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GENE FLOW AND DIFFERENCES AMONG LOCAL POPULATIONS OF THE LAND SNAIL *ARIANTA ARBUSTORUM* (LINNAEUS, 1758) (PULMONATA: HELICIDAE)

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ABSTRACT: The genetic structure of nine populations of *Arianta arbustorum* (Linnaeus, 1758) in South Poland was studied by means of allozyme electrophoresis on cellulose acetate gel. The aim of the study was to answer the following questions: (1) is there (unlike in mtDNA haplotypes) any geographic variation in allozymes among local populations of *A. arbustorum*?; (2) what are the levels and pattern of gene flow?; (3) are the subpopulations really panmictic units? To answer the questions six enzyme systems, coded by eight loci (*Hbdh*, *Iddh*, *Idh-1*, *Idh-2*, *Mpi*, *Pgdh*, *Pgm-1*, *Pgm-2*), were assayed. Neither Fisher's method (significant for four pairs of loci) nor Ohta's D-statistics applied to study linkage disequilibrium indicated close linkage caused by population subdivision. Mean number of alleles per locus was 1.98, mean expected heterozygosity 0.337. Multilocus test showed a heterozygote deficiency for all populations but one; multipopulation test showed the same for all loci, though many loci were at Hardy-Weinberg equilibrium for individual populations. f values were high, θ values moderate. Inbreeding, Wahlund's effect (mostly caused by the occurrence of several generations) and selection were considered the main sources of the observed heterozygote deficits. Mean value of θ was 0.270 and the resulting estimate of gene flow Nm=0.676. Pairwise Nm's were low (almost 5 in one case, more than 2 in two cases, and more than 1 in four cases, out of 36 comparisons) fairly differentiated and not always consistent with biological reality. Neither pairwise θ values, nor Cavalli-Sforza and Edwards arc and unbiased Nei genetic distances were statistically significantly associated with geographic distances among populations. Multidimensional scaling on θ, as well as UPGMA clustering and neighbor joining trees computed on all the three parameters, showed no geographic pattern; correspondence analysis on allele frequencies did not show such a pattern, either. It is hypothesized that the present differentiation is a combined result of the following: (1) time (the snail has probably inhabited the studied area since before the latest glaciation); (2) the low levels of migration; (3) the immigrants' poor chance of winning in the competition against the autochthons; (4) selection; (5) new mutations (possibly).

INTRODUCTION

In land helicids short-distance genetic differences among local demes, marked in mitochondrial DNA haplotypes, are unusually high. They are comparable with such differences among some genera or even families of other animals (THOMAZ et al. 1996, AVISE 2000). A few helicid species showing stable genetic polymorphism have for a long time been studied as model animals (DI CESNOLA 1907, JOHNSON 1976, 1979, JONES et al. 1977, 1980, CLARKE et al. 1978, FALNIOWSKI et al. 1993a, b, LAZARIDOU-DIMITRIADOU et al. 1994). The conclusion is that morphological polymorphism reflects ecological factors, variable selection, as well as historical constraints and stochastic phenomena like area effects (COOK 1965, COOK & KING 1966, CAMERON & PALLES-CLARK 1971, PARKIN 1971, 1972, 1973, JOHNSON 1976, 1979, JONES et al. 1977, 1980, CLARKE et al. 1978, REICHHOLF 1979, ABDEL-REHIM 1983a, b, 1984, BURLA & STAHEL 1983, BURLA 1984, ABDEL-REHIM et al. 1985, ARTER 1990, BURLA & GOSTELI 1993).

On the other hand, allozyme polymorphism is selectively nearly neutral and shows no geographic pattern apart from area effects. This has been described in *Cepaea nemoralis* (Linnaeus, 1758) (JOHNSON 1976, 1979, for review see CLARKE et al. 1978, JONES et al. 1977, 1980) and some other helicoids like *Bradybaena fruticum* (O. F. Müller, 1774) (FALNIOWSKI et al. 1993a, b, 2004), in which, however, a trace of geographic variation in gene flow is found. ARTER (1990) studied interpopulation differences in *Arianta arbustorum* (Linnaeus, 1758) along a few kilometres at the same altitude in the Alps and found a significant association between Nei genetic distances and geographic distances. Is then *A. arbustorum* a special case of a land helicoid with an "aberrant" genetic structure of its subdivided population?

The snail inhabits wet shrubs, meadows, forests, parks and cemeteries. In mountains it is common on rocks and in rock crevices, in the Polish Tatra Mountains it can be found above 2,000 m a.s.l. (URBAŃSKI 1957), and in the Alps above 2,700 m (BURLA & STAHEL 1983). Its range spans from central France, NE Ireland and Great Britain to the western part of Scandinavia in the north; to Bothnian Bay and the vicinity of Kiev in the east; to the South Carpathians and South Alps in the south (KERNEY et al. 1983). In Poland it inhabits all the territory except Podlasie with the Białowieża Forest, Wzgórza Trzebnickie and Little Poland Upland; an isolated part of its range covers Kraków–Częstochowa Upland (RIEDEL 1988).

A. arbustorum has been a popular subject of studies concerning biology, ecology, biometry, etc.; several of its biological and ecological traits have indicated differentiaton (STÖVER 1973, TERHIVUO 1978, ANDREASSEN 1981, ABDEL-REHIM 1983b, 1984, BAMINGER 1997, BAUR 1984a, b, c, 1986a, b, 1987a, b, 1988a, b, c, 1990a, b, 1992, 1993, 1994, BURLA 1984, REICHARDT et al. 1985, BAUR & BAUR 1986, 1990, 1992, 1993, BAUR & GOSTELI 1986, RABOUD 1986a, b, BAUR & RABOUD 1988, SPEISER & ROWELL-RAHIER 1991, 1993, BURLA & GOSTELI 1993, CHEN & BAUR 1993, BOJAT & HAASE 2001, BOJAT et al. 2001). The snail is hermaphroditic, but cross fertilization seems obligatory: the fecundity of isolated selfing specimens was less than 1% of that of cross-fertilizing specimens (CHEN & BAUR 1993); multiple mating is common (BAUR 1988c, 1992), sperm may be stored for more than a year (BAUR 1988c) and the snail probably can control the usage of stored sperm (BOJAT & HAASE 2001, BOJAT et al. 2001). It matures in the 2–4th years of life and lives through the next 3–4 years on average, the maximum life span being 14–17 years (RABOUD 1986, BAUR & RABAUD 1988, BAUR 1990b). Throughout its life the snail produces 43–831 eggs depending on locality (BAUR & RABOUD 1988). Actively crawling, it disperses very slowly (BAUR & BAUR 1993) mainly upstream along streams and rivers (BAUR & GOSTELI 1986). Its population density is 5–20 individuals m^{-2} (BAUR 1986a, BAUR & BAUR 1990); both density and aggregation pattern vary throughout the year (TERHIVUO 1978, BAUR 1984a). In spring it crawls more actively; its local panmictic units consist of a few hundreds of specimens (ARTER 1990). It is a generalist herbivore preferring mixed diets (SPEISER & ROWELL-RAHIER 1993). Several traits of its biology and ecology remain unchanged in specimens transported to different habitats, which suggests a genetic component of those local adaptations.

