila melanogaster*, Crozier and Crozier (1993) reported that *COII* had a higher substitution rate than did *COI* in insects. This difference in substitution rate could contribute to the higher A + T saturation for *COII*.

The ILD test between full-length sequences of *COI* and *COII* revealed no significant conflict ( $P = 0.14$ ). However, when the ILD test was performed for two and three

equal size fragments of *COI* and *COII*, following the suggestion of Downton and Austin (2002), significant phylogenetic conflict was revealed ( $P = 0.01$ ). Significant phylogenetic conflict ( $P = 0.01$ ) also was found between equal size fragments from within the *COI* gene. Given that (1) mitochondrial genes usually do not recombine (for review, see Eyre-Walker and Awadalla, 2001) and (2) actively translated mitochondrial genes evolve under similar conditions, these results were unexpected. However, the ILD test is particularly prone to type I errors (rejecting the true hypothesis; Darlu and Lecointre, 2002), different levels of homoplasy and phylogenetic noise between large data sets *per se* can also cause significant conflict (Dolphin et al., 2002) and the heterogeneity of evolutionary rates of data sets can make results of the ILD test misleading (Barker and Lutzoni, 2002). Therefore, we questioned the results of the ILD tests that detected incongruence between equal size fragments of *COI* and *COII*, and we combined the two gene fragments in our analysis.

The ratio of transitions to transversions (Ti/Tv) in third codon positions was plotted against the number of transversions in third codon positions to assess the degree of saturation due to multiple transitions in third codon positions. The Ti/Tv ratio expected at saturation (following Holmquist, 1983) was not reached (0.166; plot not shown). A partition homogeneity test (ILD) for the combined data set of *COI* + *COII* did not reveal significant conflict between first and second versus third codon positions ( $P = 0.4$ ). Therefore, we assigned equal weight to transitions and transversions for all sites in the analysis. We also performed MP analysis using various alternative weighting schemes. These gave the same strict consensus tree, except that the weakly supported clade VII became paraphyletic in some analyses (data not presented).

Heuristic search with unlimited number of trees saved per replicate in PAUP\* resulted in 7,433 MP trees of TL = 2,508, CI = 0.356, and RI = 0.719 (Figs. 1, 2). Three independent trials using perturbations of 10%, 15%, and 20% of parsimony-informative characters during 200 iterations in PAUPRat yielded 176, 140, and 125 MP trees, respectively, of TL = 2,508, CI = 0.356, and RI = 0.719. The strict consensus of 441 MP trees recovered by PAUPRat had the same topology as the strict consensus of 7,433 MP trees inferred by the heuristic search (LRT under the GTR+I+ $\Gamma$ :  $-\ln L_1 = 16076.516$  and  $-\ln L_2 = 16059.662$ ;  $\chi^2_{111} = 33.7$ ,  $P = 1.0$ ).

Four independent Bayesian analyses converged on statistically equivalent log-likelihood scores and reached stationarity at no later than 200,000 generations (plots not shown). Majority consensus trees of four rounds of Bayesian analysis were identical, and the posterior probability values supporting congruent nodes were highly correlated (not shown), providing support for the assumption that the analyses converged on a single optimum. The majority consensus trees are presented in Figures 1 and 2. Figure 1 illustrates relationships within *Agrodiætus*, whereas Figure 2 presents relationships between *Agrodiætus* and the outgroups.

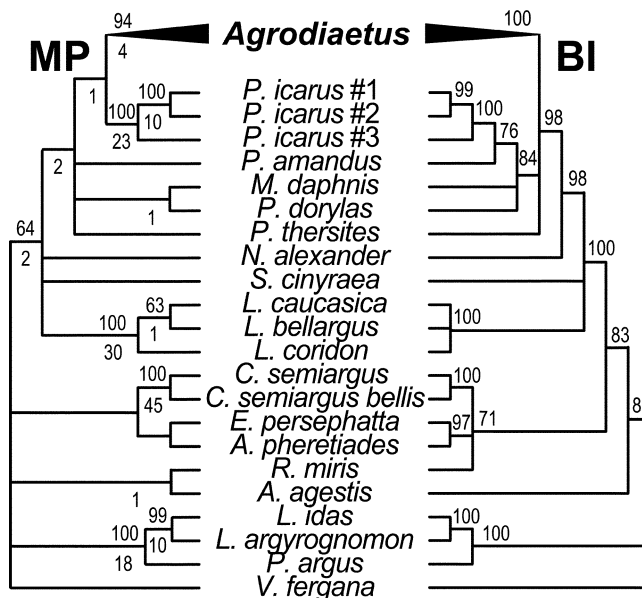


FIGURE 2. Maximum parsimony (MP) and Bayesian inference (BI) outgroup trees of *Agrodiætus* inferred from 113 sequences of *COI* and *COII*. The strict consensus tree (MP) was constructed from 7,433 MP trees: TL = 2508, CI = 0.356, and RI = 0.719. Values for bt > 50% and Br are shown above and below recovered branches, respectively. The 70% majority consensus tree was recovered from Bayesian trees sampled during four independent Bayesian analyses under the GTR+I+ $\Gamma$  model for DNA substitution: mean  $-\ln L = 16147.25 \pm 12.1$ . The pP is shown above every branch on the BI tree.

The monophyly of *Agrodiætus* is well supported by both MP (bt = 94%; Br = 4) and Bayesian inference (BI) analyses (pP = 1.00) (Figs. 1, 2). Although the MP and BI trees are similar, there are some differences at both the ingroup and outgroup levels. The most conspicuous difference is the position of clades IV + V and VI + VII + VII within *Agrodiætus* (Fig. 1). However, the placement of these clades on the MP tree was poorly supported (bt < 50 and Br = 1). The use of site-specific substitution rates for different codon positions (instead of substitution rates under the gamma distribution) with the GTR+I model did not substantially change the inferred BI tree, although clade XII appeared paraphyletic (Fig. 1). According to the MP analysis, the genus *Polyommatus* is sister to *Agrodiætus*, whereas under the Bayesian analysis, *Polyommatus thersites* and the clade ((*P. icarus*, *P. amandus*) *Meleageria daphnis*, *Plebicula doryllas*) forms an unresolved trichotomy with *Agrodiætus* (Fig. 2).

*COI* + *COII* sequences provided considerable resolution within *Agrodiætus*. The alignment of 91 ingroup sequences recovered 356 parsimony-informative characters (18% of sites). Constraints corresponding to the proposed systems (Table 1) of Hesselbarth et al. (1995) and Eckweiler and Häuser (1997) were enforced during a heuristic search in PAUP\* to test current hypotheses of *Agrodiætus* classification. Likelihood scores of the strict consensus trees of the resultant MP trees were calculated in PAUP\* under GTR+I+ $\Gamma$ . These analyses yielded

$-\ln L = 17268.669$  for Hesselbarth et al. (1995),  $-\ln L = 17241.007$  for Eckweiler and Häuser (1997), and  $-\ln L = 16059.662$  without any constraints. Thus, both prior taxonomic hypotheses were rejected by a likelihood ratio test ( $\chi^2_{111} = 2418.014$  and  $\chi^2_{111} = 2362.69$ , respectively;  $P = 0.0000$ ).

Our *Agrodiaetus* specimens could be divided into 12 clades (I–XII, Fig. 1). Each clade was recovered by both MP and BI methods except clade VII, which was monophyletic in all MP trees but paraphyletic on the BI tree (Fig. 1). Each of the clades I, III, VI, X, and XI contained members of a single species group. Three subspecies of *A. damon* form clade I (bt = 100; Br = 19, pP = 1.00; Fig. 1) at the base of the *Agrodiaetus* clade, whereas the remainder of the *damon* group are placed in clades IV, V, and IX. Most members of the *admetus* group are found in clade III (with the remainder in clade II). Most members of the *dolus* group are clustered together in clade II, but two species, *A. antidolus* and *A. kurdistanicus*, together form clade VI (Fig. 1). The three representatives of the *poseidon* group in our sample grouped together as clade X (bt = 76; Br = 3; pP = 1.00; Fig. 1). Most members of the *actis* group are found in clade XI (with the remainder in clade XII).

Our analysis recovered substantial structure among *Agrodiaetus* clades, with broad agreement between MP and BI trees (Fig. 1). Clade I was recovered as basal to the rest of *Agrodiaetus* (clades II–XII) in both analyses. The larger of these clades had bt < 50, Br = 2, and pP = 0.94. Within this clade, both analyses recovered four subclades: (II, III), (IV, V), (VI, VII, VIII), and (IX, (X, XI, XII)), with ((X, XI), XII) particularly well supported. MP and BI analyses differed as to the relationships among these subclades.

The hypothesis of phylogenetic independence between chromosome numbers and the recovered ingroup topology was tested using the test for serial independence (TFSI; Abouheif, 1999) implemented in Phylogenetic Independence 2.0 (Reeve and Abouheif, 2003). Only single representatives of *Agrodiaetus* species with known chromosome numbers were used in this test. The test rejected the hypothesis that chromosome numbers were not correlated with phylogeny ( $P = 0.0001$ ).

#### *Analysis of the COI + COII + EF1- $\alpha$ Data Set*

*EF1- $\alpha$*  has provided substantial resolution and support for recovered branches at the species and genus levels among Lepidoptera (Cho et al., 1995; Monteiro and Pierce, 2001). However, in our analysis, 29 *EF1- $\alpha$*  sequences of a 1,195-bp region provided only 88 parsimony-informative characters (7.4% of all sites; Table 3). The alignment of 14 ingroup sequences of *Agrodiaetus* provided only 19 parsimony-informative characters (1.6% of total sites), whereas 15 outgroup sequences recovered 54 parsimony-informative characters. The application of the  $\chi^2$ -test in PAUP\* did not reject the hypothesis of homogeneity of nucleotide frequencies for parsimony-informative characters in every pair of taxa ( $P = 0.87$ ).

