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IS THE EXTREMELY LOW HETEROZYGOSITY LEVEL IN *YPONOMEUTA RORELLUS* CAUSED BY BOTTLENECKS?

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Abstract.—Among the common and widespread species of the small ermine moths (*Yponomeuta*) the almost complete absence of genetic variability from a sample of 75 protein loci is a striking feature of *Y. rorellus*. Congeners exhibit normal to high *H* levels. At least for soluble proteins, this variation estimate is a real one, not resulting from an inability of the zymogram technique to detect variation nor from a biased sample of loci. The most likely explanation for the dearth of variation observed is a bottleneck at the species' origin. Moreover, ongoing population-size fluctations in the historical past of *Y. rorellus* may have prevented the recovery of equilibrium *H* levels as a consequence of genetic drift in small populations.

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Genetic variation within populations is a prerequisite for evolution, as it provides the raw material for selection of better-adapted genotypes in response to challenges by the environment. Mutation is the ultimate source of all this variation.

Rapidly accumulating information from the last two decades shows that many species have high levels of genetic variation at the enzyme level; there is, however, much heterogeneity in variability levels among taxonomic groups as well as among species within taxonomic groups (Nevo et al., 1984; Graur, 1985).

Much speculation has arisen concerning the causes of these differences in overall genetic diversity. The following potential causes have been proposed: differences in population size, differences in environmental heterogeneity, genetic or breeding systems limiting variability, directional selection for homozygosity or for general-purpose genotypes in some species, or any combination of the aforementioned factors (Futuyma, 1979; Kimura, 1982; Nevo et al., 1984). A confusing factor in comparisons of heterozygosity estimates among species can be different samples of loci, as single locus heterozygosities differ much among loci (Powell, 1975; Menken, 1980*c*; Simon and Archie, 1985).

Species of the small ermine moth genus *Yponomeuta* (Lepidoptera, Yponomeutidae) have similar life histories, morphologies, and geographic distributions, but somewhat differing feeding habits (Herrebout et al., 1976). In a group of six closely related species, heterozygosity levels per population based upon a homologous set of 51 genetic loci range from as low as 0% (*Y. rorellus*) to over 15% (*Y. evonymellus, Y. padellus,* and *Y. cagnagellus*; Table 4). In the present study, I report the near absence of any genetic variation in natural populations of *Y. rorellus* based upon a sample of 75 loci coding for soluble proteins. The reliability of the variation estimates and the possible causes of the low heterozygosity values will be discussed. Bottlenecks seem to be the major factor that account for the observed monomorphism.

MATERIALS AND METHODS

Life Cycle

Yponomeuta rorellus, a diploid, bisexual, univoltine species, is common and widespread with a palearctic distribution. Eggs are laid in July in masses on branches of willow (*Salix* spp.). Although the caterpillars hatch some three weeks after oviposition, they remain under the protection of the egg mass until the following spring. They then spin a communal web over the developing foliage and feed within or in the neighborhood of the web, extending it as the leaves are consumed. Entire willow trees may be defoliated in cases of severe infestation, a characteristic *Y. rorellus* shares with the other species of the so-called "*padellus* complex." In case of depletion of willows, few if any other hosts will be attacked (Herrebout et al., 1976). Fifth-instar full-grown caterpillars usually pupate within their web.

