

## Biochemical taxonomy and evolutionary relationships in *Polyommatus* (subgenus *Agrodiaetus*) (Lepidoptera, Lycaenidae)

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### Summary

Relationships between monomorphic species of the subgenus *Agrodiaetus* have been studied by enzyme electrophoresis. Results indicate that all species traditionally recognised on the basis of de Lesse's karyological studies may represent real species. Irrespective of problems of holocentricity it also appears likely that chromosome rearrangements may have contributed to speciation in this group of insects.

### Zusammenfassung

Die Beziehungen zwischen den monomorphen Arten des Subgenus *Agrodiaetus* wurden mit der Hilfe elektrophoretischer Methode untersucht. Die Ergebnisse zeigen, daß die Arten, die gewöhnlich nach de Lesse's karyologischen Untersuchungen anerkannt werden, echte Arten sind. Trotz des Holocentrismusproblems, haben vermutlich "Chromosomen-Rearrangements" in der Artenbildung dieser Insektengruppe eine Rolle gespielt.

### Résumé

Les relations phylogénétiques entre les espèces monomorphes appartenant au sous-genre *Agrodiaetus* ont été étudiées au moyen de l'analyse électrophorétique des enzymes. Nos résultats suggèrent que toutes les espèces traditionnellement reconnues dans ce groupe sur la base des études caryologiques conduites par de Lesse, représentent de véritables espèces. Quoi qu'il en soit de l'holocentrisme chromosomal chez les Lépidoptères, il semble aussi très probable que quelque mécanisme de réarrangement chromosomal puisse avoir contribué à la spéciation au sein de ce groupe d'insectes.

## Introduction

Members of the subgenus *Agrodiaetus* (Hübner, 1822) are widespread across Europe and Asia, and range from the Iberian peninsula to the Altai Mountains and further to the east, with a distribution characterized by extreme geographical fragmentation.

The identification of species of *Agrodiaetus* can be quite difficult. As a consequence, the systematics of this subgenus goes little beyond alfa-taxonomy and no phylogenetic reconstruction has so far been attempted. Morphological features traditionally employed in butterfly taxonomy, either do not sufficiently differ between species (e.g. male genitalia) or, although variable, are not sufficiently constant at the intraspecific level (e.g. female genitalia, shape of androconial scales, etc.).

So far, therefore, most taxonomic work has been based on characters of wing colouration and markings. In this respect, two groups are traditionally recognized. One includes the so-called dimorphic species, whose males are promptly identified by their blue wings, on the dorsal surface, as opposed to the brown wings of females. The other group includes monomorphic entities only, with dark brown wings in both sexes.

FORSTER (1956), working on this basis, was the first to attempt a broad revision of *Agrodiaetus*. Since using chromatic characters within the monomorphic forms of the *P. ripartii* complex is impossible, however, his work was almost entirely dedicated to the study of the dimorphic complex.

A major problem deriving from the use of characters such as the shade of the blue colour on the dorsal surface of the male's wings, or the extent and degree of development of submarginal markings on the ventral surface of the hindwings, etc., is that the interpretation of their relative weight may be subjective. Notwithstanding the great importance generally attached to Forster's work, therefore, it is not surprising that solutions offered by this author for some taxonomic problems remain controversial, while others have been abandoned altogether.

The extensive karyological study carried out on members of this and other subgenera of *Polyommatus* by the late Hubert de Lesse while working in the Paris Natural History Museum from the late 1950s (DE LESSE, 1957; 1959a,b,c,d,e,f; 1960a,b,c; 1961a,b; 1962a,b; 1963 a,b,c) was immediately welcomed by lycaenid specialists. Characters derived from haploid chromosome complements soon became widely employed in the taxonomy of this group, where they contributed considerable changes. On this basis, many morphologically almost indistinguishable forms of the monomorphic complex have been recognized as distinct species, whereas a number of new species have been described both within this and the dimorphic group.