Transition and transversion substitutions were treated equally at every site during MP analysis. A partition homogeneity test (ILD) conducted under a heuristic search in PAUP\* revealed a significant conflict ( $P = 0.04$ ) between the phylogenetic signals of *COI* + *COII* combined and *EF1- $\alpha$*  sequences and between equal size fragments (1,195 bp) of *COI* and *EF1- $\alpha$*  ( $P = 0.02$ ). However, the  $P$  value recovered in both tests was above the critical value ( $P = 0.01$ – $0.001$ ) suggested by Cunningham (1997) in testing for incongruence between different data sets. Therefore, we chose to perform separate and combined analyses of these gene fragments.

A heuristic search with equally weighted characters yielded 6,629 MP trees for the *EF1- $\alpha$*  data set (TL = 354; CI = 0.678; RI = 0.562). Four independent Bayesian analyses converged on statistically equivalent log-likelihood scores and reach stationarity at no later than 50,000 generation (plots not shown). The constructed majority consensus trees were identical, whereas posterior probabilities of congruent branches were almost equal. The strict consensus of 6,629 MP trees and a 70% consensus of trees sampled in Bayesian analyses are shown in the Figure 3. According to both the MP and BI methods, the monophyly of *Agrodiaetus* was well supported (bt = 90; Br = 4; pP = 1.00). Bayesian analysis of the *EF1- $\alpha$*  data set provided better resolution and support than the MP analysis at the outgroup level (Fig. 3). According to the BI majority tree, *Lysandra bellargus* and *Meleageria daphnis* form a clade (pP = 0.80), which is the sister lineage to *Agrodiaetus*. The clade containing these three taxa has pP = 0.73 (Fig. 3). Three clades comprising (1) *Polyommatus amandus* + *P. icarus* + *Sublysdandra cinnyraea*; (2) *Lysandra bellargus* + *Meleageria daphnis*; and (3) *Plebicula dorylas* formed a polytomy with *Agrodiaetus* on the BI phylogram (figure not shown). *Lycaeides argyrognomon* + *Plebejus argus* formed a clade on both the MP and BI trees (bt = 56; Br = 1; pP = 0.99).

The alignment of the 29 *COI* + *COII* sequences recovered 327 parsimony-informative characters (16.6% of the total sites; Table 3). A  $\chi^2$ -test in PAUP\* rejected the hypothesis of homogeneity of nucleotide frequencies for parsimony-informative characters ( $P = 0.0002$ ). There is little evidence that heterogeneity of nucleotide composition leads to significant phylogenetic error (Rosenberg and Kumar, 2003). However, in an attempt to rule out the possible effect of heterogeneity on tree inference, we performed a LogDet (LD) distance transformation (Lockhart et al., 1994) while accounting for invariable sites (LD+I) on the 29 *COI* + *COII* sequences in PAUP\*. The LD distance transformation was specifically designed to account for a strong bias in nucleotide frequencies among sequences (Lockhart et al., 1994). A heuristic search for a minimum evolution objective function was used to infer distance trees from the DNA distances calculated under LD+I transformation and the GTR+I+ $\Gamma$  model in PAUP\*. The null hypothesis of no difference between the two trees was not rejected by the Kishino–Hasegawa parametric test ( $P = 0.5128$ ; Kishino and Hasegawa, 1989) or by the Templeton nonparametric test ( $P = 0.5127$ ; Templeton,



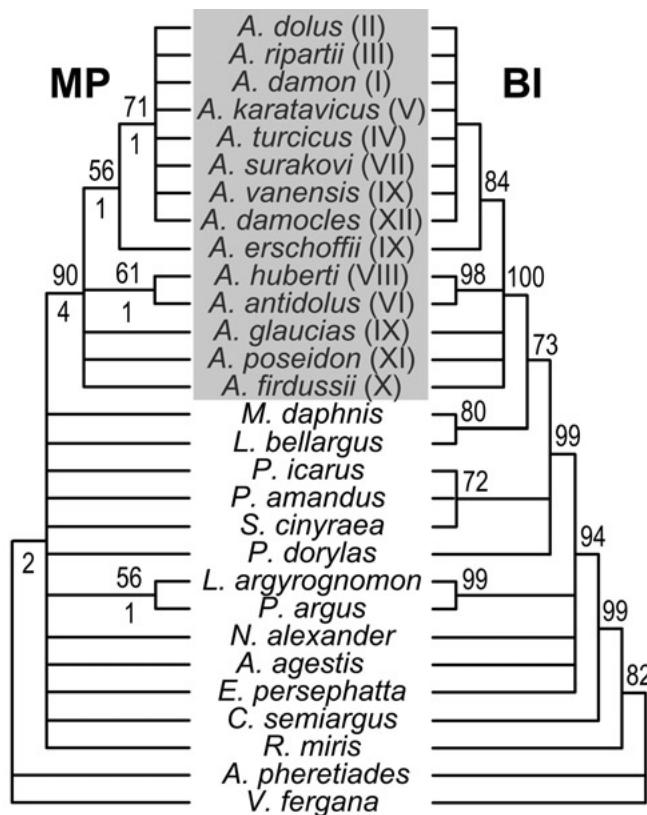


FIGURE 3. Maximum parsimony (MP) and Bayesian inference (BI) trees inferred from 29 sequences of the *EF1- $\alpha$*  gene. The strict consensus tree (MP) was constructed from 6,629 MP trees: TL = 354, CI = 0.678, and RI = 0.562. Values for bt > 50% and Br are shown above and below recovered branches, respectively. The 70% majority consensus tree was recovered from Bayesian trees sampled during four independent Bayesian analyses under the GTR+I+ $\Gamma$  model for DNA substitution: mean  $-\ln L = 3816.20 \pm 7.2$ . The pP is shown above every branch on the BI tree. The shaded block highlights sampled *Agrodiaetus* species. The Roman numerals correspond to *Agrodiaetus* clades (see Fig. 1).

1983). The distance trees built with Tajima–Nei distances (Tajima and Nei, 1982) and Kimura two-parameter distances (Kimura, 1980) were identical to the tree built with LD+I distances (TL = 1,873; CI = 0.513; RI = 0.431;  $-\ln L = 14075.3125$  under the GTR+I+ $\Gamma$  model). Therefore, we carried out our phylogenetic analysis without accounting for the heterogeneity of nucleotide frequencies.

A heuristic search performed in PAUP\* yielded 22 MP trees of TL = 1,470, CI = 0.485, and RI = 0.437 for the 29 *COI* + *COII* sequences. Although the MP trees inferred from separate phylogenetic analysis of *COI* + *COII* and *EF1- $\alpha$*  differed in branching order at both the ingroup and outgroup level, these differences were not well supported (Fig. 4). The group *Lycaeides argyrognomon* + *Plebejus argus* is well supported in both of these MP trees (Fig. 4). The main relationships among *Agrodiaetus* clades inferred from the large data set of *COI* + *COII* (113 sequences) were also recovered using the small data set of *COI* + *COII* (29 sequences). In particular, subclades (II, III), (IV, V), (VI, VII, VIII), and ((X, XI),

XII) (Figs. 1 and 4) were supported from analyses of both data sets.

The analysis of the combined data matrix of *COI* + *COII* + *EF1- $\alpha$*  (29 sequences) resulted in well-supported MP and BI trees. A heuristic search recovered 12 MP trees of TL = 1,852, CI = 0.515, and RI = 0.440. Four independent Bayesian analyses converged on statistically equivalent log-likelihood scores and reached stationarity at no later than 60,000 generation (plots not shown). The constructed majority consensus trees are identical, and posterior probabilities of congruent branches are almost equal. The monophyly of *Agrodiaetus* was again recovered with bt = 97, Br = 7, and pP = 1.00 (Fig. 4). According to the strict consensus of 12 MP trees, *Polyommatus icarus* and *P. amandus* form a sister clade to *Agrodiaetus* (bt < 50; Br = 1), whereas *P. icarus* is the sister to *Agrodiaetus* on the average-branch-length consensus of sampled Bayesian trees (topology not shown). A more inclusive group is formed by *Agrodiaetus*, *Polyommatus*, and *Plebicula dorylas* + *Meleageria daphnis* (bt = 51; Br = 2; pP = 1.00; Fig. 4). This group together with *Neolysandra alexander* and *Lysandra bellargus* + *Sublysandria cinyraea* form a clade with bt = 91, Br = 9, and pP = 1.00. The MP and BI trees differ in the position of *Rimisia miris* and *Eumedonia persephatta* (Fig. 4). However, the position of *Rimisia miris* on the MP tree is weakly supported (bt < 50, Br = 0). The clade *Lycaeides argyrognomon* + *Plebejus argus* was recovered on both the MP and BI trees (bt = 100; Br = 18; pP = 1.00; Fig. 4). The BI tree has better resolution than the MP tree at the outgroup level. Thus, *Vacciniina fergana* and *Agriades pheretiades* are the taxa most distant from *Agrodiaetus*, followed by *Rimisia miris* and *Cyaniris semiargus*.

#### Dating Main Phylogenetic Events

To calibrate a molecular clock, a branching point on a tree must be linked to a particular geological event so that substitution rate can be scaled in evolutionary time. Unfortunately, our data are not amenable to such an exercise because lycaenid butterflies lack a useable fossil record. Additionally, our current sample does not include a phylogeographic event that could be independently dated. Therefore, we used two different published estimates of the mtDNA substitution rate in arthropods. The substitution rate of *COI* sequence in *Tetraopes* beetles (Farrell, 2001) provided a slower estimate of 1.5% uncorrected pairwise distance per million years and hence older estimated ages. The substitution rate for the entire mtDNA genome of various arthropod taxa (Brower, 1994) provided a faster estimate of 2.3% uncorrected pairwise distance per million years and hence younger estimated ages. The use of two different rate estimations to calibrate the recovered tree was intended to correct partially for the potential bias generated by not taking into account the sequence divergence already presented in the ancestral population at the time of its divergence into evolutionary distinct lineages.