Apart from the wide continuous variation and polymorhism, in the Alps and West Europe there are a number of local forms of *A. arbustorum* (EHRMANN 1956), some of them reproductively isolated (BAUMGARTNER 2001, BAUMGARTNER et al. 2001). Phylogeographic studies on mtDNA (GITTENBERGER et al. 2001, HAASE et al. 2001) revealed a common lack of geographic pattern of variation (prominent differences among closely situated populations, no differences among regions). It does not agree with what ARTER (1985, 1990) has found in allozymes. Thus, this study was to find out if allozyme differences among local populations of *A. arbustorum* were really incongruent with the ones expressed in mtDNA haplotypes. The other goal was to get more data on a subdivided population of the species, especially on the level and pattern of gene flow. Our null hypothesis was that differences among local panmictic units were, in accordance with the stepping stone or isolation by distance models (WRIGHT 1978), positively, while the levels of gene flow negatively, correlated with geographic distance.

MATERIAL AND METHODS

LOCALITY DESCRIPTION

Snails were collected at nine localities (Fig. 1) in South Poland, in the vicinities of Kraków or in the city: 1. Skała Kmity, 50.103°N, 19.818°E; 2. Kraków, Hofmana St., 50.060°N, 19.889°E; 3. Kraków, Lasek Mogilski, 50.057°N 20.096°E; 4. Tyniec, 50.017°N, 19.804°E; 5. Wola Duchacka, 50.020°N, 19.971°E; 6. Radziszów, 49.939°N, 19.811°E; 7. Wola Radziszowska, 49.904°N, 19.827°E; 8. Ubrzeż, 49.867°N, 20,336°E; 9. Pcim, 49.760°N, 19.971°E.

COLLECTION AND ELECTROPHORETIC TECHNIQUES

Snails were collected by hand from plants and the litter below. Attention was paid not to mix specimens of one deme with those of another. To avoid this, thus

to avoid Wahlund's effect, snails were always picked up from within an area of a few dozens of square metres. The evolutionary effective population size in *A. arbustorum* had not been estimated yet, so we assumed that it was comparable with the figure estimated for *C. nemoralis* (GREENWOOD 1974) and applied a similar collection technique. In the laboratory, the individuals were immediately frozen in a deep freezer at –80°C and kept there until use. Specimens for electrophoresis were homogenized in Eppendorf tubes, in 125–250 µl of distilled water each. The homogenates were centrifuged at 11,000 rpm for 10 min. and used immediately for cellulose acetate electrophoresis following the protocol of RICHARDSON et al. (1986). The cellogel strips were from MALTA, Italy, the other chemicals from SIGMA, USA. The enzyme names and E.C. codes are after MURPHY et al. (1996).

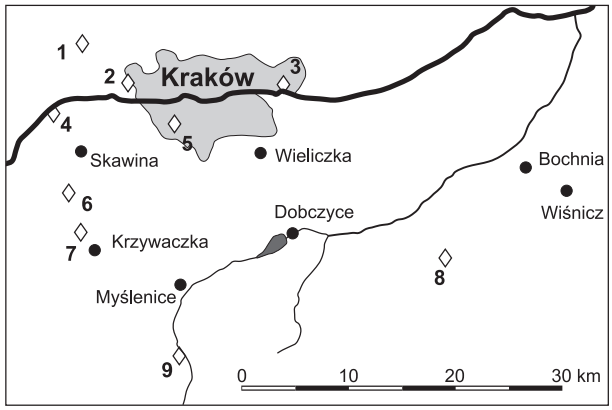

Fig. 1. Map of the study area

NUMERICAL TECHNIQUES

The data were analyzed with GENEPOP (RAYMOND & ROUSSET 1995). Exact tests for HWE (GUO & THOMPSON 1992, ROUSSET & RAYMOND 1995) with exact probabilities were done; f = F_{IS} in WRIGHT (1978) for each polymorphic population and each polymorphic locus were computed; linkage disequilibrium was calculated with Fisher's technique. F-statistics were computed over all populations, with Weir's FSTAT (WEIR 1990, GOUDET 1995), following the notation and computational procedure introduced by WEIR & COCKERHAM (1984) and WEIR (1990), the confidence intervals estimated with jackknife and bootstrapping techniques. FSTAT was also applied to calculate θ (= F_{ST} in WRIGHT 1978) and Nm values for all possible pairs of populations. D-statistics of OHTA (1982) were computed with LINKDOS (GARNIER-GERE & DILLMANN 1992). Genetic variability measures and Cavalli-Sforza and Edwards arc distances, for all the data and for each locus separately, were computed with BIOSYS-1 (SWOFFORD & SELANDER 1981). UPGMA clustering, neighbor-joining trees, nonlinear multidimensional scaling, minimum spanning tree, correspondence analysis and Mantel tests were computed with NTSYSpc (ROHLF 1998). Geographic distances were measured on a map, as straight lines with a ruler, and along the rivers and streams with a curvimeter.

RESULTS

29 enzyme systems were assayed, out of which the following six, coded by eight loci, were polymorphic and gave always consistent, interpretable results (Table 1): hydroxybutyrate dehydrogenase (HBDH, E.C. 1.1.1.30), L-iditol dehydrogenase (IDDH, E.C. 1.1.1.14), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42), mannose-6-phosphate isomerase (MPI, E.C. 5.3.1.8), phosphogluconate dehydrogenase (PGDH, E.C. 1.1.1.44) and phosphoglucomutase (PGM, E.C. 5.4.2.2.). All the subsequent analyses are entirely based on the above systems. Linkage disequilibrium calculated applying Fisher's method across populations (Table 2) was statistically significant for 4 out of the 28 possible pairs of loci: *Idh-2* and *Pgdh*, *Idh-2* and *Pgm-1*, *Pgdh* and *Pgm-2*, *Pgm-1* and *Pgm-2* (Table 2); it was statistically significant in 33.3, 16.7, 25.0, and 12.5 per cent, respectively, of the populations for which calculation was possible (Table 2). In a way similar to Fisher's F-statistics (Table 3) Ohta's D-statistics, partitioning linkage disequilibrium was computed for each pair of the seven loci. In general, $D'_{IS}{}^2$ was higher than $D'_{ST}{}^2$, and $D_{ST}{}^2$ was higher than $D_{IS}{}^2$.