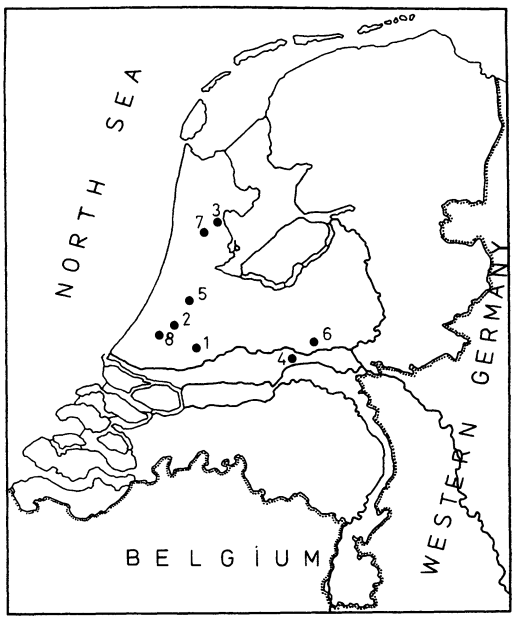

FIG. 1. Collection sites in the Netherlands for the *Yponomeuta rorellus* populations studied: 1) Haastrecht; 2) Hazerswoude; 3) Oosthuizen; 4) Schuilenburg (Kesteren); 5) Ter Aar; 6) Wageningen; 7) Zuidoost Beemster; 8) Zoetermeer.

Eclosion takes place after approximately three weeks.

Samples

Over a period of seven years (1974–1980) populations of *Y. rorellus* were sampled by collecting third- or fourth-instar larvae from the field (Table 1). They were reared in cages in the laboratory until eclosion. Mortality in the laboratory was less than 5%. Adults and, in some cases, fifth-instar larvae were deep frozen at −30°C until used for electrophoresis. Eight localities were sampled (Fig. 1). Food plants were *Salix alba* (most cases), *S. cinerea, S. viminalis, S. dasyclados, S. fragilis,* and *S. caprea.* Other host plants include *S. tenuijulis, S. babylonica, S. incana, S. oxycarpa,* and *S. australior* (Z. Gershenson, pers. comm.).

Electrophoresis

Specimen preparation, electrophoresis, isozyme specification, and enzyme assays follow Menken (1982*a*). In addition to the 51 loci studied in Menken (1982*a*) (Oosthuizen population, sampled in 1978), data are presented here on 22 more loci (Table 2). This set of loci was not included in the previous study because of inconsistently scorable variation and/or absence in one or more of the other small ermine moths investigated.

The variation reported is inferred to be Mendelian from comparison with the results from crosses in *Y. cagnagellus* (Menken, 1980*a*) and the general fit of observed phenotypic proportions to those expected under Hardy-Weinberg equilibrium.

The one heterozygote that occurs at each of the *Fudh* and *Estβ-1* loci is a single male. This observation is in agreement with the sex-linked character of these two loci (Menken, 1980*a*; Lepidoptera have female heterogamety). *A-Acph* activity is limited to males (Menken, 1982*a*).

RESULTS

Natural populations of *Y. rorellus* harbor extremely low levels of genetic variation as revealed by electrophoresis. Individual values of mean heterozygosity per population (H) range from 0 to 0.0153 (mean ± SE = 0.0045 ± 0.0042; Table 1). Most notable is the Schuilenburg population, occurring on the common food plant *S. alba,* in which not a single individual in a sample of 25 moths was heterozygous for any of 45 proteins screened.

Glyceraldehyde-3-phosphate dehydrogenase contributes most to H; out of 105 mutant alleles, 58 or 55.2% are allele $G3pdh^{96}$ (Table 1). This allele is unique to *Y. rorellus* (Table 3). Single-locus heterozygosity ranges from 0.0000 to 0.4442 with a mean of 0.1103 ± 0.1416. In contrast, *G3pdh* is extremely monomorphic in nine other *Yponomeuta* species (Menken, 1980*c*); just five heterozygotes were found in 7,000 individuals).

At 15 other loci, one or a few heterozygotes were found (Table 1). Every locus has one single mutant allele except for *Pgm* (alleles *97* and *103*) and *Aph-2* (alleles *94* and *103*). Many of these alleles are unique to *Y. rorellus* (Table 3). Variant alleles at the *Estβ-3* (see below), *Lap-3,* and *Lap-4* loci could not be classified as unique or otherwise, since they have not been studied in other *Yponomeuta* species.

Scarce information is available on two more loci not shown in Tables 1 and 2, an

TABLE 1. Sample information for *Y. rorellus*. N_I = number of individuals electrophoresed; N_L = number of loci studied; H = average heterozygosity. Numbers of heterozygotes per enzyme locus are given. For abbreviations see Table 2 and Menken (1982*a*). For adults, both adult specific enzymes and enzymes occurring in both developmental stages are given. For larvae, only larva-specific enzymes and *G3pdh* are shown.

