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Systematics and evolution of holarctic Pierinae (Lepidoptera). An enzyme electrophoretic approach

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Summary. Enzyme electrophoretic data show a remarkably high degree of genetic similarity within the European group of *napi* s.l. whereas genetic differences exist at several loci between the European and the North American taxa of *napi* s.l. It is concluded that the European taxa did not differentiate to the species level and form a phylogenetically young group. The North American taxa included in this study are specifically distinct from European *napi* and separated much earlier. Within these North American taxa *marginalis, oleracea* and *virginiensis* did undergo speciation. The data show a splitting of the genus *Pieris* into three species groups, each genetically differentiated to the same level. The splitting of *Pieris* into two genera, as suggested by earlier investigators, is not supported here.

Key words. Pieridae; Lepidoptera; systematics; evolution; enzyme electrophoresis; genetic differentiation.

Introduction

There are few groups of closely related taxa whose systematic relationships have given rise to as many discussions as the *napi*-group of the genus *Pieris* Schrank (*napi* s.l.). This group is distributed over the entire holarctic region and comprises a huge number of morphologically very similar taxa. A great variety of taxonomic methods have been used to clarify the situation with the result that the number of diverging views has steadily increased. There are two subgroups in the European region each containing several taxa: 1) lowland populations which are morphologically like *napi* and 2) populations in the mountains of central Europe and in Northern Scandinavia with a wing color like *bryoniae.* Despite the fact there are broad areas of contact between pale '*napi*' and dark '*bryoniae*'-like populations, in which interbreeding has been reported[8,33,34,42], some authors have regarded the two subgroups as specifically distinct[9,13,29] or as members of a superspecies[7,25,34,43,45]. Others[4,18,19,23] have regarded them as subspecies.

An important reason for these different opinions lies in the equivocal results of hybrid crosses[5,6,25,26]. In some crosses it was possible to maintain a *napi* × *bryoniae* stock up to the 14th generation[25] while in others it was not even possible to obtain an F_1. Remarkably high degrees of sterility have also been reported in crosses between '*napi*' populations from various places in Europe[25,27]. This has especially been true for crosses between *napi*-like populations from northern and southern Yugoslavia. As a consequence specific distinctiveness has been inferred for the southern Yugoslavian populations (*Pieris balcana* Lork.)[26].

For the interpretation of the systematic significance of the results of these hybrid crosses it would be necessary to undertake a more comprehensive survey, studying the degrees of interfertility between geographically close and distant populations covering the distribution area of several taxa in Europe.

The problems with the systematic status of the nearctic taxa of *napi* s.l. resemble those mentioned for the European taxa. In the North American literature these taxa (including *marginalis, castoria, mcdunnoughi* and *oleracea* compared in this study, table 1) are normally regarded as conspecific with *napi*[11,20,28] whereas at least in the newer European literature some authors have given them specific rank[9,46].

It is the aim of this investigation to provide a new and independent set of data for the discussion of the genetic relationships of the taxa in question by using enzyme electrophoretic techniques. Taxa of the *rapae-* and *brassicae*-species groups of *Pieris* and of the genus *Pontia* Fabricius have been included for several reasons: 1) To elaborate an internal standard for evaluating the significance of degrees of genetic differentiation. 2) To substantiate earlier observations[14] that genetic data do not support division of the genus *Pieris* into two genera, *Pieris* and *Artogeia* Verity[22,44]. 3) To reexamine the relationships between *Pieris* and *Pontia,* where earlier observations[14] have indicated a clear step of differentiation, contrary to the view of many North American authors[20].