The Bayesian topology of average-branch-length consensus reconstructed from the data set of 113 *COI* + *COII*

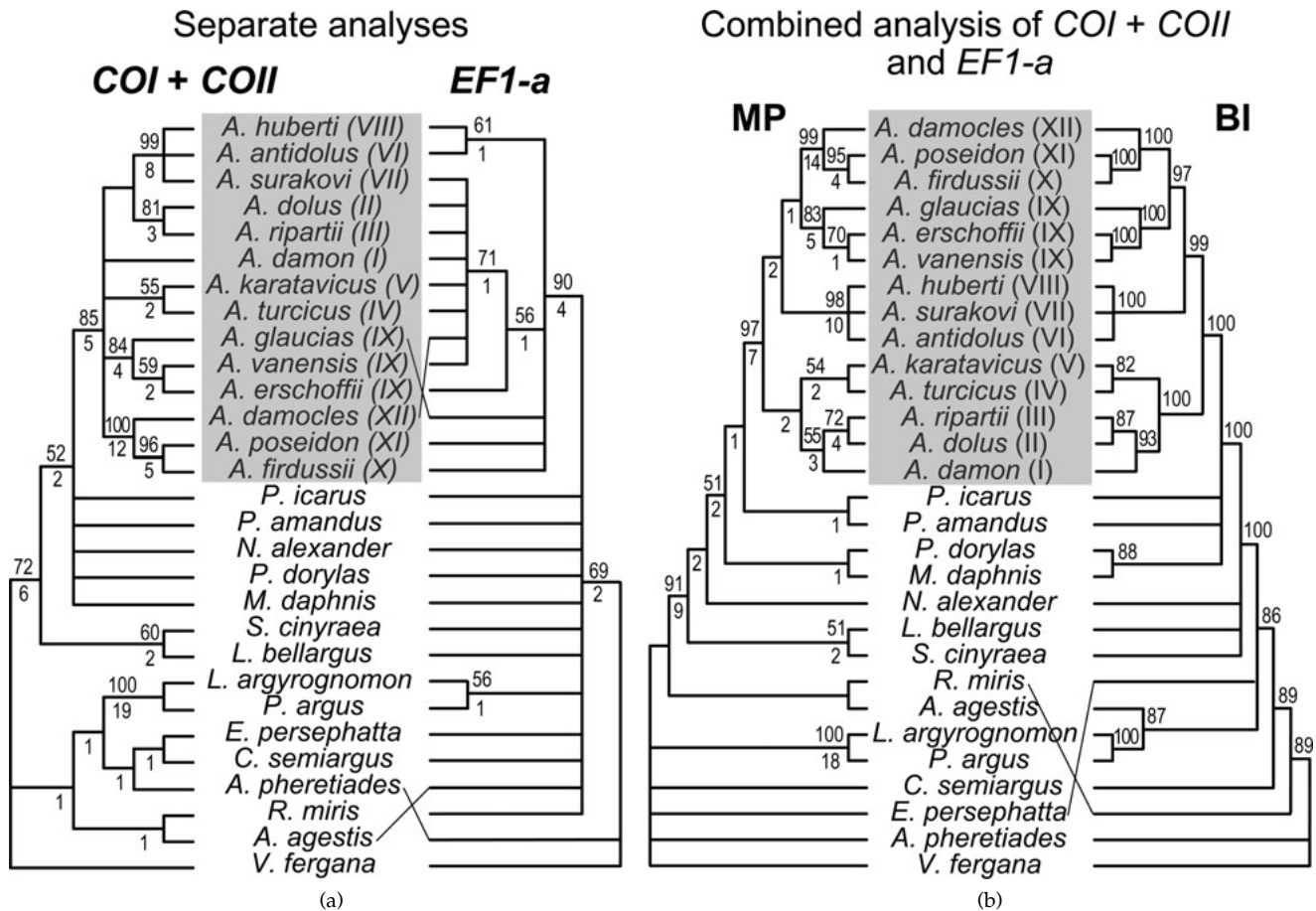


FIGURE 4. Separate and combined analyses of *COI* + *COII* and *EF1-α* genes. (a) Comparison between MP trees inferred from the separate analyses of *COI* + *COII* and *EF1-α* genes. The strict consensus tree for the *COI* + *COII* genes was constructed from 22 MP trees: TL = 1,470; CI = 0.485; RI = 0.437. The strict consensus tree for the *EF1-α* gene was constructed from 6,629 MP trees: TL = 354; CI = 0.678; RI = 0.562. Values for bt > 50% and Br are shown above and below recovered branches, respectively. (b) MP and BI trees inferred from the combined analyses of *COI* + *COII* + *EF1-α* genes. The strict consensus tree was constructed from 12 MP trees: TL = 1,852; CI = 0.515; RI = 0.440. Values of bt > 50% and Br are shown above and below recovered branches, respectively. The 70% majority consensus tree was recovered from Bayesian trees sampled during four independent Bayesian analyses under the GTR+I+Γ model for DNA substitution: mean  $-\ln L = 14098.60 \pm 6.6$ . The pP is shown above every branch on the BI tree. The shaded blocks highlight sampled *Agrodiaetus* species. The Roman numerals correspond to *Agrodiaetus* clades (for details, see Fig. 1).

sequences was used for dating phylogenetic events. Heterogeneous substitution rates across the topology were homogenized with the NPRS algorithm (Sanderson, 1997). We decided against using the basal node to estimate the age of *Agrodiaetus* because multiple substitutions and hence the error of estimation for pairwise distances would increase with overall sequence divergence (Hillis et al., 1996). Thus, the topology was calibrated using mean uncorrected pairwise distances between clade XII and clade X + XI (Fig. 5). This node (point 1, Fig. 5) was chosen because it is well supported and contains few clades. Given the substitution rate of 2.3% or 1.5% per million years, this point corresponded to 1.23–1.89 million year ago (MYA). Applying this calibration to the recovered topology, the origin of the genus *Agrodiaetus* was dated to 2.51–3.85 MYA. Figure 5 presents two dating scales applied to the Bayesian topology of average-branch-length consensus smoothed with the NPRS algorithm.

## DISCUSSION

### *Agrodiaetus*: Monophyly and Ingroup Relationships

Phenotypic differences among species within the genus *Agrodiaetus* are slight, with only one superficial character, a long white streak on the underside of the hind wings, considered a possible synapomorphy. However, this character is variable and can be undeveloped or absent in some individuals, populations, and even species. Furthermore, a white streak of similar shape and position is found in some species of *Polyommatus* (sensu stricto) and *Aricia*. Extreme karyotypic diversity also is not restricted to *Agrodiaetus*. In particular, two other genera within the section *Polyommatus*, *Lysandra* (n = 24–92) and *Plebicula* (n = 131–225), have marked variation in chromosome number. Thus, *Agrodiaetus* has been a problematic taxon because of the absence of characters that uniquely separate it from other genera in the section.

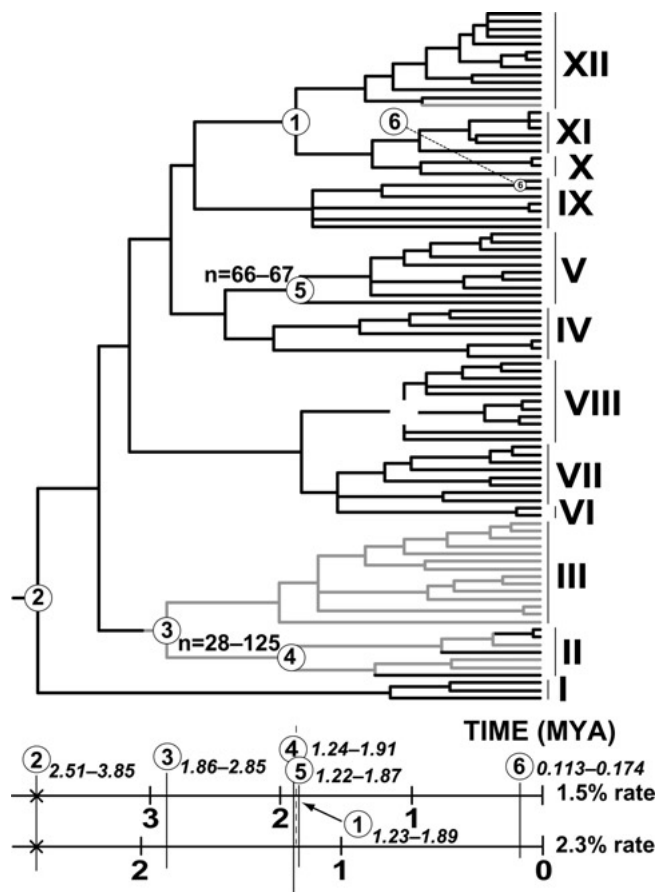


FIGURE 5. The Bayesian topology of average-branch-length consensus (ingroup only) inferred from 113 sequences of *COI* + *COII* under the GTR+I+ $\Gamma$  model for DNA substitution. Heterogeneous evolution rates were homogenized with the NPRS algorithm (Sanderson, 1997) across the topology. Two published substitution rates, 1.5% (Farrell, 2001) and 2.3% (Brower, 1994) for uncorrected pairwise distances, were used to calibrate the topology at the node 1. The clades on the topology were labeled following their order on Figure 1. Although clades II and V have similar ages (node 4: 1.24–1.91 million years ago [MYA]; node 5: 1.22–1.87 MYA), they have accumulated markedly different karyotype diversities ( $n = 28$ –125 and  $n = 66$ –67, respectively). Monomorphic *Agrodiaetus* species are highlighted in gray.