Not even this study, however, could provide a definitive solution to the biological riddle of *Agrodiaetus*. A first problem is that butterfly chromosomes are normally seen in a contracted state, when they do not show any of the

karyological details used to identify homologous regions. What is worse, it soon became apparent that Lepidoptera chromosomes may be holocentric, (see WHITE, 1973 for a review). Should this be true, it is contended, haploid chromosome complements (i.e.'chromosome numbers') would provide little or no evidence for speciation. Holocentric chromosomes, in fact, could conceivably pair with each other almost at any homologous region, and not at the centromeric region only as is usual. For similar reasons they could freely become fragmented, or bind on ends to each other, etc. without necessarily causing any major karyological imbalance. Even though views on this subject seem now to be changing again (see BIGGER, 1960 ; SUOMALAINEN, 1969 ; or LORKOVIC, 1990 for a review), haploid complements permit, at least, various interpretations. For example, whereas different haploid complements, as such, perhaps should not be considered sufficient evidence for speciation, when encountered in morphologically similar allopatric populations, the discovery of sharp karyotypic differences between parapatric or sympatric populations may represent a strong argument to assume that gene flow has been interrupted.

For all these reasons, however, the unusually broad variability in haploid chromosome complements currently known to exist within the subgenus *Agrodiaetus* (from  $n = 7$ , in *Polyommatus nephohiptamenos* to  $n = 125$ , in *P. dolus*), combined with the lack of evidence from crossing experiments to determine levels of hybrid dysgenesis, stimulate questions on whether or not the 60 odd currently recognized species may really all represent biologically distinct taxa.

The purpose of this work is therefore to i) utilize electrophoretically detectable enzyme variability to analyze levels of genetic divergence between karyotypically different sibling species of the monomorphic complex, ii) show relationships among members of this subgenus and finally to iii) suggest a possible evolutionary scenario.

## Materials and methods

### *Preparation of samples*

A total of 196 specimens from 21 natural populations of *Agrodiaetus* and 1 population of the subgenus *Lysandra* (*Polyommatus (Lysandra) corydonius* Herrich-Schäffer, 1852, otherwise known as *P. (L.) caucasicus* Lederer, 1869), included as outgroup, were collected at several localities in Italy, France, Spain, ex-Yugoslavia and Turkey (Table 1).

Since females are difficult to identify, only adult males were employed. Their wings were immediately removed with sharp scissors and the whole bodies were frozen in liquid nitrogen while still alive. Specimens were stored in this medium for several weeks, until further processing. Samples were prepared for electrophoresis as follows. Individual butterfly bodies were thawed in 250 µl of an ice-cold homogenizing solution (NADP 0.125 mM, 2-mercaptoethanol 1.14 mM ; pH range between 6-8) and macerated with an electric tissue grinder.

Table 1  
Populations sampled

Locality	Country, Region	No.	Symbol
Col de Cabre	France, Drôme	13	D1 ( <i>damon</i> )
Glassier di Ollomont	Italy, Aosta	11	D2 ( <i>damon</i> )
Tahir	Turkey, Agri	10	D3 ( <i>damon</i> )
Tragacete	Spain, Cuenca	11	D4 ( <i>damon</i> )
Les Puits d'Auzon	France, Bouches du Rhône	10	L1 ( <i>dolus dolus</i> )
L'Hospitalet du Larzac	France, Aveyron	5	L2 ( <i>dolus vittatus</i> )
Pic du Cougouille	France, Aveyron	10	L3 ( <i>dolus vittatus</i> )
Ainsa	Spain, Huesca	6	Fu ( <i>fulgens</i> )
Erzincan	Turkey, Erzurum	9	Me ( <i>menalcas</i> )
Ainsa	Spain, Huesca	2	R1 ( <i>riparti</i> )
Akşehir	Turkey, Konya	11	R2 ( <i>riparti</i> )
Col de Braus	France, Alpes Maritimes	16	R3 ( <i>riparti</i> )
Koçak	Turkey, Van	10	De ( <i>demavendi</i> )
Sinkan	Turkey, Ankara	9	A1 ( <i>admetus anatoliensis</i> )
Küru Dagi	Turkey, Çanakkale	9	A2 ( <i>admetus admetus</i> )
Nova Breznica	Macedonia	9	A3 ( <i>admetus admetus</i> )
Gevas	Turkey, Van	10	In ( <i>interjectus</i> )
Tragacete	Spain, Cuenca	14	Fa ( <i>fabressei</i> )
Oulx	Italy, Torino	7	Ex ( <i>exuberans</i> )
Pondel	Italy, Aosta	12	Hu ( <i>humedasae</i> )
Palandöken	Turkey, Erzurum	10	Ly ((L.) <i>corydonius</i> )

Centrifugation at 13,000 x g for 15 minutes permitted the separation of a clear supernatant. Care was paid to avoid overheating during both homogenization and centrifugation. Homogenates were stored at -80°C in 5-15 µl aliquots in microtubes.