Mean number of alleles per locus varied from 1.9 (populations 1, 4 and 5) to 2.1 (population 8), mean 1.98; mean expected heterozygosity varied from 0.234 (population 3) to 0.478 (population 7), mean 0.337; mean observed heterozygosity from 0.139 (population 3) to 0.237 (population 9), mean 0.194. The GENEPOP exact multilocus and multipopulation tests assuming H_1 = heterozygote excess yielded not significant levels of probability. The same tests assuming H_1 = heterozygote deficit showed significant departures from HWE (Hardy–Weinberg equilibrium) for each multipopulation (by locus) test (Table 4), and for multilocus (by population) test for all populations but 1 (Table 4). However, the above significance levels were often biased by a few extreme values. Each locus and each population considered separately, a significant heterozygote deficiency was observed in 71.4% and 66.6% of the populations in which the *Pgdh* and *Idh-1* loci were polymorphic, respectively. As to the remaining six polymorphic loci, the corresponding figures were 44.4% for *Iddh* and *Pgm-2*, 33.3% for *Hbdh*, *Mpi* and *Pgm-1*, and 22.2% for *Idh-2*.

Table 1. Gene frequencies in the studied populations of *Arianta arbustorum*

	Population								
Locus	1	2	3	4	5	6	7	8	9
Hbdh									
(N)	36	48	46	56	40	56	64	56	36
A	0.389	0.000	0.457	0.375	0.600	0.268	0.297	0.482	0.778
B	0.611	0.750	0.543	0.625	0.400	0.732	0.703	0.518	0.222
C	0.000	0.250	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Iddh									
(N)	36	48	46	56	32	54	62	52	36
A	0.167	0.188	0.196	0.607	0.438	0.167	0.323	0.269	0.500
B	0.833	0.813	0.804	0.393	0.563	0.833	0.677	0.731	0.500
Idh-1									
(N)	36	48	38	56	36	54	58	50	34
A	0.111	0.542	0.816	0.446	0.667	0.241	0.431	0.720	0.471
B	0.778	0.458	0.105	0.554	0.333	0.759	0.569	0.280	0.529
C	0.111	0.000	0.079	0.000	0.000	0.000	0.000	0.000	0.000
Idh-2									
(N)	36	40	32	56	36	42	42	32	34
A	0.111	0.500	1.000	0.268	1.000	0.238	0.548	0.469	0.706
B	0.889	0.500	0.000	0.732	0.000	0.762	0.452	0.531	0.294
Mpi									
(N)	36	48	38	56	36	52	60	52	36
A	0.944	0.083	0.789	0.000	0.167	0.173	0.667	0.673	0.139
B	0.056	0.917	0.211	1.000	0.833	0.827	0.333	0.327	0.861
Pgdh									
(N)	36	48	36	56	40	50	62	52	36
A	0.000	0.104	0.167	0.071	0.100	0.020	0.516	0.788	0.139
B	1.000	0.896	0.833	0.929	0.900	0.980	0.484	0.212	0.861
Pgm-1									
(N)	36	40	44	56	36	48	62	52	32
A	0.000	0.100	0.023	0.036	0.167	0.667	0.581	0.673	0.219
B	1.000	0.900	0.977	0.964	0.833	0.333	0.419	0.212	0.781
C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.115	0.000
Pgm-2									
(N)	32	40	44	56	36	48	62	50	36
A	0.500	0.050	0.977	0.536	0.056	0.813	0.419	0.680	0.611
B	0.500	0.950	0.023	0.464	0.944	0.188	0.581	0.320	0.389

Departures from HWE may be quantified with f (Wright's F_{IS}), and those values for each locus and population are shown in Table 4. They are relatively high even in the many instances of not significant heterozygote deficiency.

F-statistics over all populations, computed with FSTAT (Table 5, Fig. 2) showed high, statistically significant values of f and F (Wright's F_{IT}), and F not equaling θ (Wright's F_{ST}) for each locus, thus a highly significant homozygote excess for each locus. f values were not much variable among loci. For each locus but *Mpi*, and across all loci, they were significantly higher than θ. The confidence intervals for θ overlapped among *Hbdh*, *Iddh* and *Idh-1*, and among the other five loci, but not between two loci of the two different groups (Table 5). The random model of WEIR (1990) was applied for numerical resampling. Both bootstrapping over loci and permutations indicated

Table 2. Below diagonal: P-value for each locus pair across all populations (Fisher's method), in bold p<0.05. Above diagonal: percent of possible comparisons (populations) for which the null hypothesis: genotypes at one locus are independent of genotypes at the other locus was rejected (p 0.05)

locus	*Hbdh*	*Iddh*	*Idh-1*	*Idh-2*	*Mpi*	*Pgdh*	*Pgm-1*	*Pgm-2*
Hbdh	–	0.0	11.1	28.6	0.0	0.0	12.5	0.0
Iddh	0.84631	–	0.0	0.0	0.0	0.0	25.0	12.5
Idh-1	0.63422	0.83508	–	14.3	12.5	12.5	14.3	11.1
Idh-2	0.14981	0.60777	0.09738	–	0.0	33.3	16.7	14.3
Mpi	0.99998	0.96842	0.37413	0.62436	–	16.7	16.7	0.0
Pgdh	0.99632	0.25329	0.57899	**0.01597**	0.23568	–	14.3	25.0
Pgm-1	0.59939	0.10784	0.24619	**0.01156**	0.12422	0.20608	–	12.5
Pgm-2	0.46575	0.27566	0.67213	0.49427	0.84486	**0.03489**	**0.00948**	–

