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Sibling species and phylogenetic relationships of *Mysis relicta* (Crustacea: Mysidacea)

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Three putative sibling species within Fennoscandian *Mysis relicta* Lovén were detected by biochemical genetic methods (enzyme electrophoresis). Four to seven completely or partially diagnostic loci were found to discriminate each species pair. Two partially sympatric species are present in the Baltic Sea, one of them abundant in coastal and peripheral areas (*'M. relicta I'*), the other predominant in the outer sea (*'M. relicta II'*). Both species were found also in a lake that became isolated from the Baltic at the end of the Litorina period, whereas in samples from over 20 lakes isolated even from the earlier stages of the Baltic, only *'M. relicta I'* was observed. The northern lake Pulmankijärvi, isolated from the Barents Sea, is inhabited by another genetically distinct type (*'M. relicta III'*).

Genetic distances among the three sibling species, *M. mixta* and *M. litoralis* (presumably the closest marine relative of *M. relicta*) were estimated on the basis of 21 loci. The results suggest that all the nominal species examined and probably also the three sibling species diverged from each other in the Tertiary, and thus do not support the controversial view that the glacial immigrant (or glacial relict) crustaceans evolved from marine ancestors only relatively recently in connection with Pleistocene glaciations.

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1. Introduction

Ever since the first description of *Mysis relicta* Lovén 1862, the views on its taxonomic status have been closely intertwined with the disputed views on the biogeographical and evolutionary history of the glacial immigrant crustaceans (also called 'glacial relicts', or 'glaciomarine relicts'). As with other members of this zoogeographical group now inhabiting fresh and brackish waters in areas once associated with the last glaciation, *M. relicta* was generally assumed to have only recently been derived from a marine progenitor species. Therefore, up to the 1950s, no strong distinction was usually made between the arctic marine *M. oculata* and the morphologically close *M. relicta* (e.g. Thienemann 1950, Ekman 1953, Zenkevitch 1963), which was often regarded merely as an environmental modification of the former.

The studies of Holmquist (1959) finally established a full specific rank for *M. relicta*. Holmquist also took the view that *M. relicta* is actually a relatively old species, which probably diverged from its present relatives as early as in the Oligocene. However, the hypothesis of an ancient origin has not been unequivocally accepted, and e.g. Segerstråle (1962, 1982) and Dadswell (1974) have still argued for considerably later (Pleistocene) speciation and adaptation events. In addition to this remaining controversy over the evolutionary time scale, some aspects of the dispersal history of the crustaceans are still problematical, although the key role of ice-dammed, or proglacial, waters as dispersal paths (Segerstråle 1957, 1976, 1982, Ricker 1959, Dadswell 1974) is now generally recognised. The scientific debate on the 'glacial relicts' has been reviewed by e.g. Segerstråle (1957, 1962) and Holmquist (1959, 1966).

This study is a biochemical genetic approach to the evolution of *Mysis relicta*, making use of enzyme electrophoresis, now an established tool in systematic and phylogenetic research (see e.g. Avise 1974, Thorpe 1982, Ayala 1983). The method involves assessment of interspecific electrophoretic similarities and differences of a set of known proteins; data on electrophoretic differences are then converted

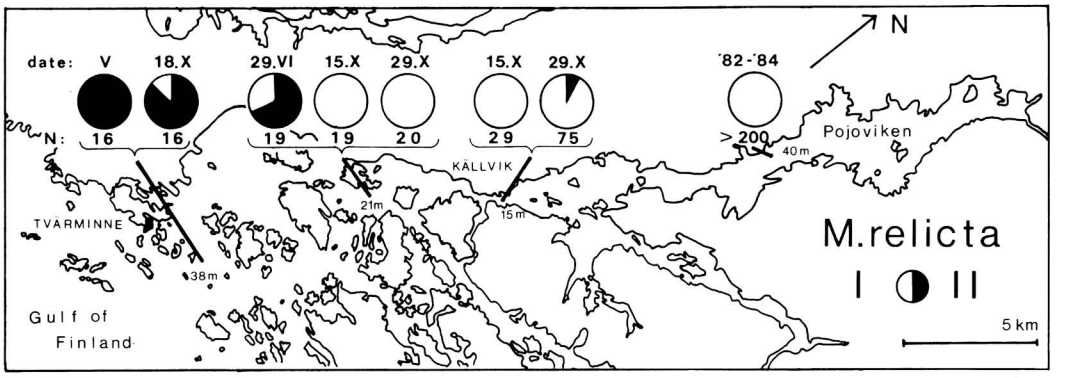

Fig. 1. The Tvärminne-Pojoviken area, SW Finland, with sampling sites and sibling species composition in some samples from 1984.

to estimates of average genetic differentiation (genetic distance) at the underlying loci. Genetic distance has been empirically found to be roughly proportional to the time since divergence of evolutionary lineages (for review, see Thorpe 1982, Nei 1986). Through the distance approach, the method can thus be used to obtain estimates of the time scale of evolutionary events, as well as to resolve the relative evolutionary affinities of species. More direct, character-based methods of phylogenetic reconstruction have also been applied to electrophoretic data (e.g. Patton & Avise 1983).

Another aspect of the use of biochemical genetics in systematics is the simple and unequivocal discrimination of morphologically similar or inseparable species. Sympatric reproductively isolated species are usually characterized by distinct allozymes of several enzymes, each constituting a diagnostic taxonomic character; other enzyme loci may show only differentiation of allele frequencies. Any hybridization would be readily detected. As regards allopatric populations, the decision of taxonomic status on the basis of genetic divergence alone may be disputable, but reviews of large amounts of data indicate a general agreement of correspondence between genetic distance and level of taxonomic rank, over different groups of organisms (Thorpe 1982, 1983). In practice, the biochemical method has been used to reveal or confirm many cases of sibling species especially in marine organisms, including crustaceans (e.g. Grassle & Grassle 1976, Thorpe et al. 1978, Hedgecock 1979).

Here, the detection of three putative sibling species within Fennoscandian *M. relicta* is described. An insight into the time scale of evolution of *Mysis* spp. is obtained through assessing genetic distances between *M. relicta* and two of its congeners, the morphologically close, arctic-subarctic *M. litoralis*, and the more distant *M. mixta*, a North Atlantic species which also coexists with *M. relicta* in the Baltic Sea.