#### *Electrophoresis*

Electrophoresis was carried out on Cellogel sheets at 4°C, as we have found the gel form of cellulose acetate an excellent support medium. An important advantage is that it requires only 0.5-1 µl per sample per enzyme run, whereas other support media require 10-50 µl: this is a remarkable advantage for projects where many enzymes must be scored (often more than once for obtaining best results) from single very small samples.

Buffer systems and staining techniques were similar to those described by MEERA KHAN (1971) and RICHARDSON *et al.* (1986). Genetically interpretable banding patterns could be obtained for: glycerol-3-phosphate dehydrogenase (E.C.1.1.1.8) ( $\alpha$ GPD), adenylate kinase (E.C.2.7.4.3) (AK), hexokinase (E.C.2.7.1.1) (HK), glucose-6-phosphate dehydrogenase (E.C.1.1.1.49) (G6PD), malate dehydrogenases (E.C.1.1.1.37) (MDh-1, MDh-2), phosphoglucose isomerase (E.C.5.3.1.9) (PGI), glutamate-oxaloacetate transferases (E.C.2.6.1.1) (GOT-1, GOT-2), malic enzyme (E.C.1.1.1.40) (ME), 6-phosphogluconate dehydrogenase (E.C.1.1.1.44) (6PGD), phosphoglucomutase (E.C.2.7.5.1)

(PGM), esterases (E.C.3.1.1.1) (ES-1, ES-2). Isozymes and alleles were designed numerically according to their decreasing mobility rate.

#### Statistical analyses

Average probabilities of interpopulation genetic distance were estimated by NEI's (1972)  $I$  and  $D$  related indexes (jackknifed according to MUELLER & AYALA, 1982), on the basis of fourteen shared loci and for pairwise combinations of all populations investigated.

#### Results and discussion

The cumulative total of alleles detected at the fourteen shared loci is 67 (range 3-9). Allele frequencies are reported in table 2. Nei's genetic index  $D$  was employed to generate the cluster shown in Fig. 1. While the distance between the subgenera *Agrodiaetus* and *Lysandra* shows, as expected, a relatively high level of genetic differentiation ( $D = 0.625$ ), the split sequence and branch lengths within the subgenus *Agrodiaetus* are rather unexpected. A second split separates *P. damon* samples ( $D = 0.460$ ) from all the rest. The separation between monomorphic and dimorphic forms occurs at  $D = 0.20$ .

From the dendrogram the following phylogenetic reconstruction can be inferred :

1. *Polyommatus (Lysandra) corydonius* lies on a different lineage with respect to all the populations of *Agrodiaetus*. The latter, accordingly, may be considered a monophyletic group (subgenus).
2. Within the phyletic line of *Agrodiaetus*, the four studied populations of *P. damon* group on a distinct branch.
3. Another branch includes all monomorphic populations, together with *P. dolus*, *P. dolus vittatus*, *P. fulgens* and *P. menalcas*. Males of both monomorphic and dimorphic forms in this phyletic line are provided with androconial scale-tufts ('sex-brands'). The close relationships within the latter lineage support an old, non phylogenetically-based suggestion by DE LESSE (1960a), who divided *Agrodiaetus* into two main groups : the *P. ripartii* complex, including both monomorphic and dimorphic forms with androconial scale-tufts in males, and the *P. damon* group, where sex-brands are absent.

Distances between members of both complexes are indeed very small, but not incompatible with those encountered between sibling species of other groups (MENSI *et al.*, 1988 ; 1992). The central point, in this respect, is represented by the pivotal position assumed in the dendrogram by *P. admetus*. This is, in fact, about the only easily identifiable species within the monomorphic complex. *P. admetus* is widely distributed from "Yugoslavia" to East Turkey and, apart from flying with many other species of the dimorphic group (*P. menalcas*, *P. hopfferi*, etc.), is often encountered in cohabitation with e.g. *P. ripartii* (Greece, Turkey, etc.), *P. demavandi* or *P. interjectus* (Turkey). Since its species-level separation from all other species of the monomorphic complex

Table 2

Allele Frequencies at 14 shared non-monomorphic loci in 21 populations. Populations are numbered as in Table 1. For some enzymes not all individuals of all populations were scored. '-' indicates that in the population scoring was not possible. In some cases numeration of alleles takes into account populations not included in this work.