Material and methods

Table 1 gives a list of the taxa, their origin, number of animals and samples investigated. Animals were usually stored at −35 °C until electrophoresis. Thoraxes were homogenized in 10 volumes of 0.1 M Tris-HCl buffer (pH 8.0) and centrifuged for 5 min at 18,000 × g. Supernatant fractions were used for vertical starch gel electrophoresis following standard procedures for electrophoretic separation and enzyme staining[14,35]. 22 loci were scored: adenylate kinase (loci AK-1 and AK-2), aldolase (ALD), arginine kinase (APK), fumarase (FUM), glutamate-oxaloacetate transaminase (GOT-1, GOT-2), glutamate-pyruvate transaminase (GPT), glyceraldehyde-phosphate dehydrogenase (GAPDH), α-glycerophosphate dehydrogenase (α-GPDH), hexokinase (HK), indophenol oxydase (IPO), isocitrate dehydrogenase (IDH-1, IDH-2), malate dehydrogenase (MDH-1, MDH-2), malic enzyme (ME-1, ME-2), phosphoglucomutase (PGM), 6-phospho-gluconate dehydrogenase (6-PGD), phosphoglucose isomerase (PGI) and pyruvate kinase (PK).

Genetic interpretation of electrophoretically detectable enzyme phenotypes is based on extensive breeding programs, analyzing the progeny of various phenotypes of each polymorphic enzyme[15]. There is strong evidence for sex-linked inheritance of the very weakly polymorphic 6-PGD in some species. The enzyme is, however, definitely autosomally inherited in the majority of taxa investigated. For calculation of allelic frequencies a possible sex linkage in some taxa has been neglected. The inheritance of 6-PGD is currently being investigated in detail.

Designation of alleles is based on mobility of the electromorphs and indicates the differences in mobility (mm) relative to the most frequent electromorph of each enzyme in *Pieris brassicae* (index 100).

Allele frequencies (table 3) will be given for pooled samples of each taxon of *napi* s.l. because of the enormous number of population samples investigated (table 1). In each population allele frequencies closely followed those reported for pooled samples (detailed documentation available from the authors upon request). The statistic $\bar{I}$[30] was used to calculate coefficients of genetic identity in pairwise comparisons of all taxa. This information served as a matrix for the construction of a dendrogram (fig. 1) by cluster analysis (UPGMA method, see[10]).

Results

The commonest alleles of each taxon at every locus investigated are listed in table 2, with the exception of four loci (AK-1, AK-2, ALD and GOT-2) where all taxa are monomorphic for the same allele. The loci are arranged in order of increasing interspecific variability which generally was found to be correlated with polymorphism. Allele frequencies observed at nine highly polymorphic loci are documented in table 3 for the taxa of the *napi*-group s.l. and the closely related *A. ergane*, *A. melete* and *A. virginiensis*.

Table 1. List of taxa (nomenclature: American taxa[23], European taxa[14,19,35,36], Asiatic taxa[15]), number and origin of animals studied

Taxa	Origin*	Number of samples	Number of animals
Pieris brassicae L.	D,F,IRL,CH	6	89
Pieris cheiranthi Hb.	E	1	17
Artogeia canidia Sp.	Taiwan	1	4
Artogeia r. rapae L.	CH,F,I,YU	9	143
Artogeia r. crucivora B.	Japan	2	7
Artogeia mannii M.	F,YU	5	43
Artogeia ergane G.	GR,YU	2	11
Artogeia melete Mén.	Japan	4	30
Artogeia n. napi L.	CH,F,I,YU	14	219
Artogeia n. meridionalis H.	F	2	22
Artogeia n. bryoniae Hb.	CH,A	7	123
Artogeia n. adalwinda Fruhst.	N,S	2	8
Artogeia n. thomsoni Warren	GB	5	112
Artogeia n. balcana Lorc.	YU	1	12
Artogeia n. marginalis Scud.	USA[48] Canada[48]	3	20
Artogeia n. mcdunnoughi Rem.	USA[48]	2	10
Artogeia n. castoria Reakirt	USA[48]	3	16
Artogeia n. oleracea Harris	USA[48]	1	4
Artogeia virginiensis Edw.	USA[48]	3	13
Pontia sp.1	E,F,M,Israel	11	60
Pontia sp.2	CH,GR,I,A, H,YU	9	53
Pontia sisymbrii Bois.	USA	2	9
Pontia occidentalis Reakirt	USA	8	27
Pontia callidice Hb.	CH	6	23
Pontia beckerii Edw.	USA	2	7

*Code: A, Austria; CH, Switzerland; D, Federal Republic of Germany; E, Spain; F, France; GB, Great Britain; GR, Greece; H, Hungary; I, Italy; IRL, Republic of Ireland; M, Morocco; N, Norway; S, Sweden; USA, United States of America; YU, Yugoslavia.