2. Materials and methods

2.1. Samples

The distribution of *M. relicta* in Finland and Sweden comprises most of the relatively deep (> 20m) lakes once flooded by the waters of the historical stages of the Baltic Sea (Segerstråle 1956). The species does not naturally occur in lakes of highlands that remained unsubmerged in late- and post-glacial times, but is found again in the north in lake Pulmankijärvi (Polmakvatn), 17 m a.s.l., which drains into and has been isolated from the Barents Sea (in the context of geological history, 'isolation' means the event when a lake becomes separated from the sea or other larger water body). *M. relicta* also lives in the brackish waters of the northern Baltic Sea. In the Tvärminne-Pojoviken area, SW Finland (Fig. 1), *M. relicta* is nowadays found only sparsely among *M. mixta* in the outer archipelago zone, near Tvärminne Zoolocial Station; in the inner archipelago, up to the strait at Källvik, *M. relicta* seems to be somewhat more frequent. In the deep part (40 m) of the relatively isolated Pojoviken bay, the population is dense; the bay is connected to the archipelago area and the open sea only through an approx. 6 km long shallow (5–10 m) passage involving a narrow strait (for details of the hydrography, morphology and biological zonation of the Tvärminne-Pojoviken area, see Niemi 1973).

M. relicta from Pojoviken has been collected repeatedly in 1982–1984, and samples from outside Tvärminne (35–40 m) were taken on several dates in 1984; furthermore, some samples along the transect between these two

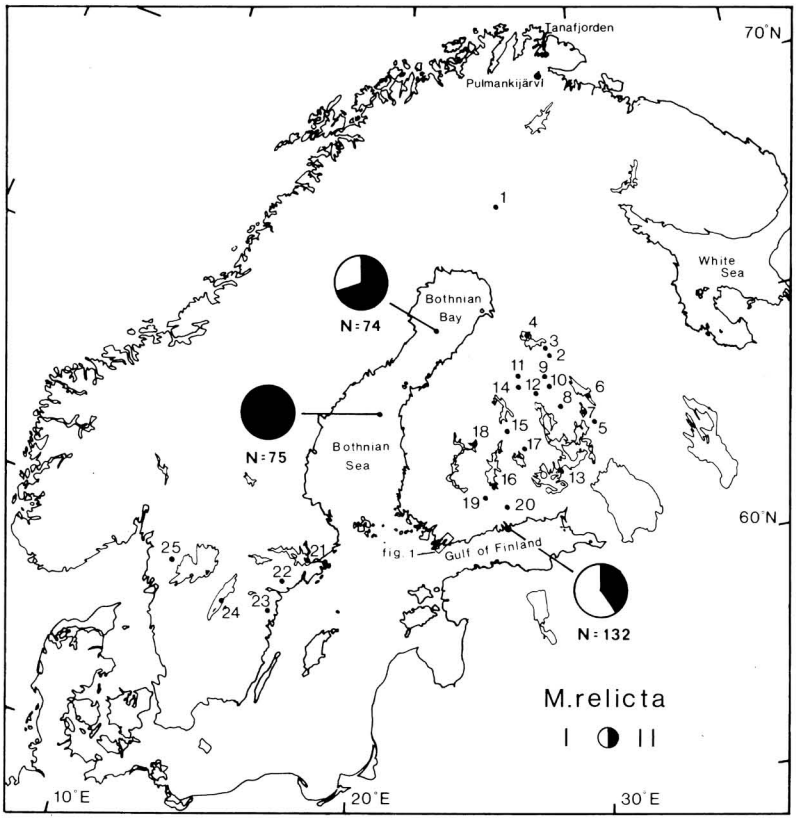

Fig. 2. Location of sampling sites in Fennoscandia, and sibling species composition in three Baltic samples. Lake numbers refer to Table 1.

points were studied (Fig. 1). Sampling was by a sled net towed horizontally about 10 cm above the bottom.

In addition to the Tvärminne-Pojoviken area, samples of *M. relicta* were studied from three other sites in the Baltic (Fig. 2): the Gulf of Finland near Loviisa (Kejvsalö östra fjärd, 60°6′N, 26°14′E, 27.VII. 1984, 20 m, sled), the Bothnian Sea (62°30′N, 20°15.5′E, 21.V.1984, 80 fn, sled) and the Bothnian Bay (64°30′N, 22°42′E, 20.11.1985, 94 m, vertical net hauls).

Samples of *M. relicta* from 20 Finnish and five Swedish lakes once affected by Baltic waters were examined for this study (Fig. 2); Table 1 gives information of the lakes, including age (since isolation) in relation to the historical development of the Baltic Sea. The late- and post-glacial history of the Baltic has been reviewed by e.g. Kvasov (1979) and Agrell (1979; also other papers in the same volume), and a short summary is given in the footnote of Table 1. A sample from Lake Pulmankijärvi, draining through the River Tana (Tenojoki) to Tanafjorden in the Barents Sea, was also studied. This lake was apparently finally isolated not earlier than some 5000 years ago (cf. Sollid et al. 1973).

Mysis mixta was collected from the Baltic near Tvärminne; *M. litoralis* was captured from the sublittoral of Tanafjorden, N.Norway (Fig. 2). It should be noted that the morphological identification of *M. litoralis* in this sample could not be entirely unequivocal, some of the main diagnostic characters of *M. litoralis* and *M. oculata* (Holmquist 1958, 1959) being ambiguous. At all events, the qualitative conclusions of this study will be the same.

The samples were usually frozen on dry ice and then stored at -20°C until electrophoresis; specimens from the Tvärminne-Pojoviken area were often kept alive in aquaria in local sea water (6–7‰ S) and analysed fresh.

2.2. Electrophoresis

Whole animals were individually homogenised in distilled water (fresh specimens) or in 1% β-mercaptoethanol (frozen animals), and subjected to standard starch gel electrophoresis followed by histochemical staining of specific enzymes (e.g. Harris & Hopkinson 1978). The particulars of applying these techniques to *Mysis* are described elsewhere (Väinölä & Varvio, in prep.).

The interpretation of stained enzyme bands on gels as products of different gene loci (isozymes) and products of different alleles at homologous loci (allozymes) follows the general practice in electrophoretic studies (see e.g. Ferguson 1980). The loci coding for different isozymes of the same enzyme are numbered according to decreasing anodal mobility of the enzyme. The most common allozyme and allele in the Pojoviken population of *M. relicta* (*'M. relicta I'*) is designated '100' at each locus, whereas

Table 1. Lakes sampled for *Mysis relicta*.

