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Biochemical systematics of the leaf mining moth family Nepticulidae (Lepidoptera). III. Allozyme variation patterns in the *Ectoedemia subbimaculella* group

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Abstract

Gel electrophoretic techniques were used to analyse patterns of variation at 12 genetic loci within and among species of the *Ectoedemia subbimaculella* group from western Europe. Geographically separated conspecific populations were similar to one another genetically, with the exception of *E. subbimaculella* where the malate dehydrogenase locus exhibited clinal variation. Genetic differences among species often concerned loci that were monomorphic or slightly polymorphic within populations. Three of the species could not be diagnosed by their allozyme content; allele distribution patterns at some loci suggest that speciation took place recently and did not involve a genetic bottleneck. Phylogenetic trees constructed from allozyme data paralleled closely the phylogeny based on morphology.

Résumé

Les techniques d'électrophorèse sur gel ont été utilisées pour l'analyse des patrons de variation à 12 loci génétiques, à l'intérieur des – et entre les – espèces du groupe *Ectoedemia subbimaculella* d'Europe occidentale. Les populations conspécifiques géographiquement séparées se sont montrées similaires au point de vue génétique, à l'exception de *E. subbimaculella*, espèce pour laquelle le locus "malate dehydrogenase" a montré une variation clinale. Les différences génétiques entre espèces concernent souvent des loci qui sont monomorphes ou faiblement polymorphes dans les populations. Trois des espèces n'ont pas pu être caractérisées par leur contenu en allozymes; les patrons de distribution des allèles à certains loci suggèrent que la spéciation a été de date récente et qu'elle n'a pas impliqué un "bottleneck" génétique. Les arbres phylogénétiques construits à partir de données sur les allozymes donnent une image parallèle à la phylogénie basée sur la morphologie.

Introduction

Allozyme analysis is a relatively long-established technique of low cost and difficulty that is especially suitable for studies of systematics, phylogeny, and population structure (Menken & Ulenberg, 1987). The so-called zymogram technique combines electrophoretic separation of enzymes from crude extracts of individual specimens with enzyme-specific staining procedures (Hunter & Markert, 1957). The great majority of species contain vast amounts of allozyme variation (Nevo et al., 1984; Graur, 1985), thus providing a nearly inexhaustible reservoir of characters that can be used in species identification and analysis of intraspecific variability patterns. The genetic basis of this variability is generally of a simple Mendelian nature and can either be reasonably inferred or readily determined through genetic crosses. The limitations of the versatile zymogram technique are rather well known and hence it is the best technique available (Loxdale & Den Hollander, 1989; Menken, 1989).

The monotrysian family of the Nepticulidae comprises some of the smallest moths known in the world; their larvae mine mainly in leaves, but also in petioles, seeds and bark of mostly dicotyledon plants. The family is cosmopolitan; it comprises two major genera (viz., *Stigmella* and *Ectoedemia*) and several minor ones. So far only morphological characters, mainly those concerning larvae and genitalia, were used to establish species groups. In the genus *Ectoedemia* (subgenus *Ectoedemia*) spe-

cies groups mirror phylogenetic relationships (Van Nieukerken, 1985).

In Nepticulidae, allozyme analysis has proved to be very successful in resolving systematic problems and discriminating sibling species in particular (Wilkinson et al., 1983; Menken & Ulenberg, 1987; Van Driel & Menken, 1988; Cronau & Menken, in press). In the present contribution the technique is applied to visualize genetic and phylogenetic relationships among members of the *Ectoedemia subbimaculella* group, a well-defined presumably monophyletic group of species that almost exclusively mine on *Quercus* (Van Nieukerken, 1985). The *E. subbimaculella* group comprises among other taxa the *E. albifasciella* complex (including *E. albifasciella* and *E. cerris*), the *E. subbimaculella* complex (three of their species are *E. heringi*, *E. liechtensteini*, and *E. subbimaculella*), and *E. nigrosparsella*. *E. ilicis* is used for outgroup comparison, because it belongs to the supposed sister group of the above-mentioned assemblage. The affinities of the *E. subbimaculella* group, however, are not yet completely clarified (Van Nieukerken, 1985).