With respect to loci with a low degree of interspecific variation it is noteworthy that the commonest allele is usually the same for taxa of *Pieris* and *Artogeia* (table 2). In the genus *Pontia* however, variation is more pronounced (e.g. MDH-1, GAPDH, α-GPDH, ME-2). Thus, even a superficial examination of enzyme variation indicates that *Pontia* is well separated from *Pieris* and *Artogeia* at the enzyme level.

In *napi* s.l. genetic differences between European and North American taxa are evident. The European taxa share the same commonest allele at every enzyme locus (table 2), with the exception of the ME-2-locus in *adalwinda* (table 3). In contrast, the North American taxa differ from the European taxa and from each other at various loci (PK, PGI, IDH-2, IDH-1, HK, GOT-1, GPT, PGM). The very close affinities between the European *napi* taxa are underlined by a comparison of allelic frequencies at polymorphic loci (table 3), which are usually very similar in all European taxa even at loci where two alleles are observed in higher frequencies (ME-2, PGI, PGM). These small differences result in very high coefficients of genetic similarity (> 0.97). As might be expected from the differences in patterns of commonest alleles, there is a remarkable heterogeneity in allelic frequencies between the North American taxa. Differences are most pronounced for *oleracea,* which diverges from *marginalis, mcdunnoughi* and *castoria* at the IDH-1-, HK-, GPT- and PGM-locus. The distribution of alleles and their frequencies are more similar between *marginalis, mcdunnoughi* and *castoria,* with the exception of PK-, PGI-, IDH-2- and IDH-1-locus.

For those taxa where several population samples (number of individuals > 10) were available, coefficients of similarity were calculated for each taxon in pairwise comparisons of the population samples, which resulted in Nei coefficients $(\bar{I}) > 0.97$. These comparisons are of particular interest with respect to the European taxa of the *napi*-group. In this group the number of the population samples available was 14 for *n. napi,* 7 for *n. bryoniae* and 5 for *n. thomsoni* (table 1). Mean values of similarity of population comparisons were 0.99 ± 0.01 for all three taxa.

When comparing population samples of these taxa the range of coefficients of similarities found between samples of different taxa did not exceed the range in comparisons within a taxon. A genetic differentiation between these three taxa is therefore not evident.

Discussion

Some problems in many systematic studies include the number of individuals, the size of the sampling area compared with the overall area of distribution of a taxon, and the number of characters compared. In this study these problems affect the estimate of the genetic relationships in all taxa in which only a few individuals have been investigated, especially *canidia* and *napi oleracea.* It has been shown[31] that confidence in the coefficients of genetic similaritiy is very high even when the

Table 2. Most common electromorphs (alleles) at all loci for all taxa investigated