Gene trees inferred from both mitochondrial and nuclear gene fragments, using both MP and Bayesian methods, all support monophyly of *Agrodiaetus* (Figs. 2–4). The fact that differently inherited genes gave concordant results increases the probability that the genus, as currently defined, is indeed monophyletic. The sister taxon to *Agrodiaetus* is likely to include species within the genus *Polyommatus* (*sensu stricto*). In our study representatives of this genus emerged close to *Agrodiaetus* on every MP tree inferred from separate and combined analyses of mitochondrial and nuclear genes and appeared as part of a basal polytomy in most Bayesian trees. The exception was the BI majority tree inferred from *EF1- $\alpha$*  (Fig. 3). This placed (*Lysandra bellargus*, *Meleageria daphnis*) as the sister taxon of *Agrodiaetus*. The clade (*Agrodiaetus*, (*Lysandra bellargus*, *Meleageria daphnis*)), however, had weak support ( $pP = 0.73$ ) and did not appear in any other tree. Thus,

all four phylograms reconstructed as average-branch-length consensus trees from four independent rounds of Bayesian analysis of the *EF1- $\alpha$*  data set did not contain this clade. Instead, they had a polytomy with four clades: (1) *Agrodiaetus* species; (2) *Polyommatus amandus*, *P. icarus*, and *Sublysandra cinyraea*; (3) *Lysandra bellargus* and *Meleageria daphnis*; and (4) *Plebicula doryllas* (not shown).

Our analysis identified 12 major clades within *Agrodiaetus* (Figs. 1, 5), labeled I–XII to facilitate discussion. Of these, clade VII is the most weakly supported. At this stage of our study, we have not thoroughly sampled some species groups. In particular, our representatives of the *A. admetus* group were biased toward species with high chromosome numbers, whereas the important species *A. alcestis* ( $n = 19$  or 20; Lesse, 1960a) and *A. interjectus* ( $n = 29$ –31; Lesse, 1960a) were not included. Although our analysis recovered a monophyletic *poseidon* group, difficult logistics (*A. deedi* occurs in Lebanon, where we have not yet collected) and the rarity of specimens prevented us from obtaining three other species considered part of the *poseidon* group (*sensu* Hesselbarth et al., 1995).

In previous classifications (Lesse, 1960a; Hesselbarth et al., 1995; Eckweiler and Häuser, 1997; Table 1), the *admetus* group contained exclusively monomorphic butterflies (both sexes brown), the *dolus* and *erschoffii* groups contained both monomorphic and dimorphic species, and the other species groups contained only dimorphic species. Mensi et al. (1994) argued that monomorphy is a derived character within *Agrodiaetus*. Bálint and Johnson (1997) proposed that monomorphy (= discoloration) had multiple origins within the section *Polyommatus*. Our analysis provides evidence for at least two origins of monomorphy within *Agrodiaetus*. Most representatives of clade (II, III) are monomorphic, and our trees suggest that monomorphy may be ancestral for this clade, with several of apparent reversals. Monomorphy appears to have evolved independently in *A. mithridates*, placed in the distant clade XII (Fig. 5).

Rose and Schurian (1977) proposed that the species within the *erschoffii* group be placed within a separate genus *Paragrodiaetus*. On the basis of its name, *Paragrodiaetus* appears to have been considered the sister genus to *Agrodiaetus*. The two representatives of “*Paragrodiaetus*” in our analysis (*A. erschoffii* and *A. glaucias*) were recovered within clade IX ( $bt = 97$ ;  $Br = 8$ ;  $pP = 1.00$ ; Fig. 1), a clade that also contained members of the *A. damon* group. Recognition of *Paragrodiaetus* would render *Agrodiaetus* paraphyletic.

Mensi et al. (1994) placed the *damon* group as the basal taxon of *Agrodiaetus* (only the *admetus*, *dolus*, and *damon* groups were sampled). Our clade I contains all the members of the group studied by Mensi et al. (1994), and consistent with their conclusion, clade I diverges at the base of *Agrodiaetus* in our analysis. However, other members of the *damon* group (*sensu* Hesselbarth et al., 1995) are placed in clades IV, V, and IX, rendering the *damon* group polyphyletic (Fig. 1). All other species groups recognized by Hesselbarth et al. (1995) are nonmonophyletic in our analysis, except the *poseidon* group (clade X), which is represented by three species.

Representatives of the same nominal species do not form a monophyletic group in three places in our inferred phylogeny. In clade III, *A. demavendi* (n = 66–72) occurs in the midst of individuals assigned to *A. ripartii* (n = 90). In clade VIII, individuals assigned to *A. huberti* (n = 33–37) and *A. turcicolis* (n = 19 or 20) are intermixed with each other and with individuals assigned to *A. ninae* (n = 33 or 34), *A. elbursicus* (n = 18), and *A. aserbeidschanus* (karyotype unknown). In clade XII, individuals assigned to *A. altivagans* (n = 20–22) are intermixed with individuals assigned to several other species (n = 16–26), including *A. damocles* (n = 25 or 26), which itself is nonmonophyletic. Leaving aside the possibility of mistaken identity, such nonmonophyletic “species” could be explained in multiple ways. All species could be reciprocally monophyletic, with these placements simple artifacts of the limited number of informative characters. Some species could retain ancestral polymorphisms that predate speciation events and thus appear paraphyletic in relation to derived species. Species boundaries could be too broad, with some nominal species containing multiple species that do not form a clade. Species boundaries could be too narrow, with continuing interbreeding between nominal species, in some cases despite differences in chromosome numbers. The available data do not allow us to discriminate among these alternatives.

#### Age of *Agrodiaetus*

The ages that we estimate for the origin of major clades are broadly consistent with those inferred from electrophoretic phenotypes by Mensi et al. (1994). Thus, we date the origin of *Agrodiaetus* around 2.51–3.85 MYA (Fig. 5; node 2), whereas Mensi et al. placed the origin of the genus at 3.1 MYA. Similarly, Mensi et al. estimated the origin of the *dolus* + *admetus* groups at 2 MYA, whereas we date the origin of clade II + III, containing species from these groups, at around 1.86–2.85 MYA (Fig. 5; node 3). Mensi et al. estimated the origin of the *dolus* group at 1 MYA, a date that falls close to our estimate of 1.24–1.91 MYA for the origin of clade II (Fig. 5; node 4), which includes four species from the *dolus* group and three species from the *admetus* group.

Three nuclear genes that have been useful for reconstruction of species-level phylogenies in Lepidoptera, 28S ribosomal DNA (rDNA; Miller et al., 1997), *wingless* (Brower and DeSalle, 1998; Campbell et al., 2000), and *EF1-α* (Cho et al., 1995; Brower and DeSalle, 1998; Monteiro and Pierce, 2001), failed to provide enough informative characters to infer relationships within *Agrodiaetus* (data for 28S rDNA and *wingless* not presented). Lineage sorting among species is likely to be a problem for nuclear markers in *Agrodiaetus* because the time until coalescence for alleles within lineages may be greater than the time between successive speciation events. *EF1-α* failed to provide sufficient phylogenetic signal to resolve relationships among karyotypically well-defined *Agrodiaetus* taxa, whereas mitochondrial genes yielded a high number of parsimony-informative characters (Table 3). Given that the substitution rate for mitochondrial genes

can be four times that for nuclear genes (Page and Holmes, 1998:127), we conclude that species of *Agrodiaetus* are younger than lepidopteran species for which both mitochondrial and nuclear genes (*COI/COII* and *EF1-α*, respectively) provide useful phylogenetic signal.

#### Karyotypic Changes within *Agrodiaetus*

Three genera in the section *Polyommatus* exhibit marked karyotype diversity; our research here has focused on relationships within *Agrodiaetus*. The other two genera, *Lysandra* and *Plebicula*, were represented in our analysis as outgroups. The three genera did not cluster together in any of our trees (Figs. 1–4), although *Lysandra bellargus*, *L. caucasica*, and *L. coridon* form a well-supported clade in the MP and BI trees inferred from *COI* + *COII* sequences (Fig. 2). Thus, our analysis suggests that accelerated rates of karyotypic diversification have at least three origins within the section *Polyommatus* (Fig. 6). Further sampling of *Lysandra* and *Plebicula*

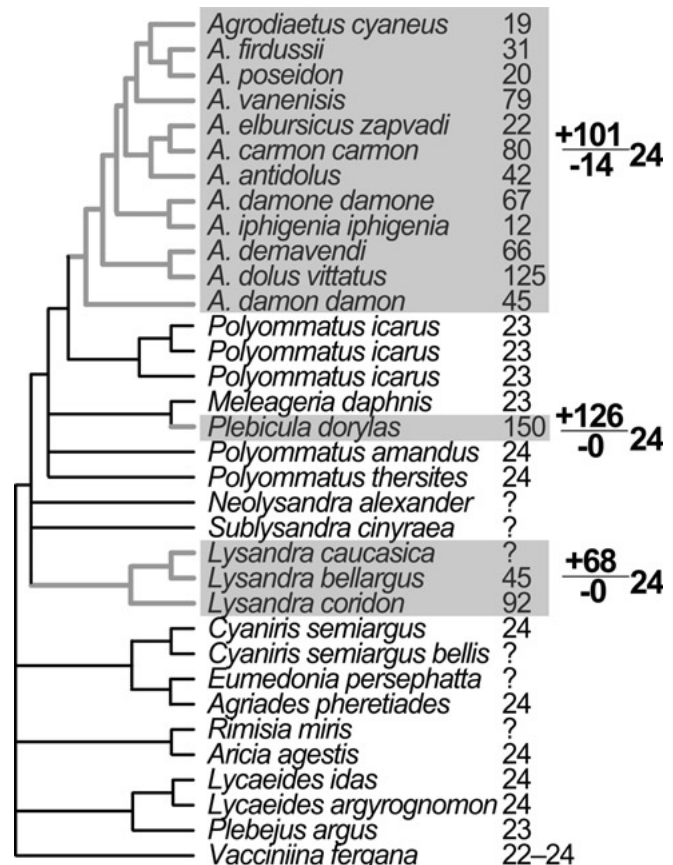


FIGURE 6. Multiple origins of interspecific karyotype diversity in the section *Polyommatus*. Chromosome numbers were mapped on the MP tree inferred from 113 sequences of *COI* + *COII* (Figs. 1 and 2). The tree was collapsed at the ingroup. Lineages with chromosome numbers that differ from the modal chromosome number of the family Lycaenidae (n = 23 or 24) are highlighted in gray. Up/down departures from the modal chromosome number (here n = 24) and extent of departure in haploid units are presented at the right of the chromosome numbers.

and the inclusion of representatives of currently unsampled genera in the section *Polyommatus* will further resolve this question.