Materials and methods

Table I lists the species investigated, their sampling locality and sample size. Additional collections were made for studying intraspecific variability patterns (Table III); these include (for abbreviations see legend to Table I) ALBI populations 81204 from Drouwen (NL) and 81374 from Noordoostpolder (NL); HERI population 83536 from Hainburg (A); LIEC population 83514 from Bezdan (Yugoslavia); and SUBB populations 81486 from Chaam (NL) and 83536 from Hainburg (A).

Larvae were collected in their mines in the field and kept in small glass jars containing a thin layer of sterilized potting ground or in plastic bags. Full-grown larvae were removed from their mines and stored at −30°C until used for electrophoretic analysis.

Electrophoretic and staining procedures essentially follow Menken (1982) and Van Driel & Menken (1988). Adequate resolution of bands in *Ectoedemia* species was not achieved for many enzyme systems, despite the various types and combinations of gel and electrode buffers, gel composition, and gel concentration as well as the large number of enzyme staining techniques applied.

The following ten enzymes, comprising 11 putative genetic loci, were studied (with E.C. number and locus abbreviations in parentheses): Malate dehydrogenase (1.1.1.37, *Mdh*), malic enzyme (1.1.1.40, *Me*), 6-phosphogluconate dehydrogenase (1.1.1.43, *6-Pgdh*), NADH dehydrogenase (1.6.99.3, *NADHdh-2*), catalase (1.11.1.6, *Cat-1*), aspertate amino transferase (2.6.1.1, *Aat*), esterase (3.1.1.2, *Est-α2*, *Est-β3*), leucine aminopeptidase (3.4.11.1, *Lap-1*), glucose-6-phosphate isomerase (5.3.1.9, *Gpi*), and phosphoglucomutase (5.4.2.2, *Pgm*). In addition one general protein was investigated (-, *Pt-2*).

All enzymes migrated toward the anode. When more than one isozyme (i.e., enzymes with identical substrate specificities encoded for by different loci) was present the least anodally migrating one was designated as "1", the next as "2" and so forth. Allozymes and their coding alleles were identified according to the migration distance in mm from the most common allozyme in *E. angulifasciella* (Stainton, 1849) (this reference band was designated "100"; see Wilkinson et al., 1983) under standard electrophoretic conditions.

Genetic interpretation of the allozyme variation patterns was inferential and found to be in agreement with Hardy-Weinberg proportions at the population level. Owing to insensitivity of the zymogram technique, allozyme bands might be heterogeneous collections of approximately identically-charged proteins. They are, therefore, designated more appropriately as electromorphs (King & Ohta, 1975), but following a suggestion by Allendorf (1977) I keep using the term allozyme.

Allele and genotype frequency data were analysed with BIOSYS-1 (Swofford & Selander, 1981) to produce an UPGMA dendrogram based on Nei's (1972) genetic distance estimate and with Jelly (Ellis, 1987; see for details Scheepmaker et al., 1988), a program for phylogenetic tree construction using the HAP algorithm of Rogers (1984).

Results and discussion

Table I lists the seven species investigated. Among the set of 12 homologous loci screened in these species, two were identically fixed for the same allozyme in all species studied, viz., *NADHdh-2* and *Pt-2*. The remaining ten loci were polymorphic in one or more of the species investigated; their frequencies are listed in Table II. Most sample sizes were small, as were the numbers of loci examined. This lowers the reliability of heterozygosity estimates as well as of dendrograms. Consequently, the results of the present analysis should be viewed with caution.

Enzyme number and choice strongly influence calculations of heterozygosity levels (Simon & Archie, 1985). Since an homologous set of 12 loci was investigated (only *E. ilicis* and *E. cerris* were not analysed at *6-Pgdh*) heterozygosities are directly comparable. Mean species heterozygosity (*H*)

Table I. Species of the *Ectoedemia subbimaculella* group, their abbreviation used in tables and figures, the food plant and the locality from which they were sampled, and the number of individuals analysed (A, Austria; H, Hungary; NL, The Netherlands; SP, Spain).