Location	Food plant	Year	N_I	N_L	H	Heterzygotes					
						G3pdh	Other				
Adults:											
Ter Aar	*Salix alba*	1974	50	12	0.0049	—	3 *Pgm*				
Ter Aar	*Salix alba*	1975	50	24	0.0041	—	1 *Pgm*	1 *Fudh*	3 *Aph-2*		
Ter Aar	*Salix alba*	1976	50	40	0.0048	4	4 *A-6Pgdh*	1 *Lap-3*	1 *Aph-2*		
Ter Aar	*Salix alba*	1977	50	50	0.0043	2	2 *Pgm*	2 *A-6Pgdh*	1 *Fudh*	2 *Aph-2*	2 *Got-2*
Ter Aar	*Salix alba*	1978	50	46	0.0033	3	3 *A-6Pgdh*	1 *Estβ-1*	1 *Lap-3*		
Oosthuizen	*Salix alba*	1977	50	49	0.0036	0	2 *A-Gluo*	1 *Mdh-2*	1 *Pt-1*	3 *Aph-2*	2 *Lap-1*
Oosthuizen	*Salix alba*	1978	39	49[a]	0.0032	3	1 *Aph-2*	1 *Pt-1*	1 *A-Gluo*	1 *Lap-4*	
Oosthuizen	*Salix alba*	1980	18	31	0.0034	2					
Haastrecht	*Salix alba*	1978	25	41	0.0009	0	1 *A-Acph*				
Haastrecht	*Salix alba*	1979	40	32	0.0050	7					
Haastrecht	*Salix cinerea*	1979	16	30	0.0114	7					
Haastrecht	*Salix viminalis*	1979	16	30	0.0000	0					
Haastrecht	*Salix dasyclados*	1979	25	34	0.0106	10	1 *A-Ao*				
Wageningen	*Salix alba*	1977	50	47	0.0008	0	1 *Pgm*	1 *Lap-4*			
Schuilenburg	*Salix alba*	1978	25	45	0.0000	0					
ZO Beemster	*Salix alba*	1980	20	31	0.0074	4	1 *Lap-4*				
Hazerswoude	*Salix fragilis*	1979	18	29	0.0096	6					
Zoetermeer	*Salix caprea*	1979	15	29	0.0153	8[c]					
Larvae:											
Oosthuizen	*Salix alba*	1975	11	18	0.0000	0					
Oosthuizen	*Salix alba*	1978	25	21[b]	0.0000	0					
Ter Aar	*Salix alba*	1977	25	22	0.0018	0	1 *L-Gluo*				

[a] Based on 36 loci from Menken (1982*a*) plus 13 loci not studied therein.
[b] Based on 15 loci from Menken (1982*a*) plus 6 loci not studied therein.
[c] One homozygote for allele $G3pdh^{96}$ was also observed.

TABLE 2. Enzyme assays and stage specifications of *Y. rorellus*. See Menken (1982*a*) for composition of buffers symbolized. Symbols for quaternary structure: M = monomer; D = dimer; — = unknown. Stage specifications: Id = identical isozymes in both stages; Ss = stage specific (referred to as L and A forms); Ls = larva specific; As = adult specific; — = studied in adults only.