Locus Taxon	MDH-1	MDH-2	APK	GADPH	α-GPDH	ME-2	FUM	ME-1	PK	PGI	IDH-2	IPO	IDH-1	HK	6-PGD	GOT-1	GPT	PGM
brassicae	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
cheiranthi	100	100	100	100	—	100	100	100	100	100	100	110	100	100	93	100	100	95
canidia	100	90	100	100	100	100	98	98	103	100	108	102	95	100	115	88	100	100
rapae	100	90	100	100	100	100	98	98	106	107	110	102	95	100	107	88	100	100
crucivora	100	90	100	100	100	100	98	98	106	107	110	102	95	100	107	88	100	100
mannii	100	90	100	100	100	100	98	98	106	100	100	102	95	100	100	88	100	100
ergane	100	90	100	100	100	100	90	103	103	100	108	102	98	109	100	100	118	107
melete	100	90	100	100	100	100	90	103	100	100	108	102	95	115	100	100	115	95
napi	100	90	100	100	100	100	90	103	100	100	100	102	98	110	100	90	115	102
meridionalis	100	90	100	100	100	100	90	103	100	100	100	102	98	110	100	90	115	102
bryoniae	100	90	100	100	100	100	90	103	100	100	100	102	98	110	100	90	115	102
adalwinda	100	90	100	100	100	100	90	103	100	100	100	102	98	110	100	90	115	102
thomsoni	100	90	100	100	100	100	90	103	100	100	100	102	98	110	100	90	115	102
balcana	100	90	100	100	100	100	90	103	100	100	100	102	98	110	100	90	115	102
marginalis	100	90	100	100	100	100	90	103	100	100	108	102	93	110	100	100	125	109
mcdunnoughi	100	90	100	100	100	100	90	103	100	110	108	102	93	110	100	100	125	109
castoria	100	90	100	100	100	100	90	103	106	110	100	102	93	110	100	100	125	109
oleracea	100	90	100	100	100	100	90	103	106	95	108	102	95	115	100	100	115	104
virginiensis	100	90	100	100	100	100	90	103	106	110	108	102	98	115	100	100	125	109
Pontia sp.1	100	90	94	100	103	102	99	106	103	107	97	96	84	108	95	93	130	110
Pontia sp.2	100	90	94	100	103	102	99	106	103	107	97	96	84	108	100	85	135	105
sisymbrii	100	100	94	110	103	104	99	100	103	107	97	94	91	108	100	83	91	108
occidentalis	89	90	94	100	103	102	99	103	106	107	97	96	91	96	85	120	86	103
callidice	89	90	94	100	103	102	99	103	106	107	97	96	91	96	85	125	86	108
beckerii	100	90	94	105	90	102	99	106	117	107	97	96	91	108	91	87	86	102

Table 3. Allelic frequencies at polymorphic loci for the taxa of the *napi* s.l. group

	MDH-1			ME-2		PGI							IDH-2		IDH-1							GOT-1			GPT		PGM								
	110	100	89	104	100	120	115	110	107	100	91	78	108	100	104	98	95	93	91	89	83	110	100	90	125	115	110	109	107	104	102	95	91	90	85
A.n.napi	0.13	0.84	0.03	0.45	0.55		0.01	0.06		0.73	0.19	0.01	0.13	0.87	0.01	0.95		0.03	0.02			0.03		0.96	0.14	0.85	0.01		0.01			0.70	0.17		0.02
A.n.meridionalis	0.05	0.91	0.05	0.50	0.50			0.07		0.55	0.34	0.05	0.16	0.82	0.02	0.82		0.05	0.09	0.02			0.02	0.98	0.07	0.91	0.02		0.05			0.66	0.25		0.05
A.n.bryoniae	0.09	0.89	0.02	0.44	0.56			0.02		0.87	0.10		0.15	0.85	0.01	0.95		0.04					0.02	0.98	0.21	0.79		0.03			0.78	0.18	0.02		
A.n.adalwinda	0.19	0.81		0.83	0.17			0.06		0.81	0.13		0.19	0.81	0.31	0.63		0.06						1.00	0.06	0.88	0.06					0.56	0.19	0.13	0.06
A.n.thomsoni	0.10	0.86	0.04	0.45	0.55		0.01		0.05	0.76	0.17		0.06	0.92		0.96	0.02		0.01				0.02	0.98	0.24	0.76	0.01		0.16			0.65	0.17		0.02
A.n.balcana	0.25	0.71	0.04	0.25	0.75		0.04			0.67	0.25	0.04	0.29	0.71		1.00								1.00	0.33	0.58	0.08		0.21			0.71	0.04	0.04	
A.n.marginalis	0.03	0.95	0.03		0.98	0.10		0.18		0.73			0.62	0.38		0.03	0.03	0.58		0.38		0.05	0.90	0.05	0.90	0.03		0.73	0.03	0.25					
A.n.mcdunnoughi	0.05	0.95			1.00	0.15		0.50		0.25	0.10		1.00			0.05		0.90		0.05			1.00		0.95	0.05		0.65	0.15	0.20					
A.n.castoria		1.00			1.00	0.03		0.69		0.28			0.09	0.91				0.91			0.09		0.97	0.03	1.00			0.94		0.06					
A.n.oleracea	0.25	0.75			1.00			0.50					1.00			0.63					0.38		1.00			1.00		0.17		0.83					
A.virginiensis	0.04	0.96			1.00	0.04		0.77		0.19			0.96	0.04			0.96		0.04				0.73	0.27	1.00			0.92	0.04	0.04					
A.ergane	0.09	0.91			1.00			0.05	0.05	0.68	0.23		1.00			0.96				0.05		0.09	0.86			0.05			0.55						
A.melete	0.12	0.85	0.03	0.10	0.90				0.02	0.90	0.08		0.96	0.04		0.02	0.97					0.07	0.87	0.02	0.08	0.92					0.02	0.48		0.27	0.18