Species	Abbreviation	Food plant	Locality	*N*
E. albifasciella (Heinemann, 1871)	ALBI	*Quercus robur*	Rockanje (NL)	33
E. cerris (Zimmermann, 1944)	CERR	*Q. cerris*	Mannersdorf (A)	2
E. heringi (Toll, 1934)	HERI	*Q. petrea*	Erd (H)	30
E. ilicis (Mendes, 1910)	ILIC	*Q. rotundifolia*	Iguatega (SP)	3
E. liechtensteini (Zimmermann, 1944)	LIEC	*Q. cerris*	Eisenstadt (A)	22
E. nigrosparsella (Klimesch, 1940)	NIGR	*Q. pubescens*	Gumpoldskirchen (A)	10
E. subbimaculella (Haworth, 1828)	SUBB	*Q. robur*	Den Haag (NL)	30

ranged from 0.064 in *E. albifasciella* to 0.204 in *E. cerris* (in the latter species only 2 individuals were studied, so that this estimate is highly unreliable) with a mean $H \pm$ S.E. of 0.126 ± 0.050 (or 0.113 ± 0.039 if *E. cerris* is excluded). This figure is very close to those for other Nepticulidae (Wilkinson et al., 1983; Van Driel & Menken, 1988; Cronau & Menken, in press), but relatively low for Lepidoptera in general (Nevo et al., 1984; Cronau & Menken, in press). This conclusion is strengthened by the fact that only 11 or 12 loci were used for calculating H levels and that a negative correlation exists between number of loci sampled and the resulting H values (Singh & Rhomberg, 1987).

To estimate the genetic differentiation between each pair of species, Nei's (1972) measures of genetic identity (I) and distance (D, with $D = -\ln I$) have been calculated for each of the 21 pairwise comparisons (data not shown). From the genetic distance matrix an UPGMA dendrogram was constructed (Fig. 1). Fig. 2 depicts a phylogenetic tree constructed with the Jelly program (Ellis, 1987). The biochemical-genetical classifications (UPGMA and phylogenetic tree; Figs. 1 & 2) corroborate Van Nieukerken's (1985) morphological classification of the *E. subbimaculella* group. The *subbimaculella* complex, consisting of the closely related species *E. subbimaculella*, *E. heringi*, and *E. liechtensteini* (the exact clustering of which depends on the populations under consideration), is the sister group of *E. nigrosparsella* and the *albifasciella* complex; the latter comprises amongst other species *E. albifasciella* and *E. cerris*. *E. ilicis* was used for outgroup comparison.

In their much-cited paper on the effect of small sample sizes on the accuracy of dendrograms Gorman & Renzi (1979) stated that one arrives at a reasonable estimate of species relationships even if only one or two individuals per taxon are analysed. After re-examining this paper Archie et al. (1989) arrived at an entirely different conclusion: few specimens might cause instable and inaccurate classifications. Nei (1978) has shown that, from a limited number of individuals, reliable genetic identity and genetic distance estimates can be calculated only if a large number of genetic markers are scored, the average heterozygosity is low, or the genetic distance is large. In the last two cases, one individual might suffice to obtain the correct topology of a dendrogram (see e.g. *Yponomeuta rorellus* [Hübner, 1832] with H less than one percent [cf. Menken, 1987] and *Y. vigintipunctatus* [Retzius, 1844], where on the average more than two allelic substitutions per locus are estimated to have occurred in their evolution from the common ancestor of the West European ermine moth species [cf. Menken, 1982]). Thus four individuals of *E. ilicis* with $H = 0.121$ and with 4 out of 11 loci diagnostic (i.e., when the probability of assigning an individual to the correct species is 95% or higher) give quite reliable estimates of relationships, while two *E. cerris* individuals with $H = 0.205$ and with one out of 11 loci diagnostic do not.