The test for serial independence (TFSI; Abouheif, 1999) rejected the hypothesis that chromosome numbers were not correlated with phylogeny ( $P = 0.0001$ ). This result is not surprising. Casual inspection of Figure 1 reveals that closely related individuals tend to have similar chromosome numbers, although there are some exceptions where sister taxa have very different numbers. To estimate visually a suggested correlation between molecular and karyotypic divergences among *Agrodiaetus* species, we plotted total pairwise distances (under the GTR+I+ $\Gamma$  model of DNA substitution) against normalized absolute pairwise differences of chromosome numbers ( $|n_i - n_j|/24$ ) using Microsoft Excel 2001 (Fig. 7). This plot showed a weak positive correlation ( $R^2 = 0.136$ ) between molecular and karyotypic divergences.

Haploid chromosome numbers ranging from 41 to 50 were recovered as the ancestral state of karyotype for the genus *Agrodiaetus* under every resolving option for character-state reconstruction in MacClade 4.0 (Maddison and Maddison, 1992): MP, accelerated transformation (ACCTRAN), and delayed transformation (DELTRAN). The recovered ancestral state of karyotype is twice as large as the modal chromosome number of the family Lycaenidae ( $n = 23$  or  $24$ ). The closest outgroup species from the genus *Polyommatus* (*P. icarus*, *P. amandus*, and *P. thersites*) have this chromosome number ( $n = 23$  or  $24$ ), as does the ingroup species *A. turcicus* ( $n = 24$ ). Only when chromosome numbers were coded as unordered character states were ancestral chromosome numbers ( $n = 22$ – $25$ ) similar to  $n = 23$  or  $24$  uncovered in MacClade using DELTRAN, whereas MP and ACCTRAN did not recover an unequivocal ancestral character state.

Setting aside questions about the accuracy of the recovered phylogeny and the subjectivity of character coding,

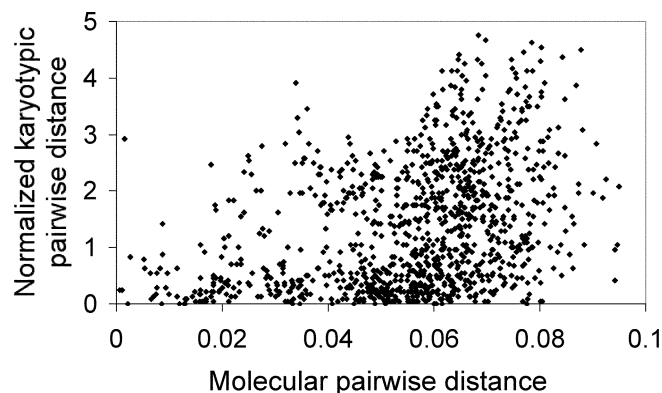


FIGURE 7. Karyotypic divergence versus molecular divergence among species of *Agrodiaetus*. Normalized karyotypic pairwise distances were measured as pairwise absolute differences of haploid chromosome numbers ( $|n_i - n_j|/24$ ) and plotted against total pairwise molecular distances. Only single individuals for each *Agrodiaetus* species with known karyotype were used in this analysis. Total molecular distances were estimated from mitochondrial genes *COI* and *COII* applying the GTR+I+ $\Gamma$  model of DNA substitution.

we have little confidence that  $n = 41$ – $50$  was the ancestral condition of *Agrodiaetus*. Algorithms of ancestral state reconstruction implemented in MacClade are based on parsimony criteria. However, parsimony ignores information about branch lengths. Furthermore, it reconstructs rapidly evolving characters with less accuracy (Felsenstein, 1973; Frumhoff and Reeve, 1994; Schluter et al., 1997) and does not provide the degree of support for its reconstruction (Cunningham, 1999). Perhaps the most distinctive feature of the genus *Agrodiaetus* is its rapidly evolving karyotype. Therefore, we think that parsimony may provide an inaccurate or even misleading method of ancestral state reconstruction of karyotypes in this group. To our knowledge, available likelihood programs of character reconstruction do not support a simple model of karyotype evolution in *Agrodiaetus*. Further research will be necessary to reconstruct the ancestral karyotype of *Agrodiaetus*: additional species of *Agrodiaetus* with distinct karyotypes must be studied, and additional characters of the karyotype (e.g., its structure, including the relative size of bivalents) must be considered to distinguish common ancestry (=homology) from convergence in chromosome number (=homoplasy).

Whether the ancestral chromosome number was 40–50 (as recovered by parsimony) or 23 or 24 (as inferred by comparative analysis with outgroup species), chromosome numbers have both increased and decreased within *Agrodiaetus*. The lowest number we observed was in *A. posthumus biruni* ( $n = 10$  or  $11$ ), and the highest was in *A. dolus vittatus* ( $n = 123$ – $125$ ) (Appendix 1). The mapping of chromosome numbers onto our phylogeny does not allow simple generalizations (Fig. 1). Clade IV ( $n = 12$ – $28$ ) and clade XII ( $n = 16$ – $25$ ) have mostly low numbers, clade III ( $n = 66$ – $90$ ) has mostly high numbers, and clade IX includes among its members *A. phyllis* ( $n = 79$ – $81$ ) and *A. posthumus biruni* ( $n = 10$  or  $11$ ) as sister taxa. This relationship is perhaps the most dramatic example of rapid karyotypic change present in our phylogeny because these sister taxa are estimated to have diverged as recently as 113,000–174,000 years ago (Fig. 5, node 6). This example should be corroborated by further karyotypic studies because the specimens VL01B205 and VL01B206 of *A. phyllis* and *A. posthumus biruni* were not karyotyped. An even greater range of chromosome numbers is found in clade II ( $n = 28$ – $124$ ), but all members of clade V have very similar karyotypes ( $n = 66$  or  $67$ ).

The main conclusion stands: chromosome numbers have undergone rapid diversification in the genus *Agrodiaetus* over the past 3.85 million years, while phenotypic characters have undergone relatively little change. Furthermore, there is evidence that the rate of karyotypic change has varied among lineages within the genus. Thus, we estimate that the roughly fourfold range of chromosome numbers ( $n = 28$ – $125$ ) found among the six identified species of clade II has arisen in <1.91 million years (Fig. 5, node 4). By contrast, the five species from clade V for which we obtained karyotypes have similar chromosome numbers ( $n = 66$  or  $67$ ) despite the fact that estimated divergence times for this clade are

similar to those of clade II (1.22–1.87 and 1.24–1.91 MYA, respectively).

#### *Karyotypic Diversification and Speciation in Agrodiaetus*

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). In its classical form, the model of chromosome speciation was based on the statement that individuals that were heterozygous for chromosome rearrangements had reduced fitness because of meiotic irregularities resulting from structural differences in maternal and paternal karyotypes (Lorkovic, 1974, 1979; Gropp et al., 1982; John et al., 1983; Piálek et al., 2001). However, meiotic irregularities also may be due to differences in genes rather than differences in chromosomes (Dobzhansky, 1933, 1937; Coyne and Orr, 1998). In general, it is hard to separate the effects of genetic (molecular) and karyotypic (chromosomal) divergences in establishing a sufficient barrier to gene flow between species. To examine this problem, we compared genetic and karyotypic divergences using both cytogenetic and molecular approaches. According to our data, the genetic divergence among *Agrodiaetus* species can be small, even when differences in chromosome numbers between the same species are large. Thus the genus *Agrodiaetus* may present a case of species radiation that has been driven by karyotype diversification. However, this supposition requires additional investigation. For example, the role of karyotypic differences as a postzygotic reproductive barrier in *Agrodiaetus* needs to be assessed. Chromosome rearrangements do not always result in hybrid sterility, and so far data regarding chromosome behavior in interspecific hybrids of *Agrodiaetus* are lacking.

The available data from interspecific crosses suggest that structural chromosome rearrangements and meiotic irregularities in Lepidoptera do not have a simple relationship. Fertile hybrids of the silkmoths *Antheraea roylei* ( $n = 31$ ) and *A. pernyi* ( $n = 49$ ) demonstrate that major differences in chromosome number are compatible with regular meiotic segregation. In  $F_1$  males, 31 chromatin bodies were observed at metaphase I. These were interpreted to be 13 bivalents and 18 trivalents (Nagaraju and Jolly, 1986). The hybrids were interbred for many generations, and in  $F_{23}$  and  $F_{32}$  males 49 chromatin bodies were observed at metaphase I. Thus, there seems to have been selection against the larger chromosomes of *A. roylei*. Nagaraju and Jolly (1983) did not provide evidence as to whether recombination had resulted in introgression of genes from *A. roylei* onto *A. pernyi* chromosomes. By contrast with these *Antheraea* hybrids, hybrids among multiple morphologically distinct *Papilio* species with indistinguishable ( $n = 30$ ) karyotypes have shown that major disturbances of synapsis, and resultant infertility, can occur in the absence of obvious structural rearrangements (Maeki and Ae, 1976, 1978a, 1978b). In fertile hybrids, 30 chromatin bodies were observed at metaphase I (interpreted as 30 bivalents formed by complete synapsis), whereas in infertile hybrids, higher

numbers of chromatin bodies were observed (interpreted as a mixture of bivalents and unpaired univalents).