Enzyme (EC number)	Abbreviation for locus	Buffer[a]	Quaternary structure	Stage specification
Oxidoreductases				
Succinate dehydrogenase (1.3.99.1)	*Sdh*	I	—	Id
Glutamate dehydrogenase (1.4.1.2)	*Gdh*	I	—	—
Monoamine oxidase (1.4.3.4)	*Mao*	I	—	—
Tyrosinase (1.14.18.1)	*L-Tyr*	II	—	Ss[a]
Transferases				
Glutamic oxaloacetic transaminase (2.6.1.1)	*Got-1*	III	—	Id[b]
Hexokinase (2.7.1.1)	*Hk-2*	I	—	Id
Adenylate kinase (2.7.4.3)	*Ak*	II	—	Id
Hydrolases				
Esterase (3.1.1.2)	*Estα-3*	III	—	As
Esterase (3.1.1.2)	*Estβ-2*	III	—	As
Esterase (3.1.1.2)	*Estβ-4*	III	—	Ls
Alkaline phosphatase (3.1.3.1)	*Aph-1*	II	—	Ls
Alkaline phosphatase (3.1.3.1)	*Aph-3*	II	—	As
Amylase (3.2.1.1)	*Amy-2*	Veronal	—	Ls
β-glucosidase (3.2.1.21)	*β-gluc*	II	—	Ls
Leucine aminopeptidase (3.4.11.1)	*Lap-2*	I	—	Ls
Leucine aminopeptidase (3.4.11.1)	*Lap-3*	I	D	As
Leucine aminopeptidase (3.4.11.1)	*Lap-4*	I	M	As
Lyases				
Fumarase (4.2.1.2)	*Fum*	I	—	Id
Aconitase (4.2.1.3)	*Acon*	I	—	Id
Isomerases				
Triosephosphate isomerase (5.3.1.1)	*Tpi*	I	—	—
Other systems				
General protein	*Pt-6*	II	—	Id
General protein	*Pt-7*	II	—	Id

[a] Adult form studied in Menken (1982*a*).
[b] Run cathodally.

esterase (*Estβ-3,* EC 3.1.1.2) and mannose phosphate isomerase (*Mpi,* EC 5.3.1.8). Both code for monomeric enzymes. Detection of *Estβ-3* is restricted to fresh adult material, and *Mpi* has been studied only once (Ter Aar population in 1981). The two enzymes seem to exhibit variation levels comparable with *G3pdh.*

The Ter Aar and Oosthuizen populations show quite constant H values over years and clearly have the highest amount of variation at loci other than *G3pdh* (Table 1). Variation at the *A-6Pgdh* locus (not investigated in 1974 and 1975) is restricted to Ter Aar. In general the other populations are somewhat more variable ($\bar{H} = 0.0061 \pm 0.0055$ versus $\bar{H} = 0.0030 \pm 0.0017$ for Ter Aar and Oosthuizen; two-tailed t-test, ns), due to the presence of a higher number of *G3pdh* heterozygotes. In Ter Aar and Oosthuizen, a slightly smaller number of loci have been investigated on the average ($\bar{N} = 32.9$), compared with the other populations together ($\bar{N} = 34.8$); hence no influence on H is to be expected (Nei and Roychoudhury, 1974; Graur, 1985). Those enzymes that appear to be polymorphic (1% criterion) in Ter Aar and Oosthuizen have been included in most other population surveys, where they appear to be monomorphic. A much more pronounced difference in H can be found between populations infesting *S. alba,* on the one hand, and all other *Salix* spp. lumped, on the other (Table 1; $\bar{H} = 0.0030 \pm 0.0022$ and 0.0094 ± 0.0057 respectively; $P < 0.01$, two-tailed t-test). Again there is not much difference in the number of loci studied ($\bar{N} = 34.9$ and 30.4 respec-

tively). The lower value for *H* on *S. alba* can be fully ascribed to a lower incidence of variation at the *G3pdh* locus, which is not compensated by more variation at loci other than *G3pdh* (see above). Population sizes are comparable on the various food plants (W. Ravensbergen, pers. comm.).

Esterases are known to be among the most polymorphic enzymes; however, *Estβ-3* seems to be the only enzyme with some variation among seven isozyme loci.

Discussion

Without genetic variability there can be no response to selection or genetic drift, and evolution cannot occur. The best single estimate of genetic variation in a population is the expected frequency of heterozygous loci per individual (*H*), assuming Hardy-Weinberg equilibrium (Nei and Roychoudhury, 1974). As these authors have pointed out, it is better to examine a large number of loci rather than a large number of individuals per locus for the reliable estimation of average heterozygosities (Archie, 1985). However, a negative correlation may exist between the number of enzymes surveyed and the magnitude of *H*, as studies often start with known polymorphic loci (Graur, 1985). Among a sample of 73 genetic loci, only *G3pdh* occasionally reaches appreciable variation levels in natural populations of *Y. rorellus*, resulting in *H* values up to 0.0153, based on various numbers of loci (Table 1).