Table 3. Ohta's D-statistics for the marker loci

Loci compared	Within subpopulation components		Between subpopulation components		Total
	D_{IS}	D'_{IS}	D_{ST}	D'_{ST}	D_{IT}
Hbdh – Iddh	0.00877	0.28541	0.06819	0.00300	0.28842
Hbdh – Idh-1	0.01705	0.33563	0.08520	0.00208	0.33771
Hbdh –Idh-2	0.03912	0.50313	0.13453	0.01984	0.52296
Hbdh – Mpi	0.01056	0.59768	0.17116	0.00426	0.60194
Hbdh – Pgdh	0.00530	0.49506	0.12271	0.00247	0.49754
Hbdh – Pgm-1	0.00886	0.54161	0.13398	0.00067	0.54229
Hbdh – Pgm-2	0.01051	0.47226	0.13455	0.00500	0.47727
Iddh – Idh-1	0.01955	0.35392	0.08220	0.00012	0.35404
Iddh – Idh-2	0.00612	0.55410	0.13599	0.00055	0.55465
Iddh – Mpi	0.01090	0.62583	0.17089	0.03046	0.65629
Iddh – Pgdh	0.00454	0.51088	0.12533	0.00015	0.51103
Iddh – Pgm-1	0.02261	0.58149	0.13317	0.00165	0.58314
Iddh – Pgm-2	0.02486	0.53545	0.14072	0.00239	0.53784
Idh-1 – Idh-2	0.03027	0.49931	0.15503	0.02268	0.52199
Idh-1 – Mpi	0.01692	0.60606	0.17249	0.00552	0.61158
Idh-1 – Pgdh	0.01156	0.51335	0.13600	0.01532	0.52866
Idh-1 – Pgm-1	0.02501	0.61040	0.15180	0.00403	0.61443
Idh-1 – Pgm-2	0.01190	0.54074	0.14820	0.00067	0.54141
Idh-2 – Mpi	0.00353	0.84467	0.25212	0.00736	0.85202
Idh-2 – Pgdh	0.02613	0.76023	0.21519	0.02308	0.78331
Idh-2 – Pgm-1	0.02527	0.89492	0.23347	0.00014	0.89506
Idh-2 – Pgm-2	0.01739	0.72420	0.22880	0.00334	0.72754
Mpi – Pgdh	0.00357	0.93888	0.26858	0.05642	0.99531
Mpi – Pgm-1	0.03243	1.11865	0.27408	0.00142	1.12006
Mpi – Pgm-2	0.01095	0.85749	0.25295	0.00325	0.86074
Pgdh – Pgm-1	0.00714	0.82714	0.20094	0.06004	0.88718
Pgdh – Pgm-2	0.05226	0.83976	0.20406	0.00009	0.83985
Pgm-1 – Pgm-2	0.06814	0.88852	0.21946	0.05710	0.94561

Table 4. F values for each locus and each population, with exact probabilities (calculated with GENEPOP by complete enumeration); D – homozygote deficit, H_o=HWE (Hardy and Weinberg equilibrium), P – probability of H_o, (computed with the assumption of H_1= homozygote deficit; with the assumption of H_1=homozygote excess all the values of P were not significant); M – monomorphic locus (if frequency of no allele exceeds 0.96; M* – locus at which more than one allele was detected, the second allele frequency <0.04); f computed: W&C – after WEIR & COCKERHAM (1984), R&H – after ROBERTSON & HILL (1984). Percent of populations not at HWE: in parentheses given percent of populations with a polymorphic locus. Multipopulation (by locus) and multilocus (by population) unbiased estimates of Hardy and Weinberg exact values, calculated by complete enumeration with GENEPOP are also given, standard errors in parentheses; H_1= heterozygote deficit; for H_1= heterozygote excess both multilocus and multipopulation tests gave not significant levels of probability

GENEPOP	*Hbdh*			*Iddh*			*Idh-1*			*Idh-2*			*Mpi*			*Pgdh*			*Pgm-1*			*Pgm-2*			multi-locus test	
population	D P	W&C	R&H	D P	W&C	R&H	D P	W&C	R&H	D P	W&C	R&H	D P	W&C	R&H	D P	W&C	R&H	D P	W&C	R&H	D P	W&C	R&H	P-val	
1.	HWE	+0.351	+0.380	HWE	-0.143	-0.150	HWE	-0.143	-0.089	HWE	-0.067	-0.070	M*			M			M			HWE	+0.548	+0.607	0.2505 (±0.0014)	HWE
2.	D 0.0122	+0.570	+0.589	D 0.0131	+0.603	+0.624	D 0.0148	+0.512	+0.529	HWE	+0.421	+0.437	HWE	+0.471	+0.486	HWE	+0.349	+0.359	HWE	-0.086	-0.088	HWE	-0.027	-0.028	0.0000 (±0.0000)	D
3.	HWE	+0.233	+0.239	HWE	+0.055	+0.057	D 0.0011	+0.683	+0.611	M			D 0.0096	+0.697	+0.730	D 0.0381	+0.618	+0.647	M*			M*			0.0000 (±0.0000)	D
4.	D 0.0143	+0.481	+0.494	D 0.0038	+0.563	+0.580	D 0.0007	+0.649	+0.669	HWE	+0.198	+0.202	M			D 0.0010	+1.000	+1.000	HWE	-0.019	-0.019	D 0.0025	+0.581	+0.598	0.0000 (±0.0000)	D
5.	HWE	+0.617	+0.671	HWE	-0.207	-0.218	HWE	+0.059	+0.063	M			HWE	+0.636	+0.700	D 0.0526	+1.000	+1.000	HWE	-0.143	-0.150	M*			0.0064 (±0.0001)	D
6.	HWE	+0.378	+0.388	HWE	+0.085	+0.087	HWE	+0.308	+0.316	HWE	+0.236	+0.243	HWE	+0.346	+0.355	M*			D 0.0030	+0.638	+0.660	HWE	+0.064	+0.065	0.0001 (±0.0000)	D
7.	HWE	-0.107	-0.109	HWE	+0.130	+0.133	D 0.0000	+0.796	+0.821	HWE	+0.349	+0.360	D 0.0300	+0.414	+0.424	D 0.0000	+0.875	+0.902	D 0.0000	+0.742	+0.764	D 0.0000	+0.742	+0.764	0.0000 (±0.0000)	D
8.	D 0.0000	+0.792	+0.819	D 0.0440	+0.430	+0.442	D 0.0001	+0.809	+0.839	D 0.0004	+0.882	+0.937	D 0.0054	+0.576	+0.594	D 0.0000	+0.889	+0.922	D 0.0001	+0.465	+0.613	D 0.0291	+0.465	+0.479	0.0000 (±0.0000)	D
9.	HWE	+0.382	+0.397	D 0.0194	+0.575	+0.601	D 0.0312	+0.549	+0.576	D 0.0888	+0.458	+0.478	HWE	+0.329	+0.342	HWE	-0.133	-0.137	HWE	+0.118	+0.122	D 0.0271	+0.553	+0.578	0.0000 (±0.0000)	D
populations not at HWE(%)	33.3 (33.3)			44.4 (44.4)			66.6 (66.6)			22.2 (28.6)			33.33 (42.9)			55.5 (71.4)			33.3 (42.9)			44.4 (57.1)				
multi--population test (P-val)	0.0000	(±0.0000)		0.0000	(±0.0000)		0.0000	(±0.0000)		0.0000	(±0.0000)		0.0000	(±0.0000)		0.0000	(±0.0000)		0.0000	(±0.0000)		0.0000	(±0.0000)		0.0000 (±0.0000)	D