		D1	D2	D3	D4	L1	L2	L3	Fu	Me	R1	R2	R3	De	A1	A2	A3	In	Fa	Ex	Hu	Ly
$\alpha$ GPD	1	0.05	0.05											1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	2	1.00	0.95	0.95	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.95	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
	3													0.05								
AK	1	0.72	0.61	0.50	1.00																	
	2	0.28	0.39	0.50		1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.75	1.00	0.94	1.00	1.00	1.00	1.00	1.00	0.80
	3													0.25		0.06						0.20
HK	1																					
	2	1.00	1.00	0.80	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
	3													0.20								
G6PD	1																					
	2													0.12								
	3	1.00	1.00	1.00	0.87																	
	4																					
	5																					
	6																					
	7																					
	8																					
	9																					
MDH-1	1													0.08				0.06			0.56	
	2	1.00	1.00	1.00	1.00	1.00	1.00	0.92	1.00	1.00	0.95	1.00	1.00	0.69	0.78	0.50	0.44	0.75		1.00	0.96	0.85
	3										0.05			0.25	0.22	0.50		0.25	1.00		0.04	0.15
MDH-2	1																					
	2	0.15	0.41		1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.79	1.00	0.87	1.00	1.00	0.89	0.50	1.00	1.00		
	3	0.85	0.59	1.00											0.12			0.11	0.50			
PGI	1													0.05								
	2	0.04												0.11	0.08	0.09	0.05					
	3																			0.06	0.05	0.21
	4	0.92	1.00	1.00	0.85	0.50	0.50	0.75	0.94	1.00	0.73	0.62	0.70	0.78	0.83	0.89	0.90	0.46	0.79	1.00	0.85	
	5											0.14	0.35	0.10								
	6	0.04										0.15	0.50	0.33	0.17	0.06	0.04	0.05	0.22	0.17	0.06	0.05
	7																			0.10		0.15

		D1	D2	D3	D4	L1	L2	L3	Fu	Mc	R1	R2	R3	De	A1	A2	A3	In	Fa	Ex	Hu	Ly	
GOT-1	1	-	0.25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	2	1.00	0.75	0.65	0.14	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.20	0.06	0.61	0.06	0.61	0.06	0.61	0.06	0.61	0.15	
	3	-	-	-	-	-	-	-	-	-	-	-	0.55	1.00	1.00	0.94	0.39	1.00	1.00	1.00	1.00	1.00	0.85
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
GOT-2	1	0.17	0.25	0.25	0.86	-	-	-	-	-	-	-	0.25	-	-	-	-	-	-	-	-	-	
	2	0.83	0.62	1.00	0.75	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.06	0.11	0.06	0.11	0.06	0.11	0.06	0.11	0.06	0.11	
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
ME	1	0.17	0.09	0.09	0.86	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	2	1.00	0.83	1.00	0.91	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
6PGD	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
PGM	1	0.92	1.00	1.00	0.75	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	2	0.08	-	-	0.25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
EST-1	1	0.14	0.25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	2	0.86	1.00	0.75	1.00	0.10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
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	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
EST-2	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
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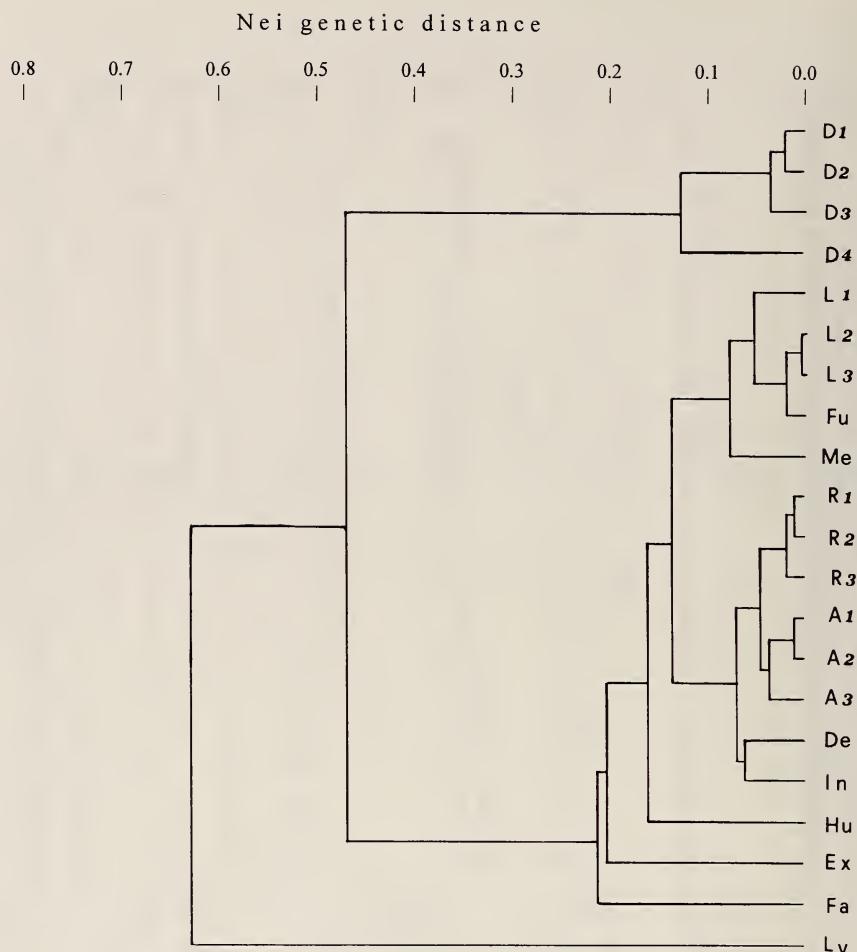