Lake	Elevation (m a.s.l.)	Isolation[a]	Present drainage	N[b]
Finland				
1. Unari	180	Y	Kemijoki	5
2. Jormasjärvi	145	Y	Oulujoki	120
3. Rehja	137	Y	"	109
4. Oulujärvi	122	M	"	95
5. Ylinen	120	Y	Jänisjoki	30
6. Pielinen	94	Y	Vuoksi	130
7. Höytiäinen	87	Y	"	56
8. Rikkavesi	101	Y	"	75
9. Sälevä	116	Y	"	11
10. Syväri	95	A	"	80
11. Rytkynjärvi	96	A	"	95
12. Maaninkajärvi	82	A	"	90
13. Saimaa, Tolvanselkä	76	A	"	120
14. Pielavesi	102	A	Kymijoki	130
15. Kynsivesi	88	A	"	99
16. Päijänne	78	A	"	100
17. Kyyvesi	101	Y	"	130
18. Keurusselkä	105	A	Kokemäenjoki	40
19. Pääjärvi	103	Y	"	120
20. Pyhäjärvi	40	A	Koskenkylänjoki	60
Sweden				
21. Mälaren	0.5	Lm	Norrström	96
22. Båven	21	L	Nyköpingsån	122
23. Yxningen	38	A	Hällaån	130
24. Vättern	89	Y	Motalaström	120
25. Ärtingen	94	(Y)	Göta älv	122

[a] Approximate chronology of the historical stages of the Baltic from which the lakes were finally isolated:

B Baltic Ice Lake (9800–8200 BC) fresh
Y Yoldia Sea (8200–7200 BC) brackish (for a short period, mainly in C. Sweden)
A Ancylus Lake (7200–6000 BC) fresh
M Mastogloia Sea (6000–5500 BC) slightly brackish (controversial)
L Litorina Sea (5500–2000 BC) more saline than the present Baltic
Lm Limnea Sea (2000 BC–) brackish

Information on lake history from Assarsson (1927), Åse (1970a, b), Saarnisto (1971), Eronen (1974) and Agrell (1979).

[b] Number of individuals examined

the others are labeled by adding to 100 the mobility difference (in mm) between the corresponding allozyme and '100' in an average electrophoretic run.

The 21 enzyme gene loci analysed in all the species studied here are (in parentheses the enzyme coded by the locus): *Aco-2* (cis-aconitase), *Apk-1*, *Apk-2* (arginine phosphokinase), *Dia-1*, *Dia-2* (NADH-diaphorase), *Got-1*, *Got-2* (glutamate-oxaloacetate transaminase) *G3pd* (glyceraldehyde-3-phosphate dehydrogenase), *Gpi* (glucose-phosphate isomerase), *Idh-1*, *Idh-2* (isocitrate dehydrogenase), *Mdh-2* (malate dehydrogenase), *Me-2* (malic enzyme), *Mpi* (mannosephosphate isomerase), *Pep-1*, *Pep-2*, (peptidase, leucyl-tyrosine used as substrate in the staining reaction), *6Pgd* (6-phophogluconate dehydrogenase), *Pgk* (phosphoglycerate kinase), *Pgm* (phosphoglucomutase), *Sdh* (sorbitol dehydrogenase), and *Tpi* (triosephosphate isomerase).

3. Results

3.1. Detection of sibling species

Comparisons between samples of *M. relicta* from some lakes of southern Finland and the brackish Pojoviken bay had earlier revealed some allele frequency differentiation among populations; this has apparently accumulated since the isolation of the lakes, during the last 9000 years (Väinölä, unpubl.). The difference between Pojoviken and the lakes is of the same order of magnitude as that between populations of individual lakes. It was suspected that *M. relicta* would probably seldom cross the shallow threshold area connecting Pojoviken to the Gulf of Finland; thus the population in the bay could be quite isolated from that in the outer archipelago and the open sea,

Table 2. Prevalent alleles (allozymes) in five *Mysis* species, and allele frequencies at polymorphic loci. – A locus is considered monomorphic if the frequency of the most common allele is >0.95. Cases in which rare variants were detected with a frequency <0.05 are indicated by an apostrophe. – The data on *M. relicta I* refer to the Pojoviken population. For most loci, data on *M. relicta II* were pooled from different localities. – Sample sizes are indicated only if data were obtained from less than 30 individuals (60 genes) for a monomorphic locus, or from less than 50 individuals for a polymorphic locus.

Locus	*relicta I*	*relicta II*	*relicta III*	*litoralis*	*mixta*
Aco-2	*104* (.08)	*104′*		*97* (.87) (*N*=4)	*92′* (.91)
	100 (.92)		*101*	*92* (.13)	*89* (.09)
Apk-1	*100*	*100*	*100*	*115*	*114*
Apk-2	*100*	*100*	*100*	*104*	*100*
Dia-1	*100*	*100*	*100*	*98*	*96*
Dia-2	*100*	*100*	*100* (*N*=8)	*100* (*N*=8)	*100*
G3pd	*100*	*100*	*100*	*100′*	*100*
Got-1	*100′*		*98* (.31)		
		92′	*92* (.69)	*92′*	*78′*
Got-2	*100′*	*105′*	*98′*	*105′*	*105*
Gpi	*100*	*100′*	*104* (.58)		*92′* (.50)
			93 (.42)	*81′*	*87* (.50)
Idh-1	*100*	*100*	*100* (*N*=19)	*100* (*N*=23)	*100*
Idh-2	*109* (.10)			*106* (*N*=18)	*106*
	100 (.90)	*100*	*100* (*N*=3)		
Mdh-2				*107* (.37)	
	100′	*100*	*100*	*100* (.63)	*94*
Me-2	*100*	*100*	*100*	*100′*	*98′*
Mpi	*107* (.34)	*107′* (.35)	*107*	*107*	
	100′ (.66)	*102′* (.65)			*84*
Pep-1	*100*	*100*	*100*	*100*	*100*
Pep-2	*100′*	*93*	*93*	*87′*	*74′*
6Pgd		*103* (.15)	*103* (.08)		
	100′	*100* (.85)	*100* (.92)	*100*	*92′*
Pgk	*100*	*100*	*100*	*100* (*N*=25)	*105*
Pgm	*104′* (.32)		*105′*	*105* (.94)	*98′* (.03)
	100′ (.68)	*100′*		*94′* (.06)	*92* (.88)
					85′ (.09)
Sdh	*100′*	*100′*	*100′*	*100′*	*102*
Tpi	*100* (.78)	*102* (.07)			
	90 (.22)	*90* (.50)	*90* (.92)		*90*
		80 (.40)	*80* (.08)	*80*	
		70 (.03)			

allowing some genetic differentiation to take place also between the local brackishwater populations. However, the electrophoretic comparison of these populations revealed a level of differentiation far higher than that between any lakes.