Table 5. F-statistics across all populations

Locus	Allele	F		θ		f	
Hbdh	1	0.479		0.159		0.380	
	2	0.447		0.078		0.400	
	3	0.667		0.223		0.571	
	All	0.475		0.125		0.400	
	Mean	0.475	(±0.114)	0.125	(±0.479)	0.400	(±0.116)
Iddh	1	0.379		0.098		0.311	
	2	0.379		0.098		0.311	
	All	0.379		0.098		0.311	
	Mean	0.392	(±0.117)	0.105	(±0.063)	0.316	(±0.093)
Idh-1	1	0.644		0.129		0.591	
	2	0.612		0.139		0.550	
	3	0.398		0.059		0.361	
	All	0.622		0.132		0.564	
	Mean	0.626	(±0.064)	0.130	(±0.074)	0.572	(±0.078)
Idh-2	1	0.571		0.288		0.398	
	2	0.571		0.288		0.398	
	All	0.571		0.288		0.398	
	Mean	0.563	(±0.089)	0.280	(±0.129)	0.397	(±0.095)
Mpi	1	0.714		0.452		0.478	
	2	0.714		0.452		0.478	
	All	0.714		0.452		0.478	
	Mean	0.711	(±0.059)	0.445	(±0.094)	0.479	(±0.054)
Pgdh	1	0.814		0.386		0.698	
	2	0.814		0.386		0.698	
	All	0.814		0.386		0.698	
	Mean	0.849	(±0.111)	0.429	(±0.180)	0.726	(±0.138)
Pgm-1	1	0.648		0.372		0.440	
	2	0.659		0.424		0.408	
	3	1.000		0.068		1.000	
	All	0.665		0.387		0.453	
	Mean	0.679	(±0.067)	0.386	(±0.076)	0.485	(±0.133)
Pgm-2	1	0.654		0.306		0.500	
	2	0.654		0.308		0.500	
	All	0.654		0.308		0.500	
	Mean	0.654	(±0.070)	0.303	(±0.157)	0.514	(±0.094)
All		0.610		0.270		0.465	
	Mean	0.609	(±0.044)	0.270	(±0.048)	0.464	(±0.039)
Bootstrapping over loci, 99% confidence intervals							
		F		θ		f	
		[0.498	0.713]	[0.161	0.385]	[0.378	0.568]

that the main source of the overall inbreeding coefficient F was f. The contribution of the population subdivision component θ was smaller, but certainly not low. Mean value of f across the loci was 0.464, and mean value of θ equalled 0.270 (Table 5). For the mean value of θ the gene flow estimate Nm=0.676 was calculated. The Nm value calculated with private allele technique, and corrected for size Nm=0.274.

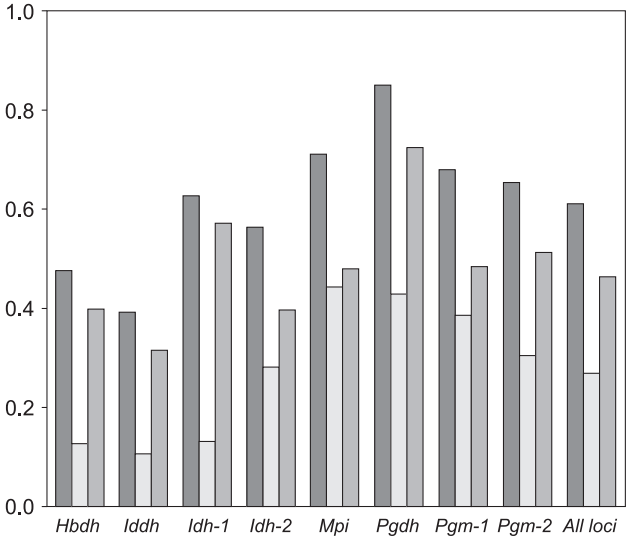

Fig. 2. F-statistics across populations for each locus and all loci, computed with FSTAT from left to right: f, θ, F

FSTAT was also applied to calculate pairwise values of θ and the resulting Nm's for each pair of populations (Table 6). The lowest values of θ (0.0478 and 0.0943, respectively) were found between populations 7 and 8, and 5 and 9; the highest (0.4572, 0.4545, 0.4435, 0.4286 and 0.4153, respectively) between populations 2 and 3, 1 and 5, 3 and 6, 1 and 3, and 3 and 4. Most of the θ values were rather high. In no case Nm values were high enough to postulate panmixia. In one case (populations 7 and 8) Nm approached 5 (Table 6), in two other cases exceeded 2, and in the other five exceeded 1. Either the UPGMA clustering, or nonlinear multidimensional scaling with superimposed minimum spanning tree (Fig. 3) showed no evidence of correspondence between a pairwise θ value and reciprocal geographic position of the localities. Mantel test for association between pairwise θ and geographic distances between the localities, measured as straight lines (r=–0.095, Z=63.547, probability that random Z would be higher than Z computed: p=0.341, 6,554 out of 9,999 permutations yielded a higher value of Z, p=0.345) did not show any statistically significant association between the two parameters. For the distances measured along the rivers and streams the association was not significant, too (p=0.426).