Figure 1. Dendrogram based on Nei's Distances. All nodes shown are statistically different from zero.

is unquestionable, its level of genetic divergence with respect to *P. ripartii*, on the one hand, and *P. demavandi* and *P. interjectus*, on the other, can be taken as a yardstick to infer that all other chromosomically- identified 'species' within the monomorphic complex may indeed qualify as biologically distinct sibling species. Conversely, even though a more detailed analysis is required, also extending to the dimorphic species group, our results are not incompatible with the hypothesis that, in the case of *Agrodiaetus*, karyological mechanisms may have been involved in speciation processes.

Based on Nei's rough estimates for the (highly controversial) molecular clock hypothesis, it may be possible to approximately date the branching events as follows : the subgenus *Agrodiaetus* originated about 3.1 Myr ago (i.e. in the late Pliocene) as dimorphic, sex brand-lacking forms (plesiomorphic characters) ; monomorphism and scale-tufts appeared later, roughly 2.3 Myr ago. Finally, the ancestors of the *Agrodiaetus dolus* group diverged only about 1 Myr ago. The dimorphic character of this complex is therefore of a secondary origin, and may be derived from a simple reverse mutation which took place within the monomorphic complex. Speciation events within the monomorphic complex are indeed very recent, generally in the 50,000-100,000 years bp interval. These distances, however, are too small to be reliable and should be confirmed by independent studies on mitochondrial DNA. The most important exceptions, in this respect, are represented by *P. fabressei*, *P. exuberans* and *P. humedasae*, which, in this order, are the most primitive taxa of the monomorphic group. It may be interesting to note that, apart from *P. admetus*, *P. fabressei* is the only other monomorphic species commonly encountered in cohabitation with another species of this same complex (with *P. ripartii* in the Montes Universales region : central Spain cf. DE LESSE, 1961c and personal observations).

### Acknowledgements

This study was financially supported by the Italian Ministry for University and Scientific Research (MURST) under 40% and 60% research funding programs, and by the Italian National Research Council. We also wish to thank Dr. V. Cameron-Curry, Prof. P. Passerin d'Entrèves and Dr. L. Giacoma for help in the linguistic revision of a first draft of this manuscript.

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Artikel/Article: [Biochemical taxonomy and evolutionary relationships in Polyommatus \(subgenus Agrodiaetus\) \(Lepidoptera, Lycaenidae\) 105-114](#)