Are these estimates an artifact caused by inadequate resolving power of the zymogram technique or biased sampling? It is now evident that electrophoresis is an imperfect detector of enzyme variants (heterogeneity within electromorphs: King and Ohta, 1975; Ayala, 1982). In order to elucidate part of this cryptic variation, some enzyme systems in *Y. rorellus* were subjected to sequential electrophoresis using two concentrations of starch (10 and 12%) and a total of 11 buffer systems with pH values ranging from 5.8 to 9.0 (unpubl.; see also Menken, 1982*a*). No additional variation was found at the *G3pdh, Pgi, Pgm, Lap-1, Estβ-1, Estβ-2,* and *A-Gluo* loci, loci that are known (with the exception of *G3pdh*) to be highly polymorphic in related *Yponomeuta* species (Menken, 1980*c*, 1981, 1982*a*). Thus the low level of variability in *Y. rorellus* seems to be a real phenomenon, not caused by inadequate technical separation of proteins. Moreover, similar electrophoretic surveys in this laboratory have found normal *H* levels in several other insect species (Menken, 1982*b*; Menken and Ulenberg, 1983, 1986; Wilkinson et al., 1983).

Table 3. Alleles unique to *Y. rorellus* are designated by +; those designated by − also occur in congeners. All loci have *100* as the most-common allele (which is also the most-common allele in *Y. cagnagellus* [Menken, 1982*a*]), with the exceptions of five loci (*) that lack such alleles and which are diagnostic, and *A-6Pgdh, Pt-1,* and *Mpi* (with most-common alleles = *98, 98,* and *95,* respectively). The diagnostic loci are fixed save for *A-Ao* (most-common allele = *98*) and *L-Gluo* (most-common allele = *104*). For abbreviations see Table 2 and Menken (1982*a*).

Locus	Allele	Unique
Fudh	*97*	+
G3pdh	*96*	+
*Idh-1**	*104*	+
Mdh-2	*106*	−
*L-6Pgdh**	*94*	+
A-6Pgdh	*97*	+
*A-Ao**	*95*	+
*A-Ao**	*98*	+
A-Gluo	*103*	−
*L-Gluo**	*104*	+
*L-Gluo**	*106*	+
Got-2	*98*	+
Pgm	*97*	−
Pgm	*103*	−
*Estα-1**	*96*	+
Estβ-1	*104*	+
A-Acph	*98*	−
Aph-2	*94*	+
Aph-2	*103*	−
Lap-1	*107*	+
Mpi	*89*	+
Pt-1	*97*	−

Bearing the above in mind, one may conclude that *Y. rorellus* is extremely monomorphic at loci coding for soluble proteins, while all species closely related to *Y. rorellus* show mean heterozygosity levels ranging from 6 to 15% (Table 4). How can this low level of polymorphism be explained?

Restricted distribution and apomixis can be excluded as possible factors reducing variation, because this is a common, widespread species which should harbor a lot of variation according to the theoretical considerations of Kimura and Crow (1964) (see Varvio-Aho [1981] for a practical example). *Y. rorellus* has a normal sex ratio (1.11, based

TABLE 4. Heterozygosity levels (H) in five species closely related to *Y. rorellus*. N_F = number of food plants; N_P = number of populations studied; N_L = mean number of loci studied per species.

Species	N_F	N_P	N_L	$\bar{H}$	H range
Y. evonymellus	1	3	41.3	0.1380 ± 0.0123	0.1270–0.1513
Y. cagnagellus	1	7	29.0	0.1253 ± 0.0196	0.1004–0.1523
Y. mahalebellus	1	3	41.7	0.0587 ± 0.0167	0.0444–0.0771
Y. malinellus	2	6	34.7	0.0809 ± 0.0095	0.0704–0.0888
Y. padellus	>10	9	30.1	0.1272 ± 0.0149	0.1135–0.1554

on 1,214 individuals). The general fit with Hardy-Weinberg proportions indicates an amphimictic breeding system as well.