The Pojoviken and Tvärminne populations of *M. relicta* are practically fixed for different alleles at the loci *Pep-2*, *Got-1*, *Got-2* and *Aco-2*. At the last two, the characteristic allele of

the Tvärminne population is also found at a low frequency in Pojoviken, but these loci are still diagnostic at the 99% level (i.e., an individual could be assigned to its own population on the basis of its electrophoretic phenotype of the particular enzyme with a probability of 99%). Furthermore, the predominant alleles of *Mpi* and *Tpi* are also different, although there is polymorphism for common alleles (Table 2); these loci are diagnostic at well over the 90% level. Most of the other loci are identically monomorphic though rare variants generally occur. *Pgm* is polymorphic in one population, the more common allele being the same as in the other.

The observed differentiation between these two nearby populations is so high that it is most unlikely to have arisen during late- and post-glacial times, i.e. during the recent history of the Baltic. The average genetic identity (Nei 1978) of the two populations, calculated for the 21 loci studied here, is 0.75, a value characteristic of populations that have been separated for millions rather than for thousands of years. This level of genetic differentiation has usually been found between morphologically close species and subspecies of other organisms (e.g. Ayala 1983).

The degree of differentiation or time since isolation as such are not necessarily sufficient grounds for deciding the taxonomic status of allopatric populations. However, the two types of *M. relicta* described above have also been found sympatrically in several areas of the Baltic (see next section). No F_1 hybrid genotypes or clear traces of introgression were detected in the present material; apparently, no considerable natural interbreeding between the two sympatric, genetically distinct populations occurs. Therefore, this makes a strong case for two good biological species. For the present, in the absence of a morphological description, we designate the sibling species prevalent (or exclusive) in Pojoviken and Tvärminne *'Mysis relicta I'* and *'M. relicta II'*, respectively.

All individuals of the conventional *M. relicta* studied from the Baltic and the lakes of southern and central Finland and Sweden could be assigned to either *M. relicta I* or *II* on the basis of the electrophoretic phenotypes of the enzymes mentioned above. However, the population of the northern Lake Pulmankijärvi constituted another genetically distinct type, approximately as different from the two southern species as these are from each other. The Pulmankijärvi population is characterized by its own alleles at the loci *Gpi*, *Pgm*, *Got-2* and *Aco-2*. Furthermore, it differs from *M. relicta I* (but not *II*) at *Pep-2* and *Got-1*. The polymorphic *Tpi* locus is also almost diagnostic (97%) between the Pulmankijärvi population and *M. relicta I*, whereas both the alleles of the Pulmankijärvi population are common in *M. relicta II*. In Pulmankijärvi, *Mpi* is monomorphic for an allele found as polymorphic in both *M. relicta I* and *II* (Table 2). The average genetic identities of the Pulmankijärvi population with the Baltic *M. relicta I* and *II* were estimated to be 0.68 and 0.78, respectively.

There is no direct evidence of reproductive incompatibility between the Pulmankijärvi population and either of the southern sibling species. However, the similar level of genetic differentiation suggests that the population most probably belongs to another (sibling) species of the nominal *M. relicta*. Here, this putative species will be designated *'M. relicta III'*.

3.2. Geographical distribution

For species identification, the samples from southern and central Finnish and Swedish lakes (Table 1) were electrophoretically examined for at least three of the loci *Pep-2*, *Mpi*, *Got-1*, *Got-2*, *Pgm* and *Tpi*. All these lakes proved to be inhabited by *M. relicta I*. The lakes include Vättern (no. 24 in Fig. 2) as well as Ärtingen (25), which is closely connected to Vänern. Thus, *M. relicta I* is probably the original *M. relicta* described by Lovén (1862) from Vättern and Vänern.

The only lake where two species were observed was Båven (22): five individuals of *M. relicta II* were identified among 117 *M. relicta I*.

In the Baltic, *M. relicta I* and *II* seem to coexist quite commonly. The sibling species composition of some samples from the Tvärminne area in 1984 are shown in Fig. 1: *M. relicta I* is exclusive in Pojoviken, but it is also abundant and predominant in the inner archipelago zone, i.e., outside the shallow threshold and area of major environmental change. In the outer archipelago zone, near Tvärminne, only a few specimens of *M. relicta I* have been found among *M. relicta II*. At least occasionally, *M. relicta II* is also found in the

Table 3. Estimates of genetic identity (I, below diagonal) and genetic distance ($D = -\log I$, $\pm SE$, above diagonal) for *Mysis* spp.

	relicta I	*relicta II*	*relicta III*	*litoralis*	*mixta*
M. relicta I	–	.28 (±.12)	.38 (±.14)	.79 (±.24)	1.33 (±.37)
M. relicta II	.75	–	.25 (±.11)	.57 (±.19)	1.12 (±.32)
M. relicta III	.68	.78	–	.57 (±.19)	1.21 (±.35)
M. litoralis	.45	.56	.56	–	1.19 (±.34)
M. mixta	.26	.33	.30	.30	–

inner archipelago and coastal zones, though it is rare compared with *M. relicta I*.

The sample from the northern Bothnian Sea was pure *M. relicta II*, whereas those from the more peripheral areas in the Gulf of Finland and the Bothnian Bay were mixtures of the two sibling species (Fig. 2). At the Bothnian Bay station, both species were present in several individual vertical net hauls.

Summing up these preliminary observations on geographical distribution, *M. relicta I* seems to be exclusive in Finnish and Swedish lakes that have obtained their populations from the early stages of the Baltic Sea (cf. Table 1). This species is also common in coastal and peripheral parts of the northern Baltic. *M. relicta II* has so far been found mainly in the Baltic Sea, coexisting with *M. relicta I* in peripheral areas, and with *M. mixta* in the open sea. The only freshwater record of *M. relicta II* is from a lake that was still covered by the Litorina Sea (i.e. a historical stage of the Baltic with a higher salinity than the present). *M. relicta III* was found only in the northern lake Pulmankijärvi.

3.3. Phylogeny of *Mysis* spp.

On the basis of allele frequencies in *M. relicta I–III*, *M. litoralis* and *M. mixta*, given in Table 2, standard measures of interspecific genetic identity and distance (Nei 1978; standard errors according to Nei & Roychoudhury 1974) were calculated (Table 3), and a dendrogram (Fig. 3) summarising the distance matrix was constructed by the UPGMA method (Sneath & Sokal 1973). The tree is taken as a rough estimate of the phylogeny of the species examined.