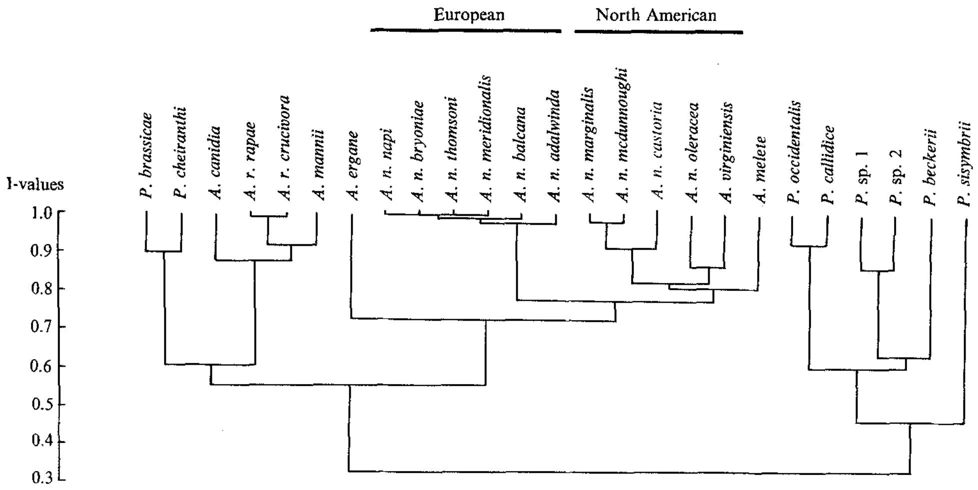

Figure 1. Dendogram of the 25 taxa (nomenclature as in table 1) investigated.

number of individuals used for estimating genetic distance is very small (one or two individuals) when a large number of loci are studied (50 or more) or the genetic distance is large and the average heterozygosity of the two taxa compared is low. Obviously, these assumptions are not fulfilled in these two taxa. Nevertheless, the data allow at least a rough estimate of the genetic relationships, because even if only four individuals (eight alleles) were compared and the observed allelic frequencies at two loci were to diverge from the 'true' distribution as much as 86% at each locus, the calculated $\bar{I}$-value would differ from the 'true' one by 0.05. The chance that this will happen is less than 1‰ if we assume that the taxon under consideration shows the same pattern of allelic distribution as has been recorded for all pierid taxa, of which a number of population samples have been compared from a significant part of their distribution area; low intrasubspecific variation, as has been reported here for *n. napi*, *napi bryoniae* and *napi thomsoni* ($\bar{I} = 0.99 \pm 0.01$) (and own unpublished data, manuscript in preparation).

The systematic value of electrophoretic data has been discussed by various investigators[2,3,36]. For systematic purposes it is important to demonstrate whether there is reproductive isolation between two populations or taxa. To do so directly is not possible either with 'classical' morphological data nor with enzyme electrophoretic data. But because the expression of the genes is much more direct in enzymes than in morphological features one can directly and easily compare the genetic composition at a set of loci. If it is possible to show that at an enzyme locus two different alleles are fixed in two taxa, this is a strong argument for the assumption that the two taxa do not share a common gene pool and that they are reproductively isolated[16,17]. On the other hand, if one finds in two taxa the same alleles in the same frequencies at all loci investigated this may (but does not necessarily) indicate the existence of gene flow and that the taxa are still in reproductive contact.