Because of regular segregation of trivalents and the difficulty of distinguishing trivalents from bivalents cytologically the question arises as to whether fission/fusion polymorphisms within species could go unrecognized. Most meiotic chromosomes of Lepidoptera are dotlike under standard microscopy. Diploid chromosome numbers are usually obtained by doubling the number of chromatin spots observed on metaphase plates during male meiosis I. The spots are thus assumed to be bivalents. Heterozygosity for a chromosomal fission would result in a trivalent rather than a bivalent without changing the observed number of spots, whereas fission homozygotes would have one more spot on metaphase plates. Microscopic discrimination of trivalents or higher order multivalents from bivalents may be difficult for many lepidopteran cells.

The rates of karyotype evolution we infer in *Agrodiaetus* appear inconsistent with every chromosomal rearrangement having been associated with a substantial heterozygote disadvantage. Therefore, the possibility that chromosomal rearrangements may not have imposed absolute barriers to gene flow (at least at early stages of karyotype divergence) should be considered as a possible explanation of apparent inconsistencies between the recovered molecular phylogeny and chromosome numbers. This view would call into question the practice of describing new *Agrodiaetus* "species" solely on the basis of sympatric occurrence of individuals with different chromosome numbers but similar morphology. One would need evidence that the observed karyotypic differences are stably maintained and that the local population does not contain individuals with intermediate numbers. This evidence is available in some cases. For example, *A. interjectus* was described by Lesse (1960a) on the basis of its profoundly different karyotype ( $n = 29$ – $32$ ; later this taxon was included into *A. erivanensis* with  $n = 34$ ; Lukhtanov et al., 2003) and was separated from the phenotypically similar and sympatrically distributed *A. ripartii* ( $n = 90$ ), *A. demavendi* ( $n = 67$ – $70$ ), and *A. alcestis* ( $n = 20$ ). After these species were described by Lesse, they have been routinely collected by many researchers from sympatric populations (e.g., Lukhtanov et al., 2003). Therefore, the impact of karyotype changes in preventing gene flow between these taxa and their validity as reproductively isolated species seems to be well supported. Lukhtanov (1993) presented an evolutionary model of karyotype diversification in *Agrodiaetus* to explain such examples of highly divergent, but stable chromosome differences within sympatric populations. In this model, chromosomal differences between closely related populations accumulate in allopatry and constitute barriers to hybridization when descendants are secondarily brought into sympatry.

Lepidopteran genera with wide ranges of chromosome numbers have also been described outside of the Lycaenidae. Haploid chromosome numbers in *Leptidea* (Pieridae) range from 26 to 104 (Lorkovic, 1941; Lesse, 1960b) and in *Erebia* (Nymphalidae) from 8 to 52 (Lesse,

1960a; Lorkovic, 1972; Lukhtanov, 1987). An extreme range, comparable to that observed in *Agrodiaetus*, has recently been described in scale insects of the genus *Apiomorpha* (Hemiptera: Eriococcidae). Haploid complements in this genus range from 2 to 96 (Cook, 2000). It is probably no coincidence that *Agrodiaetus* (Lepidoptera) and *Apiomorpha* (Hemiptera) both belong to insect orders that have holocentric chromosomes (Hughes-Schrader and Schrader, 1961; Kuznetsova, 1979; Maeki 1980a, 1980b; Wolf et al., 1997). Centromeric activity of holocentric chromosomes is not localized to a single site but is spread throughout the chromosome. As a consequence, the fragments from a chromosomal fission can attach to mitotic and meiotic spindles and be protected from loss at cell division.

Despite the above arguments for a possible role of holocentric chromosomes in karyotypic diversification, karyotypes of most Lepidoptera are highly conservative (Robinson, 1971). The existence of well-defined modal chromosome numbers, for the Lepidoptera as a whole and for the family Lycaenidae, suggests that chromosome numbers are stable in most evolutionary lineages. Either bursts of karyotype diversification, such as the one documented here for *Agrodiaetus*, are rare and when they occur leave few long-term descendant species, or perhaps some selective force favors a return to the modal chromosome number.

Our conclusion that karyotypic instability has originated at least three times in the section *Polyommatus* suggests that polyommatus may possess some factor predisposing them to chromosomal rearrangement. Transposable elements cause chromosome rearrangements in plants (Bennetzen, 2000) and animals (Lyttle and Haymer, 1992; Cáceres et al., 1999; Lönnig and Saedler, 2002) and may be horizontally transferred between species. For example, *P* elements are absent from all strains of *Drosophila melanogaster* collected before 1950 but are present in most strains collected since the 1950s (Kidwell, 1983). *P* elements appear to have been transferred to *D. melanogaster* from *D. willistoni* during the 20th century and have rapidly spread through the global population of *D. melanogaster* (Clark and Kidwell, 1997). During their initial spread into previously uninfected genomes, *P* elements cause a suite of genetic disturbances known as hybrid dysgenesis, including a greatly increased rate of chromosome rearrangement (Engels and Preston, 1981). In the *virilis* group of *Drosophila*, transposons have been implicated in the origin of many chromosome rearrangements, both those that are fixed differences between species and those that are polymorphic within species (Evgen'ev et al., 2000). It is tempting to speculate that karyotypic diversification in *Agrodiaetus* may have been driven by the spread of a transposable element, perhaps with horizontal transfer to or from the related genera *Lysandra* and *Plebicula*.

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- Note:* Two specimens [Genbank AY502109–AY502112], *Agrodiaetus hamadanensis* (VL02N555) and *Albulina atys* (AD03B064) representing the previously unsampled *dama* species-group of *Agrodiaetus* and an outgroup genus, respectively, have been obtained and added to the dataset since the manuscript was submitted for publication. According to both mitochondrial and nuclear genes, *A. hamadanensis* appears at the base of the clade (VI + VII + VIII; see Fig. 1), whereas *Albulina atys* clusters with *Agriaes pheretiades* (NK00P777; see Figs. 2 and 3).

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APPENDIX 1. *Agrodiætus* and outgroup species sampled in this study. *COI* and *COII* were sequenced for every specimen.