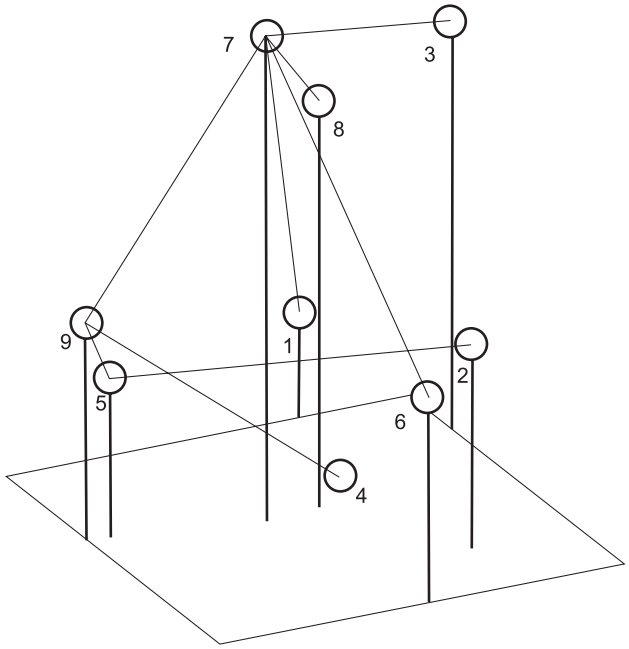

Fig. 3. Multidimensional scaling of the populations, based on pairwise θ, with superimposed minimum spanning tree, Stress 1 (ROHLF 1988) = 0.1399

To deal with such a subdivided group of populations, their history rather too short for mutations to become an important factor of interpopulation differentiation, Cavalli-Sforza and Edwards arc distance seems the most appropriate. ARTER (1990) has, however, applied Nei distances, thus for comparison we also calculated unbiased Nei distances. Both distances were computed for all loci together and for each locus separately. Then, Mantel tests for association between the genetic distances and geographic distances was computed along with UPGMA clustering and neighbor-joining trees.

Cavalli-Sforza and Edwards arc distances calculated for all loci were not significantly associated with geographic distances either measured as straight lines

Table 6. Pairwise θ (below diagonal) and Nm estimates (above diagonal) between all pairs of populations

	1	2	3	4	5	6	7	8	9
1	–	0.452	0.333	0.580	0.300	0.565	1.076	0.522	0.5472
2	0.3563	–	0.297	1.197	1.216	0.576	0.910	0.441	0.801
3	0.4286	0.4572	–	0.352	0.400	0.314	0.698	0.582	0.663
4	0.3014	0.1727	0.4153	–	0.799	0.933	0.787	0.473	2.115
5	0.4545	0.1705	0.3849	0.2383	–	0.376	0.969	0.517	2.402
6	0.3068	0.3028	0.4435	0.2113	0.3994	–	1.129	0.688	0.869
7	0.1885	0.2154	0.2637	0.2412	0.2052	0.1813	–	4.983	1.196
8	0.3239	0.3620	0.3007	0.3459	0.3259	0.2666	0.0478	–	0.775
9	0.3137	0.2378	0.2738	0.1057	0.0943	0.2233	0.1729	0.2439	–

(p=0.4556), or along the rivers and streams (p=0.3982). Similarly, they were not significant for particular loci, except that the associations between Cavalli-Sforza and Edwards arc distance and geographic distance measured as straight lines were nearly significant for *Pgdh* (p=0.0530), and *Pgm-1* (p=0.0525). Nei unbiased distances calculated for all loci were not significantly associated with geographic distances either measured as straight lines (p=0.0956), or along the rivers and streams (p=0.2326). From among particular loci, an only statistically significant association was found between Nei unbiased distances calculated for *Pgdh* and geographic distances measured as straight lines (p=0.0273).

The UPGMA clustering and neighbor-joining trees based on Cavalli-Sforza and Edwards arc distances calculated for all loci clustered population 2 and 5, both from the city. They also clustered population 4 with 9 (both close to a river) and 6 with 8 (neither close nor similar localities). For particular loci, the UPGMA and neighbor joining clusterings were as follows: *Hbdh*, both techniques: (5, 9); *Iddh*, both techniques: (1, 6), (2, 3), (4, 5, 9); *Idh-1*, UPGMA: (2, 3, 5, 8), (1, 4, 6, 7, 9); *Idh-2*, both techniques: (3, 5), (1, 4, 6) (rather close to each other and westernmost); *Mpi*, both techniques: (1, 3, 7, 8), (2, 4, 5, 6, 9); *Pgdh*, neighbor-joining: (1, 2, 4, 5, 6) situated rather close to each other (Fig. 1): this may explain the nearly significant association for this locus. The other populations, however, were not grouped geographically: for example populations 7 and 8 were clustered together, but UPGMA did not show even that, partly geographic, cluster. Similarly, the nearly significant result of Mantel test for *Pgm-1* may be explained by the clustering of populations 1, 2, 3, 4 and 5 by neighbor joining, only partly confirmed by UPGMA. Both techniques clustered the geographically close populations 6 and 7. Finally, for *Pgm-2* both techniques clustered populations 2 and 5 (situated in the city, not far from each other).

The UPGMA clustering and neighbor-joining trees based on Nei unbiased distance calculated for all loci clustered population 7 with 8; the other populations were grouped variously depending on the technique, but still without a geographic pattern. UPGMA clusterings for particular loci gave more than one (to more than 200) trees each, thus they are not given comments here. Neighbor-joining trees for particular loci clustered the populations as follows: *Hbdh*: (5, 8, 9), (1, 2, 3, 4, 6, 7); *Iddh*: (4, 9 of a riverside), (1, 2, 3, 5, 6, 7, 8); *Idh-1*: (2, 3, 5, 8), (1, 4, 6, 7, 9); *Idh-2*: (3, 5, 9), (1, 2, 4, 6, 7, 8); *Mpi*: (1, 3, 7, 8), (geographically close 2,4), 5, 6, 9); *Pgdh*: (geographically close 1, 2, 4, 5, 6 reflecting the statistically significant result of Mantel test), (geographically distant populations 7, 8); *Pgm-1*: (relatively close 1, 2, 3, 4 , 5, distant 9), (distant 6, 7, 8); *Pgm-2*: (2, 5 of the city, 7), (1, 3, 4, 6, 8, 9).

The genetic distances, as well as pairwise θ, are based on numerous assumptions, which in most cases are doubtful. Thus in a real subdivided population they will be more or less biased. To circumvent those restrictions we used allele frequencies directly to compute correspondence analysis. The populations projected in the space of the first three factors (Fig. 4) did not reveal any geographic pattern once more.