A severe bottleneck when *Y. rorellus* arose may have caused the drop in the level of variation (Nei et al., 1975; Chakraborty and Nei, 1977). After a population reduction a bimodality in h levels of different loci can be observed (Nei et al., 1975; Berlocher, 1976). This has not been the case in *Y. rorellus*. It seems that the founders did not carry over genetic variation in the form of individual heterozygosity. Most of the variation found in *Y. rorellus* is unique (Table 3) and probably resulted from mutations after its original bottleneck. After a bottleneck, it takes more than $2N_e/(4N_ev + 1)$ generations (with N_e = effective population size and v = mutation rate/generation/locus) for the original H level to be restored.

Reliable estimates of population sizes in *Yponomeuta* are not available, let alone effective population sizes. Locally, *Y. rorellus* numbers can exceed 10^5, but population size is known to fluctuate heavily over years (Herrebout et al., 1976; A. M. Emmet, pers. comm.). Moreover, population differentiation and substructuring are difficult to assess, as the low level of variation does not allow the calculation of F_{ST} values (Wright, 1951), except in the case of *G3pdh*. The mean F_{ST} value over three years (0.0304 ± 0.0304) indicates little differentiation among populations, although there is a striking difference in H between populations on *S. alba* and those on other *Salix* species (Table 1).

The time since *Y. rorellus* shared a common ancestor with its closest relative can be estimated from the genetic distance (Nei, 1971) between them to be 1.4×10^6 to 5.7×10^6, depending on the formula used (Thorpe, 1982; Menken, 1982*a*). Bottlenecks lead to overestimates of divergence times (Chakraborty and Nei, 1977); the higher estimate is probably the more reliable one (Thorpe, 1982). Because *Y. rorellus* is univoltine, there have thus been several million generations for H to have been restored. Using the crude estimates $N_e = 10^5$ and $v = 10^{-7}$, the number of generations required is about 1.92×10^5. A single bottleneck when *Y. rorellus* arose cannot, therefore, explain sufficiently its low H value. The difficulty of estimating the crucial quantities of D, N_e, v, and t means that it is hard to choose among various possible explanations, but some remarks can still be made. Thus, recurrent population-size fluctuations (which do occur frequently in *Y. rorellus*) may have kept effective population sizes small, preventing populations from building up H. The absence of differential allelic fixation counts against this scenario though: it is possible that the present populations derived from a single recent population of restricted size, a bottleneck which may have been caused by a recent ice age (some 10,000 years ago). However, congeners probably experienced similar bottlenecks as they feed on plants belonging to the same plant community (Herrebout et al., 1976). The finding that *Y. rorellus* populations from Darmstadt and Bayreuth (West Germany) show the same dearth of variation (Menken, unpubl.), together with the extraordinarily simple sex-pheromone (Löfstedt et al., 1986) and the reduced chromosome number (n = 29) (Thorpe, 1929; Gershenson, 1967) of *Y. rorellus* compared with other *Yponomeuta* species (n = 31), all hint at a bottleneck at the origin of this species.

Other factors, both genetic and environmental, may reinforce the low incidence of new variation in *Y. rorellus*.

The number of food-plant species is an important component of niche breadth (environmental heterogeneity). A positive correlation between number of food plants and

genetic variation has been found for *Rhagoletis* (Berlocher, 1976), but not for a number of forest lepidopterous species (Mitter and Futuyma, 1980). In related *Yponomeuta* species, no clear correlation between *H* level and number of food plants could be found. *Y. rorellus* infesting various *Salix* species has by far the lowest *H* level; *Y. cagnagellus* and *Y. evonymellus* (both strictly monophagous), and *Y. padellus* (oligophagous) have *H* levels between 10 and 15% (Table 4). The importance of this and of factors such as low mutation rates, trophic stability (Levins, 1968), and effective population size is difficult to assess in the absence of critical data. They all remain in the realm of conjecture.

Yponomeuta species can be hybridized easily in the laboratory, but the monomorphic nature and genetic homogeneity of natural populations of *Y. rorellus* provide additional evidence of the effectiveness of its reproductive isolation from sympatric relatives in the *padellus* complex (Menken, 1980*b*).

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