With respect to morphology, *E. subbimaculella*, *E. liechtensteini*, and *E. heringi* are easily discriminated in the larval stage; adults of *E. liechtensteini*, however, are not separable from those of *E. heringi* (Van Nieukerken, 1985). However, the three species are biochemical-genetically undifferentiated (no diagnostic loci present) and thus appear to be of recent origin. They exhibit the following allozyme patterns:

Table II. Allele frequencies at 10 polymorphic loci in 7 species of the *Ectoedemia subbimaculella* group; *h* indicates single locus heterozygosity and *H* the average heterozygosity.

Species		ALBI	CERR	HERI	ILIC	LIEC	NIGR	SUBB
Locus and alleles								
Mdh								
Sample size		66	4	50	6	44	20	60
103				1.00	0.17	0.82		0.07
110						0.02		
114		1.00	1.00		0.83	0.16	1.00	0.93
	h	0.00	0.00	0.00	0.28	0.31	0.00	0.12
Me								
Sample size		66	4	30	6	38	12	56
76		0.03					0.08	
84		0.94	1.00				0.92	
88		0.03			1.00			
100						0.03		0.02
108				1.00		0.97		0.98
	h	0.12	0.00	0.00	0.00	0.05	0.15	0.04
6-Pgdh								
Sample size		10		46		36	14	40
86		1.00					1.00	
88								0.02
96				0.76		0.75		0.83
98				0.02				
100				0.20		0.22		0.12
103				0.02				0.03
104								
106						0.03		
	h	0.00		0.38		0.39	0.00	0.30
Cat-1								
Sample size		60	4	52	6	44	14	56
86					1.00			
89			1.00					
91		0.02					1.00	
96		0.98						
106				1.00		0.95		0.96
111						0.05		0.04
	h	0.03	0.00	0.00	0.00	0.09	0.00	0.07
Aat								
Sample size		66	4	44	4	44	12	56
91		1.00	0.50	1.00		1.00		0.98
95					1.00			0.02
96			0.50					
110							1.00	
	h	0.00	0.50	0.00	0.00	0.00	0.00	0.04
Est-α2								
Sample size		66	4	20	6	20	14	60
108					0.83			
114		1.00	1.00	1.00	0.17	1.00	1.00	1.00
	h	0.00	0.00	0.00	0.28	0.00	0.00	0.00

Table II. (continuation)

Species		ALBI	CERR	HERI	ILIC	LIEC	NIGR	SUBB
Est-β3								
Sample size		66	4	44	6	40	20	56
86								0.04
90				0.09				
94				0.91		1.00		0.96
96			0.25					
100					1.00			
104		1.00	0.75				1.00	
	h	0.00	0.37	0.16	0.00	0.00	0.00	0.07
Lap-1								
Sample size		66	4	44	4	42	20	56
88							0.10	
100							0.75	
108								
109			0.25	0.02		0.12	0.15	0.25
114			0.75					
116				0.95		0.86		0.70
120				0.02		0.02		0.05
125		1.00			1.00			
	h	0.00	0.38	0.09	0.00	0.25	0.41	0.45
Gpi								
Sample size		66	4	50	6	44	20	56
80				0.02				
82			0.50					
83		0.04				0.04		
87						0.02		
90		0.94	0.50	0.96		0.77	0.85	0.86
93					0.17			
95						0.02		
97				0.02	0.67	0.14		0.14
98							0.15	
104					0.17			
z		0.02						
	h	0.12	0.50	0.08	0.50	0.38	0.26	0.25
Pgm								
Sample size		58	4	60	6	44	20	54
46				0.08				
60				0.07		0.04		0.04
68				0.13		0.70		0.02
70		0.52						
78				0.43		0.18		0.61
79		0.48	0.50					
85				0.08				0.33
87						0.05		
88					0.83			
89				0.02				
91				0.15		0.02		
93			0.50					
94				0.02	0.17		1.00	
97				0.02				
	h	0.50	0.50	0.75	0.28	0.47	0.00	0.51
H		0.064	0.204	0.122	0.121	0.161	0.068	0.142

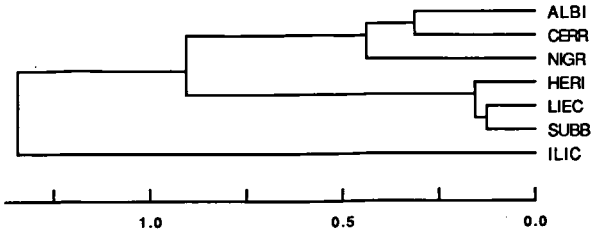

Fig. 1. UPGMA dendrogram of Nei's (1972) genetic distance (*D*) based on 12 genetic loci.