Taxon	Locality and date	Chromosome no. (n)	Accession no.
<i>Agrodiætus</i>			
<i>actis</i> species group			
<i>A. altivagans</i> <sup>a</sup>	Armenia, Aiodzor Mts., Gnishyk, 7/1998	20 or 21 (Lukhtanov and Dantchenko, 2002b)	VL99Q075
<i>A. altivagans</i> <sup>b</sup>	Turkey, Gümüşhane, 7/2001	21 (Lukhtanov, unpubl.)	VL01L111
<i>A. altivagans</i> ssp. <sup>b</sup>	Turkey, Van Prov., Çatak, 7/2001	22 (Lukhtanov, unpubl.)	VL01L222
<i>A. altivagans</i> <sup>b</sup>	Turkey, Erzurum Prov., Tortum, 7/2001	21 (Lukhtanov, unpubl.)	VL01L450
<i>A. firdussii</i> <sup>c,d</sup>	Iran, Demavend, 7/2001	31–33 (Lesse, 1962a)	VL01B199
<i>A. pseudactis</i> <sup>a</sup>	Armenia, Aiodzor Mts., Gnishyk, 6/2000.	29 (Lukhtanov and Dantchenko, 2002b)	AD00P263
<i>A. bilgini</i> <sup>b</sup>	Turkey, Gümüşhane prov., Torul, 7/2001	25 (Lukhtanov and Dantchenko, 2002a)	VL01Q140
<i>A. haigi</i> <sup>b</sup>	Turkey, Van Prov., Çatak, 7/2001	26 (Lukhtanov and Dantchenko, 2002b)	VL01L224
<i>A. haigi</i> <sup>b</sup>	Turkey, Van Prov., Çatak, 7/2001	25 (Lukhtanov and Dantchenko, 2002a)	VL01L340
<i>A. haigi</i> <sup>b</sup>	Turkey, Van Prov., Çatak, 7/2001	25 (Lukhtanov and Dantchenko, 2002a)	VL01L220
<i>admetus</i> species group			
<i>A. admetus anatoliensis</i> <sup>b</sup>	Turkey, Gümüşhane, 7/2001	≈80 (Lukhtanov, unpubl.)	VL01L101
<i>A. admetus</i> ssp. <sup>c</sup>	Armenia, Aiodzor Mts., Gnishyk, 7/2000	79 (Lukhtanov and Dantchenko, 2002b)	AD00P016
<i>A. dantchenkoi</i>	Turkey, Van Prov., Çatak, 7/2001	40–42 (Lukhtanov et al., 2003)	VL01L342
<i>A. demavendi</i> <sup>c</sup>	Armenia, Aiodzor Mts., Gnishyk, 7/2000	66–72 (Lukhtanov and Dantchenko, 2002b)	AD00P014
<i>A. eriwanensis eriwanensis</i> <sup>a</sup>	Armenia, Aiodzor Mts., Gnishyk, 6/00.	34 (Lukhtanov and Dantchenko, 2002b)	AD00P303
<i>A. fabressei</i> <sup>c</sup>	Spain, Puerto de la Losilla, Albarracín, 8/1999	90 (Lesse, 1960b, 1961)	MAT99Q984
<i>A. ripartii budashkini</i> <sup>c,d</sup>	Ukraine, Crimea, Karabi yaila, 7/2000	90 (Kandul, 1997)	NK00P859
<i>A. ripartii paracestis</i> <sup>b</sup>	Turkey, Gümüşhane, 7/2001	≈90 (Lukhtanov, unpubl.)	VL01L103
<i>A. ripartii paracestis</i> <sup>b</sup>	Turkey, Gümüşhane Prov., Dilekyolu, 7/2001	90 (Lukhtanov, unpubl.)	VL01L166
<i>A. ripartii paracestis</i> <sup>c</sup>	Armenia, Pambak Mts., Dzhur-dzhur Pass, 6/2000	Unknown	AD00P337
<i>A. ripartii</i> ssp. <sup>c</sup>	Russia, Tula Reg., Tatinki, 7/2000	Unknown	AD00P033
<i>A. ripartii ripartii</i> <sup>c</sup>	Spain, Lleida Prov., Tremp, 7/1999	90 (Lesse, 1961)	MAT99Q878
<i>A. ripartii sarkani</i> <sup>b</sup>	Kazakhstan, Dzhungarian Alatau Mts., Kolbai, 7/2000	90 (Lukhtanov and Dantchenko, 2002a)	NK00P829
<i>A. ripartii sarkani</i> <sup>b</sup>	Kazakhstan, Tarbagatai Mts., Taskeskan, 7/2000	90 (Lukhtanov and Dantchenko, 2002a)	NK00P848
<i>A. ripartii colemani</i> <sup>b</sup>	Kazakhstan, West Tian-Shan, Ugamski Mts., 6/2000.	90 (Lukhtanov and Dantchenko, 2002a)	NK00P822
<i>A. specimen 1</i>	Armenia, Aiodzor Mts., Gnishyk, 7/2000	Unknown	AD00P430
<i>A. specimen 2</i>	Armenia, Aiodzor Mts., Gnishyk, 7/2000	Unknown	AD00P475
<i>carmon</i> species group			
<i>A. carmon carmon</i> <sup>b</sup>	Turkey, Gümüşhane, 7/2001	≈80–82 (Lukhtanov and Dantchenko, 2002a)	VL01Q129
<i>A. carmon munzuricus</i> <sup>b</sup>	Turkey, Erzincan Prov., Munzur Daglari Mts., 7/2001	≈80 (Lukhtanov and Dantchenko, 2002a)	VL01L262
<i>A. arasbarani neglectus</i> <sup>a</sup>	Armenia, Zangezur Mts., Megry, 7/2000	25 (Lukhtanov, unpubl.)	AD00P325
<i>A. ciscaucasicus</i> <sup>a</sup>	Russia, N. Caucasus, Kislovodsk, 8/1998.	16 (Lukhtanov, 1989)	VL99Q074
<i>A. cyaneus</i> <sup>b</sup>	Turkey, Van Prov., Gevas, 7/2001	19 (Lukhtanov, unpubl.)	VL01L237
<i>A. huberti</i> <sup>b</sup>	Turkey, Erzurum, 7/2001	34 or 35 (Lukhtanov, unpubl.)	VL01L315
<i>A. huberti huberti</i> <sup>a</sup>	Armenia, Aiodzor Mts., Gnishyk, 6/2000	35–37 (Lukhtanov and Dantchenko, 2002b)	AD00P260
<i>A. huberti</i> ssp. <sup>b,d</sup>	Turkey, Gümüşhane Prov., Dilekyolu, 7/2001	33 or 34 (Lukhtanov unpubl.)	VL01L123
<i>A. pierceae</i> <sup>b</sup>	Turkey, Van Prov., Güzeldere Geçidi, 7/2001	22 (Lukhtanov and Dantchenko, 2002a)	VL01L365
<i>A. kendeveni</i>	Iran, Demavend, 7/2001	Unknown	VL01B209
<i>A. merhaba</i> <sup>b</sup>	Turkey, Artvin Prov., Kilickaya, 8/2001	17 (Lukhtanov, unpubl.)	VL01L458
<i>A. surakovi surakovi</i> <sup>a,d</sup>	Armenia, Aiodzor Mts., Gnishyk, 7/2000	50 (Lukhtanov et al., 2002b)	AD00P006
<i>A. surakovi sekercioglu</i> <sup>b</sup>	Turkey, Van Prov., Çatak, 7/2001	50 (Lukhtanov et al., 2002a)	VL01L196
<i>A. turcicus</i> <sup>a,d</sup>	Armenia, Pambak Mts., Dzhur-dzhur Pass, 6/2000	24 (Lukhtanov and Dantchenko, 2002b)	AD00P135
<i>damon</i> species group			
<i>A. damon</i> <sup>c,d</sup>	Spain, Pirenees, Urús, 7/1999	45 (Lesse, 1960b)	MAT99Q841
<i>A. damon kotshubeji</i>	Armenia, Aiodzor Mts., Gnishyk, 7/1998	Unknown	VL99Q076
<i>A. damon</i> ssp. <sup>a</sup>	Russia, St. Petersburg Reg., Luga, 7/2000	45 (Kandul, unpubl.)	NK00P156
<i>A. baytopi</i> <sup>b</sup>	Turkey, Van Prov., Güzeldere Geçidi, 7/2001	27 or 28 (Lukhtanov, unpubl.)	VL01L383
<i>A. darius</i>	Iran, Demavend, 7/2001	Unknown	VL01B193
<i>A. iphigenia araratensis</i> <sup>b</sup>	Turkey, Van Prov., Çatak, 7/2001	13 (Lukhtanov, unpubl.)	VL01L322
<i>A. iphigenia araratensis</i> <sup>a</sup>	Armenia, Aiodzor Mts., Gnishyk, 6/2000	14 (Lukhtanov and Dantchenko, 2002b)	AD00P293
<i>A. iphigenia iphigenia</i> <sup>b</sup>	Turkey, Gümüşhane, 7/2001	12 (Lukhtanov, unpubl.)	VL01Q138
<i>A. phyllides kentauensis</i>	Kazakhstan, Karatau Mts., Turpan Pass, 6/2000	Unknown	NK00P773
<i>A. phyllides</i> <sup>c</sup>	Kazakhstan, Dzhambul Reg., Kirgizski Mts., 6/2000	≈66 (Lukhtanov and Dantchenko, 2002b)	NK00P672
<i>A. phyllides</i> ssp. <sup>c</sup>	Kazakhstan, Shymkent Reg., Karzhantau Mts., 6/2000	≈66 in <i>A. phyllides</i> (Lukhtanov and Dantchenko, 2002b)	NK00P808

(continued on next page)