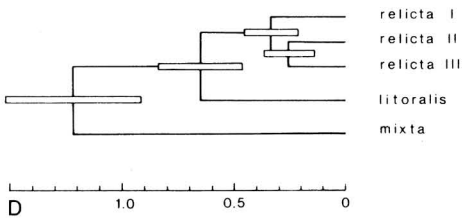

Fig. 3. UPGMA-dendrogram of genetic distances (Table 3) for *Mysis* spp. Boxes indicate ±1 *SE* of the branching points, calculated according to Nei et al. (1985).

As regards the three nominal species, the topology (branching order) of the tree is as would be expected on morphologigal grounds. *M. mixta* has a distance of about 1.2 (estimated obervable substitutions / an average gene) from both *M. litoralis* and the *M. relicta* species group. This is a value typical of members of closely related genera in other invertebrates (Thorpe 1983). Holmquist (1959) refers *M. mixta* to another subgenus (*Michteimysis*), although even a generic division has earlier been suggested. The distances between *M. litoralis* and *M. relicta I–III* are in the range characteristic of congeneric species, whereas distances among members of the *M. relicta* group resemble those observed between morphologically described sibling species of other taxa.

The branching order of the sibling species remains unresolved by the distance approach, as is illustrated by the standard errors of branching points in Fig. 3. The uncertainty arises from the restricted number of loci examined; the role of small sample sizes at some loci is negligible in this respect (cf. Nei & Roychoudhury 1974.)

Another approach to resolving the evolutionary branching sequence is the Hennigian method based on direct character analysis (see e.g. Patton & Avise 1983). Using *M. litoralis* as an outgroup for the *M. relicta* species group, only the allelic distribution of *Pgm* gives cladistically relevant information on the intersibling relationships in the present case. Pgm^{100} seems to be a synapomorphy of *M. relicta I*

and *II* and thus suggests them as being the most recently diverged species pair. Other features uniquely common to these species are Pgi^{100} and also $Aco\text{-}2^{104}$, but the direction of character state changes (polarity) at these loci cannot be decided.

On the other hand, *M. relicta II* and *III* have retained largely similar polymorphisms at *Tpi* and *6Pgd*, which might be considered an improbable occurrence, unless the divergence of lineages was a relatively recent event. This retention is also an illustrative example of why the Pgm^{100} 'synapomorphy' cannot be taken as conclusive evidence of the branching sequence: polymorphism for Pgm^{100} and Pgm^{105} may well have been preserved from one branching event to another, with a subsequent loss of one morph in each lineage. *M. relicta II* and *III* also uniquely share the allele $Pep\text{-}2^{93}$. No corresponding features connecting *M. relicta I* and *III* at the individual locus level are apparent; this is also reflected in the distance matrix, even though the clustering method is not able to illustrate the point. At the set of loci studied, *M. relicta I* appears more apomorphic (relative to *M. litoralis*) than the other sibling species.

4. Discussion

4.1. On the time scale of evolution

Changing opinions on the prehistory of the glacial immigrant crustaceans have always reflected the contemporary ways of thinking about adaptational evolution and the speciation process. As early as 1860, when first reporting on the newly found peculiar faunal element of two Swedish lakes, Lovén (1862) indirectly suggested an evolutionary relationship between arctic marine and the remnant lacustrine species. Subsequently, a view of a recurrent origin of *M. relicta* following the isolation of *M. oculata* into different freshwater basins was entertained for most of the following century. Accompanying this concept of morphological modification associated with a salinity change, morphological intermediates were expected to be found in intermediate environments. Particularly, the occurrence of a largely similar 'relict' fauna both in Eurasia and North America has caused discussions of possible parallel origins of the species (see Segerstråle 1957, 1962, 1982, Ricker 1959, Holmquist 1959, 1966, for history and discussion of the views).

Holmquist's (1959) confirmation of the specific status of *M. relicta* and rejection of the idea of ancestor–descendant relationships among extant species was connected with the recognition of two other close relatives of *M. oculata*, i.e *M. litoralis* and *M. polaris*. *M. relicta, M. litoralis* and *M. oculata* are circumpolar species, all of which have been found sympatrically. *M. litoralis* is morphologically even closer to *M. relicta* than is *oculata*; the species is found mainly in diluted coastal environments, and has also been met in fresh water (Holmquist 1959). Under the early supposition of recurrent evolution of the 'relicts', *M. litoralis* was actually suspected of being an intermediate stage in the transition from *M. oculata* to *M. relicta* (see Holmquist 1959). However, the four endemic *Mysis* species of the Caspian Sea are apparently the closest living relatives of *M. relicta* (Holmquist 1959, 1966).

Along with arguments concerning geological history (see below), the widespread distribution and geographically rather uniform morphology of *M. relicta* were the main grounds leading Holmquist to suggest that it was an old, well established species, dating back to the mid-Tertiary. There has been some reluctance to abandon the role of the Glacial epoch in the inception of the glacial relicts, though; Dadswell (1974) suggests an evolution in early Pleistocene or late Pliocene brackish-water seas, whereas a hypothesis of speciation associated with a forced freshwater adaptation upon isolation of marine ancestors into a Siberian ice-lake, dammed up by the ice sheet of one of the last glaciations (perhaps the very last one), has been advocated by Segerstråle (1957, 1982).

Holmquist (1949, 1959) actually found some morphological variation among populations of *M. relicta*, e.g. between Baltic and lake populations and also between samples from different parts of the Baltic. However, as no geographical or salinity related pattern could be revealed on a worldwide scale, she concluded that there has not been differentiation even to the level of geographical races within *M. relicta*. The present results certainly do not agree with this view of species coherence, but Holmquist's opinions of Tertiary evolution seem to be better supported by the genetic data.

We can try to estimate the time since diver-

gence of the lineages leading to the present *Mysis* spp. by applying to our data the observed relationship between genetic distance and time in taxa of 'known' evolutionary history. There have been two principal ways of calibrating the 'electrophoretic clock' of molecular evolution. The first, indirect one is based on the correlation of electrophoretic distances with immunological distances, which in turn have been calibrated with the fossil record; the other method uses distances between taxa whose divergence can be directly related to events of geological history (or to fossil records). The correlations obtained by the first method have suggested rates of one electrophoretic substitution per gene (unit genetic distance) in 15−20 million years (see Thorpe 1982). With the present estimates of genetic distances in *Mysis*, this would correspond to a ca. 5 Myr age for the radiation of the sibling species, ca. 10 Myr for their divergence from *M. litoralis* and about 20 Myr for the divergence from the *M. mixta* lineage.