(1) Fixed for the same allozyme (*NADHdh-2, Est-α2, Pt-2*);
(2) Fixed for an allozyme, that is the most common in the other species (*Me, Cat-1, Aat, Est-β3*, and probably *Mdh*, but see below);
(3) Share major polymorphisms (*6-Pgdh, Lap-1, Gpi*, and *Pgm*).

Especially at loci of the last category allelic variation within the three species was large compared to variation between them. It looks like the daughter species inherited much of the polymorphisms present in their common ancestors and these polymorphisms are persisting after speciation. A comparable situation has been found in three species of *Yponomeuta* at several enzyme loci (Menken, 1982 and unpublished results). Recently, DNA restriction maps (Solignac & Monnerot, 1986) and DNA sequence analysis (Coyne & Kreitman, 1986) in *Drosophila* showed the same kind of shared polymorphisms. In such cases large data sets are needed to get to an unambiguous phylogeny. Alternatively, introgression between species may also be contributing to these patterns of polymorphism. If ancestral polymorphism is indeed passed on to daughter species, it implies that the new founder populations were not very small. In case of small founder groups either monomorphic or essentially diallelic loci are to be expected in newly formed species, due to the almost complete loss of rare and low-frequency alleles during a severe genetic bottleneck (Chakraborty et al., 1980; Huetell et al., 1980; Menken & Ulenberg, 1987). At least for *Gpi* another explanation is possible. Various studies (e.g. Harrison, 1977; Watt et al., 1986; Howard & Shields, 1990) produced evidence that patterns of variation at this locus might be explained by some form of natural selection. Therefore, selection regimes common to the three species in the *S. subbimaculella* complex might cause their similar allozyme composition.

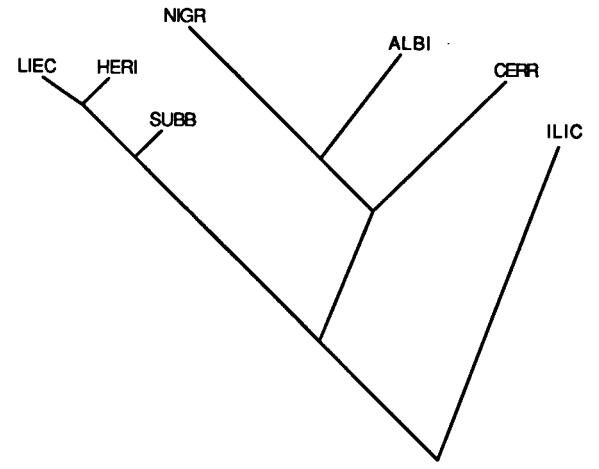

Fig. 2. Jelly network (Ellis, 1987) based upon Rogers' HAP algorithm (Rogers, 1984), represented as a "Hennigian" tree.

The supposition that, on the assumptions of the neutral theory (Kimura, 1982), proteins that tolerate more variation accumulate more intraspecific variability, and thus would diverge more rapidly than those with less variability (Ward & Skibinski, 1985) is not confirmed: differentiating and/or diagnostic loci appear to be the very ones with minor polymorphisms (e.g., *Cat-1*, *Aat*, and *Est-β3* for *E. ilicis*). The finding that differences among species often concern loci that are monomorphic within species is in agreement with data from *Chauliognathus* beetles (Howard & Shields, 1990), *Yponomeuta* (Menken, 1982; Menken & Ulenberg, 1987), and *Drosophila* (R.S. Singh, pers. comm.). Altukhov (1982) observed similar patterns of genetic differentiation among salmonid fish species; this would suggest that speciation does not involve gene frequency changes at polymorphic loci, but saltational changes at monomorphic loci instead. Alternatively, as has been suggested by Howard & Shields (1990), the common ancestor was variable at many loci and this variation could have been lost owing to speciation through founder events and subsequent genetic drift or to different directional selection regimes. This alternative is not very likely to apply to nepticulids, since the monomorphic