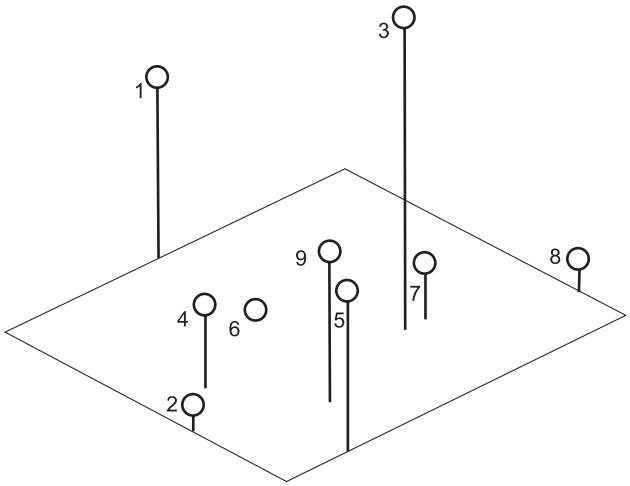

Fig. 4. Correspondence analysis of the allele frequencies, with populations projected in the space of first three factors, whose cumulative inertia equalls 72.73% of the total variation

DISCUSSION

Calculated with Fisher's method across loci, a significant disequilibrium was found among only four, out of the 28 possible, pairs of loci. Significant values were found in only one third of populations for one pair of loci; in one fourth of populations for another pair, and in much less of them for the remaining two pairs of loci. Therefore, the linkage cannot be close, and reflects rather population subdivision (HARTL & CLARK 1997). $D'_{IS}{}^2$ is the expected variance of correlation of two alleles at two loci in one gamete in a subpopulation, which is relative to that in the total population. It is the variance of the correlation of two alleles at two loci on one chromosome in a population, which is relative to that in the total population (OHTA 1982). The general pattern, in which the values of $D'_{IS}{}^2$ are always higher than those of $D'_{ST}{}^2$, and the values of $D_{ST}{}^2$ are always higher than those of $D_{IS}{}^2$, points to this being a nonsystematic disequilibrium (OHTA 1982, BLACK & KRAFSUR 1985). The main source of such disequilibrium is again limited

migration, certainly not the natural epistatic selection that favours gametes with the same allele combinations in every population, or strong selection of this kind in only a part of the populations (OHTA 1982).

The mean number of alleles per locus, rather high and little interpopulation-varied, suggests that no population showed signs of population decline. The number of alleles is a measure of genetic variability, which can be much more sensitive to the effects of population decline than e.g. heterozygosity (LEBERG 1992, BROOKES et al. 1997). The mean expected heterozygosity we found in *A. arbustorum* (0.337) is higher than the average for molluscs (0.148: LAZARIDOU--DIMITRIADOU et al. 1994) yet lower than the figures found in many molluscan species (up to about 0.64: SELANDER & OCHMAN 1983, NEVO et al. 1984).

Statistically significant departures from HWE , usually a homozygote excess, is just a rule in molluscan populations (LAZARIDOU-DIMITRIADOU et al. 1994, see FALNIOWSKI et al. 1999 for references, also FALNIOWSKI et al. 2002a, b). In the present study both multipopulation (except population 1) and multilocus exact tests showed a significant homozygote excess, but many particular populations and loci were at HWE, which often was despite relatively high values of f. Inbreeding and/or selfing are the phenomena usually invoked to explain homozygote excess, although the real background may be more complex (WEIR 1990, 1996, HARTL & CLARK 1997, FALNIOWSKI et al. 1999, 2002a). In populations of such little mobile and not gregarious animals inbreeding and/or selfing must be as much inevitable as evolutionarily advantageous (JARNE et al. 1993). There is evidence that *A. arbustorum* avoids inbreeding (e.g. CHEN & BAUR 1993). On the other hand, in none of our South Polish populations population density was as high as that described for Swiss populations (unpublished observations). Then there may be more inbreeding in the former than in the latter. The values of f are correlated among loci, which confirms inbreeding. However, moderate values of θ, are rather unusual for inbreeding/selfing populations (HILLIS 1989). Moreover, the confidence intervals for three of the studied loci did not overlap the ones for the other five loci. Hence, apart from inbreeding, some disruptive forces must have contributed to the observed homozygote excess. One of them is Wahlund's effect. Despite the collection technique, admixtures of snails from other panmictic units cannot be excluded; on the other hand, the longevity of *A. arbustorum* may have resulted in the collection of snails of different generations. In general, in a subdivided population one should expect a homozygote excess, even if the species is panmictic (HARTL & CLARK 1997). Another factor, not uncommon in such a highly polymorphic species, may be disrupting selection. In general, most allozymic variation, except in some loci, seems selectively neutral, or nearly neutral (SZAROWSKA et al. 1998). Our UPGMA and neighbor-joining trees show some ecological grouping for a part of the studied loci, which may support that some alleles are subject to selection.

Perhaps the most striking result are the relatively low values of θ (0.270), which are yet high enough to result in low Nm values. The mean value of θ is similar to the ones obtained for *Partula taeniata* and *Helix aspersa*, the geographic scale of the former being comparable (JOHNSON et al. 1988) while that of the latter much bigger (2,900 km: GUILLER et al. 1994). It is noteworthy that the Nm estimate calculated from private alleles is about half the one calculated from the mean value of θ, so the two estimates do not differ much from each other. In general, the relatively high number of individuals assayed per sample, eight loci considered only, makes the estimate based on analytical theory more realistic than the one based on private alleles (see FALNIOWSKI et al. 1999). In the present study the pairwise values of θ and the resulting Nm's are fairly variable among pairs of populations.

Indirect Nm estimates, those based on analytical theory included, have been much criticised. In fact, many of the assumptions of the model are violated (COCKERHAM & WEIR 1993, SLATKIN 1993, FALNIOWSKI et al. 1998, 1999, 2002a, WHITLOCK & MCCAULEY 1999) . Firstly, most populations are not at HWE. Secondly, sample sizes are not big enough. Thirdly, there probably is no equilibrium or quasi-equilibrium between genetic drift and interpopulation gene flow. To approach such an equilibrium, it may need a number of generations of the order of evolutionary effective population size at low migration rates (BOSSART & PROWELL 1998, AVISE 2000). The curvilinear relationship between Nm and θ is such that along the main part of the curve small differences in one variable will cause considerable differences in the other (TEMPLETON 1998). Theoretically, Nm is related to the θ parameter that is not the same as the empirical statistics θ. Thus, sampling variance in any empirical data must in many cases contribute to unrealistic estimates of Nm. Finally, selection on some alleles cannot be excluded.

In general, θ is just one of the parameters that describe the structure of a subdivided population. It quantifies relatedness between the genes of different individuals, thus it reflects relatedness of individuals within a population: it is a co-ancestry coefficient. Under the strict neutral model $\ln(1-\theta)$ it will increase linearly with time, and indicating the extent of differentiation it may serve as a measure of distance for a pair of populations (REYNOLDS et al. 1983). On the other hand, θ must reflect all differences between two populations, which are effect of all the various evolutionary forces that have been acting in these populations.