## APPENDIX 1. (CONTINUED)

Taxon	Locality and date	Chromosome no. (n)	Accession no.
<i>A. phyllis</i> ssp. <sup>c</sup>	Iran, Demavend, 7/2001	79–81 (Lesse, 1963)	VL01B205
<i>A. posthumus biruni</i> <sup>c</sup>	Iran, Demavend, 7/2001	10 or 11 (Lesse, 1960b)	VL01B206
<i>A. rovshani</i>	Iran, Sabalan, 7/2001	Unknown	VL01B200
<i>A. tanker</i> <sup>c</sup>	Turkey, Erzurum, 7/2001	20 or 21 (Lesse, 1960b)	VL01L429
<i>A. vanensis</i> <sup>c</sup>	Armenia, Zangezur Mts., Gumoratz, 7/2000	78 (Lesse, 1957)	AD00P332
<i>A. vanensis sheljuzhko</i> <sup>a,d</sup>	Armenia, Aiodzor Mts., Gnishyk, 7/2000	79 or 80 (Lukhtanov and Dantchenko, 2002b)	AD00P010
<i>damone</i> species group			
<i>A. damocles damocles</i> <sup>c</sup>	Russia, South Urals, Verblyuzhka Mt., 7/2001	≈25 (Lukhtanov, unpubl.)	VL01B001
<i>A. damocles damocles</i> <sup>c</sup>	Russia, South Urals, Adaevo, 7/2001	≈25 (Lukhtanov, unpubl.)	VL01B170
<i>A. damocles kanduli</i> <sup>b</sup>	Turkey, Erzincan Prov., Munzur Daglari Mts., 7/2001	25 (Lukhtanov and Dantchenko, 2002a)	VL01L180
<i>A. damocles krymaeus</i> <sup>c,d</sup>	Ukraine, Crimea, Kurortnoe, 6/2000	26 (Kandul, 1997)	NK00P103
<i>A. damocles rossicus</i> <sup>a</sup>	Russia, Volga Reg., Volsk, 7/2001	25 (Lukhtanov et al., 1997)	VL01B371
<i>A. damone altaicus</i> <sup>c</sup>	Russia, Altai, Kuraiski Mts., Aktash, 8/2000	67 (Lukhtanov et al., 1997)	NK00P893
<i>A. damone damone</i> <sup>a</sup>	Russia, South Urals, Guberli Mts., Adaevo, 7/2001	67 (Kandul, 1997)	VL01B184
<i>A. damone irinae</i> <sup>a</sup>	Russia, Volgograd Reg., Kamyshinsky, 7/2000	≈67 (Kandul, unpubl.)	AD00P077
<i>A. pljushtchi</i> <sup>a</sup>	Ukraine, Crimea, Ai-Petri Mt., 7/2000	67 (Kandul, 1997)	NK00P120
<i>dolus</i> species group			
<i>A. ainsae</i> <sup>c</sup>	Spain, Lleida Prov., Tremp, 7/1999.	108–110 (Lesse, 1962b)	MAT99Q894
<i>A. antidolus</i> <sup>b,d</sup>	Turkey, Erzincan Prov., Munzur Daglari Mts., 7/2001	42 (Lukhtanov, unpubl.)	VL01L270
<i>A. dolus vittatus</i> <sup>c,d</sup>	France, Languedoc Reg., Mende, 7/1999	123–125 (Lesse, 1962b)	MAT99Q923
<i>A. fulgens</i> <sup>c</sup>	Spain, Santa Coloma de Queralt, 7/1999	103 (Munguira et al., 1994)	MAT99Q910
<i>A. kurdistanicus</i> <sup>b</sup>	Turkey, Van Prov., Çatak, 7/2001	62 (Lukhtanov, unpubl.)	VL01L190
<i>A. menalcas menalcas</i> <sup>b</sup>	Turkey, Gümüşhane Prov., Dilekyolu, 7/2001	85 (Lukhtanov, unpubl.)	VL01L122
<i>A. mithridates</i> <sup>b</sup>	Turkey, Erzincan Prov., Tercan, 7/2001	22 (Lukhtanov, unpubl.)	VL01L300
<i>erschoffii</i> species group			
<i>A. erschoffii</i> <sup>c,d</sup>	Iran, Gorgan Prov., Shahkuh, 7/2002	13 (Lukhtanov, unpubl.)	AD02L274
<i>A. glaucias</i> <sup>c,d</sup>	Iran, Gorgan Prov., Shahkuh, 7/2002	≈56 (Lukhtanov, unpubl.)	AD02M278
<i>iphigenides</i> species group			
<i>A. iphigenides</i> ssp.	Kazakhstan, Shymkent Reg., Ugamski Mts., 7/2000	Unknown	NK00P823
<i>A. juldusus kasachstanus</i> <sup>a</sup>	Kazakhstan, Dzhungarian Alatau Mts., Kysylagash, 6/2000	67 (Lukhtanov and Dantchenko, 2002b)	NK00P617
<i>A. karatavicus</i> <sup>b,d</sup>	Kazakhstan, Shymkent Reg., Karatau Mts., 6/2000	67 (Lukhtanov, unpubl.)	NK00P738
<i>poseidon</i> species group			
<i>A. hopfferi</i> <sup>b</sup>	Turkey, Erzincan Prov., Munzur Daglari Mts., 7/2001	15 (Lukhtanov, unpubl.)	VL01L263
<i>A. putnami</i> <sup>b</sup>	Turkey, Erzurum Prov., Kayabasi, 7/2001	26 (Lukhtanov and Dantchenko, 2002a)	VL01L416
<i>A. poseidon</i> <sup>b,d</sup>	Turkey, Gümüşhane, 7/2001	≈20 (Lukhtanov, unpubl.)	VL01L108
<i>transcaspicus</i> species group			
<i>A. aserbeidschamus</i>	Armenia, Kajaran, Katnarat, 8/2001	Unknown	VL01B033
<i>A. specimen 3</i>	Iran, Demavend, 7/2001	Unknown	VL01B210
<i>A. elbursicus zapvadi</i> <sup>b</sup>	Turkey, Van Prov., Çatak, 7/2001	18 (Lukhtanov, unpubl.)	VL01L195
<i>A. ninae</i> <sup>a</sup>	Armenia, Aiodzor Mts., Gnishyk, 7/2000	33 or 34 (Lukhtanov and Dantchenko, 2002b)	AD00P004
<i>A. ninae</i> ssp.	Armenia, Pambak Mts., Dzhur-dzhur Pass, 8/2000	Unknown	AD00P345
<i>A. ninae firuze</i> <sup>b</sup>	Turkey, Gümüşhane Prov., Dilekyolu, 7/2001	≈34 (Lukhtanov, unpubl.)	VL01L169
<i>A. turcicolus</i> <sup>b</sup>	Turkey, Van Prov., Çatak, 7/2001	20 (Lukhtanov, unpubl.)	VL01L348
<i>A. turcicolus</i> <sup>b</sup>	Turkey, Van Prov., Curubas Geçidi, 7/2001	19 (Lukhtanov, unpubl.)	VL01L228
<i>A. turcicolus</i> ssp. <sup>b</sup>	Turkey, Van Prov., Güzeldere Geçidi, 7/2001	20 (Lukhtanov, unpubl.)	VL01L352
Outgroup taxa			
<i>Agriades pheretiades</i> <sup>d</sup>	Kazakhstan, Dzhambul Reg., Kirgizski Mts., 6/2001	Unknown	NK00P690
<i>Aricia agestis</i> <sup>c,d</sup>	Kazakhstan, Dzhambul Reg., Kirgizski Mts., 6/2001	24 (Lorkovic, 1941)	NK00P712
<i>Cyaniris semiargus bellis</i>	Armenia, Zangezur Mts., Akhtchi, 7/2000	Unknown	AD00P369
<i>Cyaniris semiargus semiargus</i> <sup>c,d</sup>	Russia, Low Volga, Volgograd Reg., Kamytsinsky, 6/2000	24 (Lorkovic, 1941)	AD00P206
<i>Eumedonia persephatta minshelkensis</i> <sup>d</sup>	Kazakhstan, Shymkent Reg., Karatau Mts., 6/2000	Unknown	NK00P743
<i>Lycaeides idas anna</i> <sup>c</sup>	USA, California, Donner Pass, 6/1992	24 for <i>Lycaeides</i> in Europe (Lesse, 1960a)	AS92Z040
<i>Lycaeides argyrognomon</i> <sup>c,d</sup>	Russia, Tula Reg., Tatinki, 8/2000	24 (Lorkovic, 1941)	AD00P560

## APPENDIX 1. (CONTINUED)

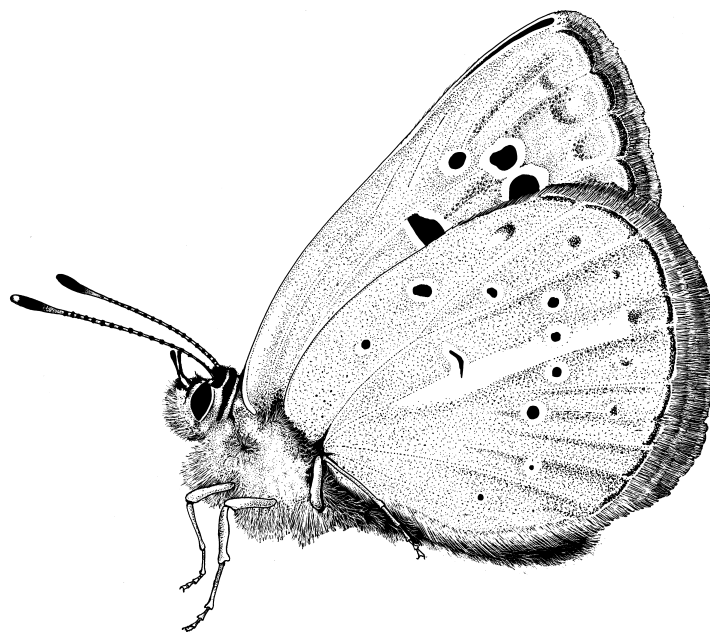
Taxon	Locality and date	Chromosome no. (n)	Accession no.
<i>Lysandra bellargus</i> <sup>c,d</sup>	Armenia, Aragatz Mts., Amberd, 6/2000	45 (Lesse, 1960b)	AD00P129
<i>Lysandra coridon borussia</i> <sup>c</sup>	Russia, Tula Reg., Tatinki, 8/2000	88–92 (Lesse, 1960b)	AD00P192
<i>Lysandra caucasica</i> <sup>c</sup>	Armenia, Aiodzor Mts., Gnishyk, 7/2000	84 (Lesse, 1960b)	AD00P435
<i>Meleageria daphnis</i> <sup>c,d</sup>	Ukraine, Crimea, Kurortnoe, 7/2000	23 (Lesse, 1960b)	NK00P108
<i>Neolysandra alexander</i> <sup>d</sup>	Armenia, Gegamsky Mts., Gegadyr, 5/2000	Unknown	AD00P092
<i>Plebejus argus</i> <sup>c,d</sup>	Ukraine, Crimea, Ai-Petri Mt., 7/2000	23 (Lorkovic, 1941)	NK00P135
<i>Plebicula dorylas armena</i> <sup>c,d</sup>	Armenia, Aiodzor Mts., Gnishyk, 6/2000	147–150 in <i>P. dorylas dorylas</i> (Lesse, 1960b)	AD00P312
<i>Polyommatus amandus</i> <sup>c,d</sup>	Kazakhstan, Altai, Oktyabrsk, 6/2000	24 (Lorkovic, 1941)	NK00P596
<i>Polyommatus icarus</i> 1 <sup>c</sup>	Armenia, Sevan, Shorzha, V/2000	23 (Lorkovic, 1941)	AD00P118
<i>Polyommatus icarus</i> 2 <sup>c</sup>	Russia, St. Petersburg Reg., Luga, 7/2000	23 (Lorkovic, 1941)	NK00P164
<i>Polyommatus icarus</i> 3 <sup>c,d</sup>	Kazakhstan, Altai, Oktyabrsk, 6/2000	23 (Lorkovic, 1941)	NK00P562
<i>Polyommatus thersites</i> <sup>c</sup>	France, Languedoc Reg., Mende, 7/1999	24 (Lorkovic, 1941)	MAT99Q947
<i>Rimisia miris</i> <sup>d</sup>	Kazakhstan, Altai, Oktyabrsk, 6/2000	Unknown	NK00P575
<i>Sublysandra cinyraea</i> <sup>d</sup>	Armenia, Zangezur Mts., Akhtchi, 7/2000	Unknown	AD00P389
<i>Vacciniina fergana</i> <sup>c,d</sup>	Kazakhstan, Shymkent Reg., Karatau Mts., Turpan Pass, 6/2000	22–24 for the genus <i>Vacciniina</i> (Lesse, 1960b)	NK00P777

<sup>a</sup>Karyotype was obtained for a different individual from the same population.

<sup>b</sup>Karyotype and gene sequence data were obtained from the same specimen.

<sup>c</sup>Karyotype was obtained from the literature.

<sup>d</sup>*EF1- $\alpha$*  was sequenced in addition to *COI* and *COII*.



*Agrodiaetus surakovi* (Dantchenko and Lukhtanov, 1994), female, Transcaucasia, Armenia, Aiotdzorskyi range, Gnishyk village, 2000 m., July 1998. Illustration by Christopher Adams.