A linear relationship between divergence time and standard genetic distance is theoretically predicted if the rate of amino acid substitution is constant in time, and also constant over loci. However, the substitution rate is known to vary considerably in different proteins. Nei (e.g. 1986) has suggested that a more linear relationship to evolutionary time in such a situation may be obtained with the measure $D_v=(1-I)/I$ (rather than $D=-\log I$, where I is the genetic identity), at least for $D_v < 1$; with larger distances the resolution will be rather poor, whatsoever. Furthermore, Nei suggests that the relationship divergence time $t = 5 \times D_v$ Myr, originally based on considerations of protein structures and observed mean rates of amino acid substitution, is in agreement with most data pertinent to the second, 'direct' method of calibrating the electrophoretic clock. However, reconsidering existing data on such distances (references in Nei 1986, and Pashley et al. 1985), a relationship of $t=7-14 \times D_v$ Myr seems more reasonable to me, particularly in the range $D_v > 0.2$, which is relevant to this study. The D_v estimates for the three main divergences discussed here are 0.29−0.40, 0.92 and 2.4, which would then correspond to divergence times of some 2−6, 6−13 and 17+ million years for *M. relicta I−III* from each other, from *M. litoralis*, and from *M. mixta*, respectively.

In any case, such estimates are prone to several sources of error and possibly also to bias due to non-generalizability of results from one case (taxon and laboratory) to another (see e.g. Thorpe 1982, Nei 1986). Even if the methods and average evolutionary rates were comparable, the 'sampling' of a restricted number of loci from a set with a wide variance of substitution rates may lead to a systematic error in relation to other studies. However, considering the set of loci studied here in relation to reviews of published electrophoretic data on invertebrates (Hedgecock et al. 1982, Ward & Skibinski 1985), this is not likely to be a source of important upward bias of divergence estimates in the present case. The proportion of diverged loci may even have been underestimated (on the scale used in most other studies). In addition to the 21 loci reported here, a number of other enzymes were also examined in *Mysis* spp. but not included in the results, as they could not be reliably scored in all of the five species. Several of them showed species specific phenotypic differences between some or all of *M. relicta I−III*; these included esterase-1 and -3, PEP-4 (phenylalanyl-proline substrate), ME-1, hexokinase, hexosaminidase-2 and pyruvate kinase.

In conclusion, I consider the above estimates of evolutionary time acceptable, with broad confidence limits, though. In the light of the results, then, it seems plausible that even the divergence of the sibling species dates back to pre-Pleistocene times, most probably to the Pliocene, and thus obviously was not connected with Ice Age conditions. As it is most reasonable to assume a monophyletic origin for both the characteristic morphology and ecology (i.e. freshwater life) of the *M. relicta* group, the time since the radiation of *M. relicta I−III* should also give a lower bound for the controversial age of (the conventional) *M. relicta*.

On the other hand, on the basis of a couple of morphological characters linking *M. relicta* with the four *Mysis* spp. of the Caspian Sea, Holmquist (1959, 1966) has argued that these five species diverged from each other only after their divergence from (e.g.) *M. litoralis*. Furthermore, as she regarded the late Oligocene or early Miocene as the latest possible time for a northern fauna to enter the Caspian basin (other authors have suggested an introduction via ice-dammed lakes in the Pleistocene), Holmquist concluded that *M. relicta* must have diverged from its closest extant relatives

around those times. The evolutionary time scale suggested by the present results would perhaps be somewhat narrower, as a Miocene date for even the *M. relicta*—*M. litoralis* divergence would seem more plausible in the light of the genetic distances.

As a particular example illustrating the level of differentiation found among the sibling species of *M. relicta*, we may still make a direct comparison with the case of three morphologically close species of the amphipod crustacean genus *Gammarus* (*G. zaddachi, G. salinus* and *G. oceanicus*), which also inhabit the Baltic Sea. In a study by Kolding & Simonsen (1983), based on 17 loci, the genetic distances of these species ranged from 0.11 to 0.20, and were thus only about half of those found among *M. relicta I—III*; the ratio is even much smaller (approx. 1 8) if only the nine loci (presumably) common to the two sudies are considered. None of the loci *Pgi, Pgm, Got-1* and *-2* and *Pep*, diagnostic in *Mysis*, were properly diagnostic between the *Gammarus* species, although most were polymorphic. The distances of the *G. zaddachi* group to two other congeners, *G. duebeni* and *G. locusta*, were similar to those between the *M. relicta* species group and *M. litoralis*.

As another comparison, relevant to the discussion on the evolutionary time scale of the 'relict' crustaceans, we note that the genetic distance of another glacial immigrant *Pontoporeia affinis* (Amphipoda) to its once presumed marine ancestor *P. femorata* has turned out to be even greater than any of those reported in this study (Väinölä & Varvio, unpubl.)

4.2. On ecology and life histories

Two apparently very similar species of the *Mysis relicta* group inhabit the Baltic, coexisting in many parts of the sea, but each also having areas of its own. A problem readily calling for attention is, what are the ecological factors regulating the incomplete spatial separation and allowing partial sympatry? The question has been pointed out also as concerns the 'pair' *M. mixta* and *M. relicta*, which exhibit a comparable pattern of partial overlap in the Baltic (Salemaa et al. 1986); the problem might be more compelling in a case of morphologically hitherto unseparable species.

Environmental salinity is the most often invoked physical factor in explaining distribution limits of brackishwater organisms (e.g. Zenkevitch 1963). In the light of the scanty data, there seems to be some, although not complete, correlation of salinity and the distribution of the sibling species. Bottom salinities of 6.5—7‰ are characteristic of the two sites where *M. relicta II* was found to predominate, whereas that of the Bothnian Bay station with a mixed population was approx. 4‰ (data of the Marine Research Institute). However, the salinity in the Pojoviken deep (*M. relicta I* exclusive) is also as high as 4—5‰, and at the Tvärminne inner archipelago sites usually over 6‰, although surface values are generally considerably lower (cf. Niemi 1973). As already noted, the transition zone of *M. relicta I* and *II* in the Tvärminne archipelago does not seem to coincide with the zone of major environmental change between Källvik and Pojoviken. Moreover, both species are capable of living in fresh and brackish water, as indicated by their distribution (apparently also *M. relicta III* must have tolerated at least brackish water before the isolation of Pulmankijärvi). Also, lake- and estuary-born *M. relicta (I)* thrive well in aquaria at 7‰, a salinity typical of the central Baltic (Holmquist 1959, and own observations), and the holeuryhalinity of *M. relicta* has been stressed also on the basis of experimental acclimatization of freshwater specimens to more dramatic salinity changes (Dadswell 1974). The distributions are apparently not regulated by differential salinity tolerances, but it remains possible that the salinity optima differ and have a role in determining the relative success of the species in a given environment.