One argument against the gene flow indirect estimates is that there are techniques of direct study on migration, whose results are more reliable. However,

the argument considers migration capabilities as the only factor determining levels of gene flow. A migrant must not only reach a new population but also survive, reproduce, and its progeny fitness must be at least not much lower than the one of the local inhabitants. Assortative mating or the lower fitness of hybrids may be responsible for the low gene flow despite migration. This aspect is commonly overlooked in debates on the dispersal of gastropods (FALNIOWSKI et al. 1999). In *A. arbustorum*, as mentioned in the Introduction, several traits of biology and ecology will remain unchanged if specimens are transported to different habitats, so their fitness to a new habitat must be far from the optimum. Thus the estimates based on the genetic data, biased and imprecise as they are, reflect not only dispersal capabilities of the studied organisms but also the real gene flow that influences the genetic structure of populations.

In *A. arbustorum* some Nm estimates look unrealistic: the highest value, (>4) was found between populations 7 and 8, situated 34 km from each other. As stressed by ENDLER (1977), adaptive differences among populations can be maintained by natural selection even under high levels of gene flow, as well as selection may mimic effects of gene flow. This high Nm may also have been due to numerous stochastic factors, area effects, etc. It is yet far too low to postulate panmixia. The figure found between populations 1 and 7 (>1) is similarly biased, the same concerns Nm about 2.4 between populations 5 and 9, but the not much lower value between 4 and 5 seems reliable. The other three estimate values higher than 1 (for the pairs 2 and 4, 2 and 5, 6 and 7), concern spatially close localities. All the other Nm's were below 1. Thus the studied populations of *A. arbustorum* seem nearly isolated: the levels of gene flow among them are negligible.

It must be stressed that none of the applied techniques detected a geographic pattern of interpopulation differentiation. Neither θ, nor Cavalli-Sforza and Edwards arc distance and Nei unbiased distance were significantly associated with geographic distances between populations, with the exception of the genetic distances for *Pgdh* and, perhaps, *Pgm-1*. The question is: how precisely, if at all, may the distances measured as straight lines on a map reflect the routes of gene flow? However, association with the distances measured along the rivers and streams as the presumed ways of migration suggested by ARTER (1990) was even less significant. Perhaps such distances are appropriate for spatially close populations, but with the scale applied in our study or larger there is no rationale of other ways of measurement than along straight lines. One must believe than the errors would sum up to approach zero (FALNIOWSKI et al. 2002a). Another question is what geographic scale one needs to detect geographic differentiation. In many cases no such differentiation was found as long as the scale was not large enough. In *A. arbustorum,* however, ARTER (1990) has detected a geographic pattern along a few kilometres. The scale in our study is larger, but on the other hand, no geographic differentiation has been found where *A. arbustorum* studies covered a bigger area than that of the present study (GITTERBERGER et al. 2001, HAASE et al. 2001).

Nonlinear multidimensional scaling computed on pairwise θ, UPGMA clustering and neighbor-joining trees on θ, Cavalli-Sforza and Edwards arc and unbiased Nei genetic distances, and correspondence analysis run directly on the allele frequencies, all showed no geographic pattern of interpopulation differences. The same was shown by the trees calculated on genetic distances for each locus separately. Thus, our results are in accordance with the results on mtDNA of GITTENBERGER et al. (2001) and HAASE et al. (2001) concerning *A. arbustorum* but also in accordance with data on *Cepaea* (JOHNSON 1976, 1979, CLARKE et al. 1978, JONES et al. 1977, 1980). They, however, are in contrast with the results of ARTER (1990) who found a geographic pattern in *A. arbustorum*. In *Bradybaena fruticum* (FALNIOWSKI et al. 1993a, b and unpublished data) geographic variation is fine yet stronger than in the present study.

The most probable explanation of such divergent results is in different time scales. As can be inferred from its phylogeographic structure, as well as from its present occurrence in cold habitats in Finland (TERHIVUO 1978), *A. arbustorum* probably survived the last glaciation in the permafrost conditions of nunatak-like refugia in the Alps (GITTENBERGER et al. 2001, HAASE et al. 2001). It probably inhabited also the territory of Poland during the last glaciation, the ice-cover being then rather far from this area. ARTER (1990) deals with a territory that undoubtedly was under ice. Therefore, the populations covered by GITTENBERGER et al. (2001), HAASE et al. (2001) and the present study must have existed for more than 10,000 years, while the ones studied by ARTER (1990), for a much shorter period. As concerns *B. fruticum*, its present distribution range and habitat requirements considered, it could not occur in South Poland during the last glaciation.

Semi-sedentary animals like land snails will colonize a new territory slowly, step by step, to result in demes more or less geographically differentiated. Such a relatively recently colonized area is that studied by ARTER (1990). Then the local populations of it, many of them of a relatively high evolutionarily effective size, will be evolving in isolation. Their low mobility coupled with phenomena like homing will result in low numbers of migrants, their alleles easily lost due to the high evolutionarily effective population size. Physiological, ecological and behavioral adaptations to local conditions, some of them genetically determined, counteract the success of immigrants in a new habitat: the latter are usually eliminated by competi-

tion. In such a population, due to its high evolutionarily effective size, random events like genetic drift cannot be more significant. Selection, however, may be an important factor. It is acknowledged that over a time as short as 10,000 years mutations cannot be a significant source of interpopulation differentiation. On the other hand, *A. arbustorum,* like several other helicids, is characterized by unusually high allozyme polymorphism. In the opinion of some authors, heterozygotes are more mutable than homozygotes. During meiosis, pairing between homologous chromosomes may lead to the "repair" of heterozygous sites through events resembling gene conversion (SZOSTAK et al. 1983, BORTS & HABER 1989, BORTS et al. 1990). In homozygous inbred lines in *Drosophila* mutation rate is lower by two orders than in other *Drosophila* (SCHUG et al. 1997). The phenomenon is better known for microsatellites (AMOS et al. 1996, AMOS 1999), but it has been confirmed for allozymes: the land snails included (WOODRUFF 1989), new alleles known as "hybridizymes" are often found in hybrid zones (BARTON et al. 1983, BRADLEY et al. 1993). DNA sequencing indicates that these tend to be new mutations (HOFFMAN & BROWN 1995). Thus we postulate that a significant contribution to interpopulation variation among the local populations *A. arbustorum* is that of new mutations.

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