Bearing these qualitative criteria in mind, two different situations can be distinguished in comparisons of the taxa investigated in this study: 1) groups of taxa that show at all loci the same alleles in almost identical frequencies *(rapae* and *crucivora; napi, bryoniae, thomsoni, meridionalis, balcana* and *adalwinda; marginalis* and *mcdunnoughi)*. These are the taxa which cluster in the dendrogram (fig. 1) at a level of $\bar{I} > 0.97$. 2) one taxon differs from another in having at least at one locus a common allele that can not be found in the other taxon or only at very low frequencies (comparisons between all other taxa).

With respect to the interpretation of systematic relationships of taxa in category 1 one might imagine they were reproductively isolated and genetically differentiated, but such a differentiation was not detected from the sample of 22 loci studied (nine of which exhibited polymorphism). However, if gene flow were interrupted between these taxa there would be a good chance of major differences arising in allelic frequencies

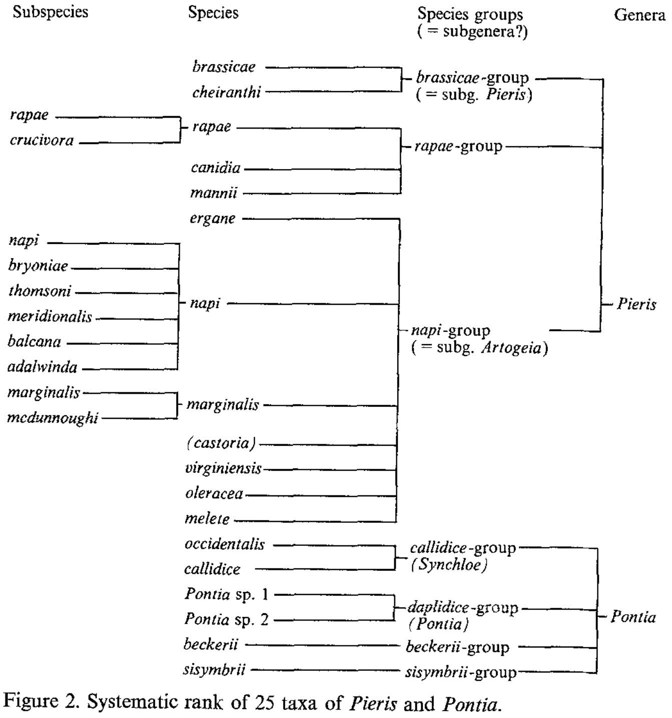

Figure 2. Systematic rank of 25 taxa of *Pieris* and *Pontia*.

at one of the nine polymorphic loci. The fact that this is not observed is a strong argument for considering the taxa as conspecific. It is of note that there are several taxa in the material investigated which are generally regarded as subspecies *(rapae* and *crucivora; napi, thomsoni* and *meridionalis).* In all these inter-subspecific comparisons these taxa show only small differences in allelic frequencies. On the other hand, in comparisons of taxa that are generally regarded as specifically distinct, but closely related *(canidia, rapae* and *mannii; melete, napi, ergane, virginiensis)* the conditions described in category 2 were fulfilled.

The North American *napi*-group taxa form a cluster with *A. virginiensis* and with the Asian *A. melete* (fig. 1). In this cluster, the biochemical-genetic data suggest subspecific rank for *marginalis* and *mcdunnoughi* and specific rank for *oleracea* and *virginiensis.* The data presented here seem to favor the opinion that *castoria* is also separated specifically from the other taxa included in this study. But this needs further examination especially in Northern California where complex transition zones may exist (Shapiro, personal communication).

Enzyme electrophoretic data show a small degree of differentiation within European *napi*-group taxa but a clear step of differentiation between European and North American taxa of that group (fig. 1). A similarly substantial level of differentiation has been found in interspecific comparisons with the *napi*-group s.l. taxa and the Asian *A. melete* and European *A. ergane.* Thus the biochemical data clearly indicate that the application of the super- or semispecies concept[7, 24, 43] is not necessary in *napi* s.l. Interspecific comparisons between other taxa support this interpretation; for example there are closer biochemical affinities in the related *rapae* species group (*rapae, mannii* and *canidia,* see fig. 1) than within the *napi*-group s.l.