Lindström & Nilsson (1984 and personal communication) have observed physiological differences in eye function and light tolerance of *M. relicta* from the Tvärminne outer archipelago site, Pojoviken and Lake Pääjärvi (19). As light is a key factor in controlling the diurnal vertical migrations of *Mysis* (Beeton & Bowers 1982), a difference like that could be involved in microspatial niche partitioning of the two Baltic sibling species. Salemaa et al. (1986) observed such differences in vertical migrations of *M. relicta* and *M. mixta*.

In sympatric populations of similar species, a possible mechanism for resource partitioning and avoidance of interspecific competition could be the differential timing of life cycles. The Pojoviken—Tvärminne populations of *M. relicta I* are at least predominantly winter breeding, young are released in the spring. In contrast, the reproduction of *M. relicta II* does not seem to be bound to season. In the Tvärminne area, I have found ovigerous females and males with well developed IV pleopods in spring, summer and autumn. Hessle and Vallin (1934) have also observed throughout-year reproduction of *M. relicta* in the Bal-

tic. According to them, this independence of season is restricted to the central areas of the sea; winter breeding is the rule in the Gulf of Bothnia. This geographical variation is probably not merely an effect of species composition, as *M. relicta II* seems to be abundant up to the Bothnian Bay. In my sample from that area, taken in February, embryo-bearing females of both species were present. Moreover, the length distributions of *M. relicta I* and *II* in this small sample (N=22+52) were quite similar, both consisting of two distinct size classes (mean lengths about 12 and 17.5 mm, from rostrum to the apices of the telson). This would suggest a similar two-year life cycle with winter breeding for both species in this area. Other reports on the reproductive dynamics of *M. relicta* in the Baltic include the observation of both one- and two-year-old winter breeding cohorts (Salemaa et al. 1986) and of a prolonged reproductive period in the Gulf of Riga (Shvetsova 1980).

Apparently all the populations studied in Finnish lakes (including Pulmankijärvi) are winter breeding, even though the generation time may vary. In Sweden, the populations of Mälaren (21), Båven (22) and Vättern (24) are also exclusively winter breeding (one-year cycle), but Ärtingen (25) and Yxningen (23) belong to a set of central Swedish lakes harboring both winter and summer breeding populations (Fürst 1972). Finding no current ecological correlates for the occurrence of summer breeding, and concluding that sympatric asynchronous populations are probably reproductively isolated, Fürst suggested they belonged to two sibling species, apparently derived from a period older than the history of the present habitats.

My sample from Yxningen, taken in late August, also contained a proportion of summer breeding individuals (mature females bearing embryos or having recently released offspring) in addition to the main mass of winter breeding animals. However, all diagnostic loci indicated that both cohorts belonged to *M. relicta I*. The result does not necessarily dispute the claim of reproductive isolation between the sympatric but asynchronous populations; the sample size was also too small to reveal slight gene frequency differences. In any case, this life history differentiation has apparently evolved during a considerably shorter time than the genetic differentiation of sibling species reported in this study. The observation concurs with that of Vuorinen et al. (1981) of little enzyme genetic differentiation between asynchronously breeding supposed sibling species of the *Coregonus albula* complex fish (Svärdson 1979).

There thus seems to be a wealth of geographical variation in the life histories of both *M. relicta I* and *II*, not only in the length of life cycles but also in the seasonal timing of reproduction. Both winter and summer breeding occur in each species, but no general tendency of strong interspecific differentiation of breeding time in sympatric populations is apparent, even though such differentiation may occur within a species. For instance, in lake Båven, with predominant one-year winter breeding of *M. relicta I*, apparently the *M. relicta II* population also breeds in winter (as judged from the sizes of four late summer individuals). Whether there are any summer breeding brackish water populations of *M. relicta I* (or lacustrine of *M. relicta II*) is not known.

4.3. On immigration hypotheses

Due to their restricted means of dispersal (inability to migrate upstream or with external agencies), the glacial immigrant crustaceans have provided an ideal object for the zoogeographical exercise of relating present patterns of animal distribution to Quaternary geological history. Some aspects of distribution, such as the occurrence of these crustaceans only in the Finnish lakes once flooded by the Baltic waters, have been easy to understand, but others concerning dispersal over greater distances and in more remote times have evoked divergent hypotheses and a long-lasted scientific debate (see Segerstråle 1957, for a thorough review), and there are problems that still await a final solution.

As regards the arrival of the animals in Fennoscandia, the original idea of Lovén (1862) was that they had been left behind by an arctic sea that extended to the area from the northeast. The late- or post-glacial marine connection between the Baltic and the White Sea was itself debated by geologists up to the last decades (the distribution of the crustaceans was actually used as a strong argument for the existence of such a connection), but this idea has now been dismissed. A rival hypothesis was that of a western immigration route,

around the Scandinavian Peninsula in waters diluted by the melting continental ice, and through a central Swedish strait into the Yoldia Sea stage of the Baltic.

As these hypotheses could not explain all aspects of the distribution of the crustaceans in Northern Europe, Segerstråle (1957) elaborated a theory of an eastern immigration along a freshwater pathway. This was an extension of an idea put forward by Högbom of dispersal in waters dammed up in front of an advancing ice cap. Segerstråle suggested that the immigrants of the Baltic basin had been sluiced up from the White Sea estuaries into an ice lake of the Onega River valley, which subsequently flooded over the watershed and brought the animals to the continental areas southeast and south of the Baltic, and into the Baltic (the Baltic Ice Lake) itself. The crustaceans would have arrived in the White Sea region from more eastern areas along a pathway of similar proglacial lakes, during an earlier, or even the same, i.e. Weichselian glaciation (see Segerstråle 1982). A parallel model for the dispersal in North America has been worked out (Ricker 1959, Dadswell 1974) and the principle of proglacial transport seems to be widely accepted, although details of the reconstructions are not always relied on (e.g. Holmquist 1966).