Table III. Intraspecific variation patterns of *E. albifasciella*, *E. heringi*, *E. liechtensteini*, and *E. subbimaculella* at the polymorphic *Mdh*, *Gpi*, and *Pgm* loci. Ref refers to the respective populations in Table I.

Species	ALBI			HERI		LIEC		SUBB		
Population	ref	81204	81374	ref	83536	ref	83514	ref	81486	83536
Locus and alleles										
Mdh										
Sample size	66	76	60	50	20	44	26	60	80	36
88							0.12			
103		0.01		1.00	1.00	0.82	0.85	0.07	0.04	1.00
110						0.02				
114	1.00	0.97	1.00			0.16	0.04	0.93	0.96	
128		0.01								
Gpi										
Sample size	66	76	60	50	20	44	26	56	90	36
80				0.02	0.05					
83	0.04	0.03				0.04	0.12		0.03	
87						0.02				
90	0.94	0.95	0.93	0.96	0.85	0.77	0.81	0.86	0.79	0.81
95			0.03			0.02				
97				0.02	0.10	0.14	0.04	0.14	0.18	0.19
100		0.03	0.03							
104							0.04			
z	0.02									
Pgm										
Sample size	58	64	60	60	20	44	26	54	70	30
46				0.08	0.05					0.03
60			0.03	0.07	0.20	0.04	0.12	0.04	0.04	0.17
68				0.13	0.25	0.70	0.42	0.02	0.06	0.03
70	0.52	0.59	0.53							
78				0.43	0.25	0.18	0.12	0.61	0.60	0.37
79	0.48	0.30	0.42							
85				0.08	0.15		0.23	0.33	0.24	0.13
87						0.05				
89				0.02	0.05				0.01	0.07
91				0.15		0.02	0.08		0.04	0.13
93		0.11	0.02							
94				0.02	0.05		0.04			0.07
97				0.02						

diagnostic loci very seldom are variable in nepticulid species (Van Driel & Menken, 1988; Cronau & Menken, in press).

Mdh is a peculiar locus in that it exhibits clinal variation in *E. subbimaculella* (Tables II & III; Menken & Ulenberg, 1987). This means that its discriminatory power depends on the population that is concerned (Menken & Ulenberg, 1987). In Fig. 1 the *E. subbimaculella* population with *Mdh-114* as most common allele is strongly differentiated from *E. liechtensteini* and *E. heringi*, but shares most of the variation at this locus with *E. albifasciella*. *E. subbimaculella* population 83536, on the contrary, is alternatively fixed (allele 103) to *E. albifasciella*, but almost identical with *E. liechtensteini* and *E. heringi*. Depending on the populations being considered, genetic identities between *E. subbimaculella*, *E. liechtensteini*, and *E. heringi* surpass 0.950

(i.e., genetic distances smaller than 0.05). Although not a law of the Medes and the Persians, populations having identities of 0.95 and higher are normally conspecific (Thorpe, 1983; Menken & Ulenberg, 1987). Yet the species seem to retain their morphological and biological distinctness in sympatry (Van Nieukerken, 1985). It is exceptional for biological species not to be distinguishable by their allozyme composition (Throckmorton, 1977; Stock & Castrovillo, 1981; Menken & Ulenberg, 1987). The three species must have been speciated recently indeed.

Overall genetic distances in the *E. subbimaculella* group were small compared to values for other *Stigmella* and *Ectoedemia* species (see discussion in Cronau & Menken, in press). This suggests that, with the exception of some species on oak, recent speciation is a rare event in western European Nepticulidae (Menken, in press).

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