As is evident from the dendrogram (fig. 1), there are three clusters in *Pieris* s.l. which comprise the *brassicae-*, *rapae-* and *napi*-species groups, respectively. The *(Artogeia) rapae*-species group, however, shows slightly closer relationships to the *(Pieris) brassicae*-species group and not to the *(Artogeia) napi*-species group as would be expected on the basis of the proposed generic classification[22, 44]. Therefore it is suggested that the use of *Artogeia* as a generic name be discontinued. It could be useful to underline the splitting of *Pieris* into three species groups by giving these three groups subgenus rank. To do so on the basis of enzyme electrophoretic data is not possible at present, for it would be necessary to investigate a greater number of taxa.

A further step of divergence can be observed between the *Pieris* and *Pontia* taxa, which are regarded as congeneric by some authors[20]. The taxa have diverged to the same extent as has been reported for comparisons between genera in *Pieridae*[14]. The biochemical data therefore strongly support the separation of *Pieris* and *Pontia* as different genera.

On the basis of enzyme data specific rank can also be assigned to the *Pontia* taxa *occidentalis* and *callidice* (fig. 1). This is consistent with breeding experiments[37, 38] which have shown reproductive barriers between these two taxa.

Within *Pontia* high levels of differentiation have been found between some of the taxa (fig. 1): these are of the same order as those found between the species-groups (subgenera?) in *Pieris.* European *callidice* has been ranked by some authors in a genus *Synchloe*[13, 40] or in a subgenus (*Synchloe*[21], *Parapieris*[4]). North American *sisymbrii* and *beckerii* have also been placed in a different genus (*Pontieuchloia*[40]) or subgenus (*Synchloe*[21], *Pontieuchloia*[4]). The high levels of genetic differentiation are therefore consistent with the morphological data.

The taxonomic and systematic interpretation of the enzyme electrophoretic data is summarized in figure 2 where only the hierarchical ranks of subspecies, species, species groups (subgenera?) and genera are used.

Enzyme electrophoretic data have uses apart from taxonomy. The degree of genetic divergence seems to depend on the time that has elapsed since the interruption of gene flow between groups of populations[3, 12, 32, 39, 41, 47]. The main evolutionary trends in the genera *Pieris* and *Pontia* (fig. 1) can then be summarized as follows: In the genus *Pieris* the three species groups (fig. 2) evolved at approximately the same time. The radiation of the species within the *brassicae-* and *rapae*-species groups took place at approximately the same time, but this event must have occurred much later than the separation of *ergane, napi, melete* and the North American taxa of the *napi*-species group. Within the *napi*-species group it is interesting to note that the biochemical-genetic data suggest that the separation of the European and North American groups occurred a relatively long time ago. It has been assumed that the radiation within the European *napi*-group is correlated with the last glaciations[43], an idea that is consistent with the very small differences reported in this study. In the genus *Euchloe* the same high degree of genetic similarity has been found between the disjunct populations of *E. simplonia* from the Alps and the Pyrenees that have probably been separated since the end of the last glacial period[16]. Similar high $\bar{I}$-values have been found for North American *marginalis* and *mcdunnoughi* whereas the $\bar{I}$-values between the other North American taxa are lower. This observation is a strong argument to postulate that they were separated much earlier from the European *napi*-group and have undergone differentiation since then. In the genus *Pontia* the separation into the four species groups (fig. 2) seems to be as old as the splitting of the three species groups in *Pieris* (fig. 1). The separation of the gene pools of *occidentalis* and *callidice* is of more recent origin.

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48 The origin of the North American *Pieris* samples may be critical. We give here a list of the localities for these taxa. A complete list with the sampling sites for all taxa is available from the authors upon request: *P. marginalis:* Cascade Head, Lincoln Co, Oregon; Clearwater Valley, Idaho; Revelstoke, B.C., *P. marginalis mcdunnoughi:* Gallatine Mtn, Montana; Goodrich Creak, Adams Co, Idaho. *P. castoria:* Gates Canyon, Solano Co; Lang Crossing, Nevada Co; American River Canyon, Placer Co, all California. *P. oleracea:* Vivian Forest, York Co, Ontario. *P. virginiensis:* Creekside, Indiana Co, Pennsylvania (2); Halton Co. Forest, Ontario.

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