The distribution of even the traditional immigrant species in Northern Europe are not similar to each other but in some cases strikingly different. Ecological conditions in the past have been invoked to explain the distributional differences. The original immigration hypothesis of Segerstråle (1957) actually involved two introduction 'events' (from the same source); the first, more southern continental pathway would have been impassable for some of the species (e.g. *Saduria entomon, Gammaracanthus lacustris*), which then never reached the northern lakes of continental Europe.

Besides the problems concerning the biological history itself, the definition of the term 'relict' and its application to the crustaceans under consideration has been a controversial issue (e.g. Holmquist 1959, 1966, Dadswell 1974, Segerstråle 1976, Koli 1984). Not touching upon this debate in this paper, the term 'glacial immigrant', suggested by Dadswell (1974) and Koli (1984), was adopted here to designate animals which have attained their present distribution in connnection with glacial conditions. Whether all of the sibling species should be regarded even as glacial immigrants, is not immediately clear.

The observed distribution of *M. relicta II* is suggestive of a new subelement in the distributional pattern of the crustaceans concerned. Along with the possibly salinity-related distribution of the species in the Baltic, the location of the only *M. relicta II* lake is tempting as regards the tradition of relating the distribution of these crustaceans to geological history. Except Mälaren (21), Båven (22, with *M. relicta II*) is the youngest of the lakes studied, and these are the only two lakes that have once been covered by the waters of the relatively saline Litorina Sea (ca. 5000–2000 BC). Båven was apparently isolated at the end of the Litorina period, approximately 4000 years ago (cf. Åse 1970a, b), whereas Mälaren became independent not earlier than 1000 years ago (Åse 1970a), probably under conditions rather similar to the present. The observed distribution of *M. relicta II* could be a consequence of the absence of the species in earlier stages of the Baltic, or to its inability to penetrate marginal areas in those times.

In the first case, we would be dealing with an introduction later than most of those proposed earlier for the glacial immigrant species. Such a late arrival would not have been directly dependent on glacial phenomena. Although an introduction from a continental lake could have been possible, the idea of a western marine introduction calls naturally for reconsideration. However, in contrast to suggestions of immigration into the Yoldia Sea through central Sweden, this would mean a Mastogloia or Litorina time penetration through the Danish waters. The main theoretical objection against a marine immigration path, i.e. the restricted salinity tolerance of some of the crustaceans (e.g. Segerstråle 1957), would not be a strong argument as regards *M. relicta II*, keeping in mind the present environment of the species in the Baltic and the suggested holeuryhalinity of *M. relicta* in general (cf. Holmquist 1966, Dadswell 1974).

The data available on *M. relicta II* (and *III*) are so scarce, though, that it may be premature to make final conclusions of even the pattern of distribution, let alone dispersal. In principle, as the species coexists with (some of) the other immigrants both in freshwater and brackish environments, it might also have invaded the area simultaneously with them, along the same route. As the sampling in each lake was made in a very restricted area, the species may well have remained undetected where it actually was present. The absence (or

scarcity) of *M. relicta II* in most lakes may also be attributable to competetive exclusion or other ecological factors (cf. distribution in the Baltic). Furthermore, if the distributional pattern of *M. relicta II* in the earlier stages of the Baltic resembled the present one (absent in an isolated bay, concentrated in open areas), the species might not have been present in the peripheral areas to be isolated as lakes; the same may have concerned Lake Mälaren in more recent times. Of course, the negative evidence for Mälaren is not very strong, but in contrast to most other localities, the material examined from this lake still came from two different samples; moreover, Fürst & Nyman (1969) have earlier studied esterases in a sample of 200 *M. relicta* from Mälaren, and no deviations were reported, although the species should be easily separated on the basis of EST-1 at least.

It may be noted that according to present views, most if not all of the Finnish lakes studied have not been subject to any considerable saline influence during post-glacial times (Eronen 1974). This does not hold for the lakes in Sweden, which were flooded by brackish waters during the Yoldia stage at least. If postulating a marine post-Ancylus period immigration for *M. relicta II*, would it not seem likely that the species was present in the Kattegat area also in the Yoldia period, thus capable of entering and possibly also surviving in at least some western or central Swedish lakes to be isolated in those times? A more thorough screening of lakes in southern Scandinavia is clearly necessary to cast any light on the Holocene history of *M. relicta II*.

An empirical argument against a western immigration path has been the absence of the immigrant crustaceans in lakes of the western and northern Scandinavian coasts (e.g. Segerstråle 1957). The only exceptions are the SW Norwegian populations of *M. relicta* and *Pontoporeia affinis*, for which both eastern and western origins have been postulated, and the *M. relicta* of Lake Pulmankijärvi in the north. The latter has not been discussed in a biogeographical context. After the discovery of the two Baltic sibling species, this locality first seemed to have an interesting position with respect to elucidating their dispersal paths. The northern population could have been derived from a stock that also reached the Baltic along a marine route after travelling around the Scandinavian Peninsula. On the other hand, it might have come from the White Sea area along with the problematical populations of the Kola Peninsula, and originate from the same stock that contributed the eastern invaders of the Baltic (cf. Segerstråle 1957, 1982). However, as we have seen, the Pulmankijärvi population bears no late-glacial relationship to either of the Baltic sibling species.

Actually, the detection of the new species does not yet in any considerable way alter the known pattern of distribution, on which the existing reconstructions of dispersal pathways have been built. It is still probable that the immigration history of *M. relicta I*, at least, was similar to that of the other traditional glacial immigrants in the Baltic area. The restricted data available on *M. relicta II* and *III* just suggest that there are more patterns to be explained.

On the other hand, the immigration hypotheses have always been strongly based on concepts of Pleistocene geological history, the details of which are still subject to change and controversy (e.g. Kvasov 1979, Grosswald 1980, Velichko et al. 1983), thus necessitating reconsiderations also in the reconstructions of the zoogeographical history (Segerstråle 1982). While perhaps rendering some aspects of biological history more comprehensible, or supporting parts of existing dispersal hypotheses, changing views on the geological past also tend to confuse the understanding of other aspects of distribution. So, actually the details of the late-glacial history of even the continental European 'relicts' are still obscure, and the interrelationships of some more distant populations (e.g. those of Ireland and North America) have never been satisfactorily understood.

In the future, we hope to be able to elucidate the zoogeographical history of the glacial immigrants by studying the relative genetic affinities of conspecific populations from different geographical areas. The discovery of new species may increase the number of problems to be solved, but on the other hand it may also considerably increase the power of the genetic approach by potentially revealing hitherto unknown discontinuities that readily indicate which aspects of distribution should not be explained by the same dispersal events.

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