Electrophoretic Confirmation of the Species Status of Pontia protodice and P. occidentalis (Pieridae)

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Abstract. Electrophoretic study of sympatric and allopatric populations of the taxa Pontia protodice and P. occidentalis demonstrates unequivocally that they represent closely related but independent gene pools. Each is genetically very homogeneous over its geographic range, strongly suggesting high levels of migration, colonization, and/or gene flow. P. protodice is less like European P. callidice than is Californian occidentalis, suggesting a possible phylogeny which agrees with previous inferences from morphology and biogeography.

Introduction

The taxa Pontia (or Pieris or Synchloe) protodice Bdv. & LeC. and P. occidentalis Reak. have posed an ongoing problem for Lepidopterists; though Chang (1963) demonstrated morphological differences between them and Shapiro (1976) summarized the by then copious biological and distributional information on hand—all of which tended to support their status as separate species—many workers, including some professionals, have remained unconvinced and profess to be unable to classify many specimens to one species or the other. The present study was undertaken in the hope of further clarifying their status by comparing population samples of both from areas of sympatry and allopatry, using electrophoresis to quantify genomic similarities and differences. An ancillary objective was to test the prediction that both species would show very little inter-populational differentiation, due to their apparent pattern of colonization and their epigamic behavior.

Materials and Methods

The sources of our samples are listed in Table 1. All animals were transported alive and immediately stored at -70°C until electrophoresis. Only 1984 and 1985 catches were used. All animals were determined as protodice or occidentalis by AMS, using wing phenotype, and all wings were saved for post-electrophoresis verification. Only one possibly ambiguous specimen was used in the study. The head and thorax of each
butterfly were homogenized in 4 volumes of Tris-HCl buffer (0.05 M, pH 8.0). We used horizontal starch gel electrophoresis, following slightly modified standard procedures (Ayala et al., 1972; Geiger, 1981). Twenty-three enzymes were scored: acid phosphatase (locus ACPH), adenylate kinase (AK-1, 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 oxidase (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).

Analysis of the progeny of parents with different phenotypes in *Pieris brassicae* L. (Geiger, 1982) was the basis for interpreting zymograms of polymorphic loci. No deviation from the pattern observed in *P. brassicae* has been found in any individual investigated in this study. The distributions of alleles are also in good accord with Hardy-Weinberg expectations.

The most frequent allele ("common allele") in *P. brassicae* was used as a standard. This allele is designated with the index 100. Electromorphs with different mobilities are designated in relation to this standard; an allele
Table 1. Localities for samples used in this study, with notes on sympatry.

California: Lassen County: 2.5 km S Adin, 1500 m, 11.viii.1985 (n=24)(AD), occidentalis abundant, protodice unrecorded but possible infrequent immigrant. Siskiyou County: Ball Mountain, 2175 m, 10.viii.1985 (n=28)(BM), occidentalis only, very abundant. Sierra County: Sierra Valley, 4 km NE Sierraville, 1500 m, 25-30.vii.1985 (nprot. =55, nocc. =46)(SV), both abundant and permanently sympatric. Placer and Nevada Counties: Donner Pass, 2100 m, 15.viii.1985 (nprot. =6, nocc. =4)(DP), occidentalis common resident, protodice frequent immigrant, overwintering once in 14 yr. Nevada County: Castle Peak, 2750 m, 6.vii.1985 (n=26)(CP), occidentalis only (14 yrs. of observation). Alpine County: Leviathan Peak, 2800 m, 25.vii.1984 (nprot. =2, nocc. =17)(LP), occidentalis common resident, protodice immigrant. Mono County: nr. Mono Lake, 1800 m, 2.vii.1985 (nprot. =17, nocc. =2)(ML), protodice common, occidentalis infrequent (but commoner at higher elevations). Kern County: Lake Isabella, 780 m, 16.viii.1985 (n=19)(LI), protodice only. San Bernardino County: Route 38 N Onyx Summit, elevations not available, 15.vi.1985 (n=3)(OS), protodice only.

Nevada: Churchill County: vic. Fallon, 1250 m, 13.viii.1984 (n=30), protodice abundant, occidentalis very rare (none taken).

Florida: Broward County: vic. Davie, metropolitan Miami, 25.iv.1984 (n=9), protodice only.

Mexico: Distrito Federal: Xochimilco-San Gregorio, 27.vi-2.vii.1984 (n=12), protodice only.

105, then, codes for an enzyme that migrates 5 mm faster than the P. brassicae variant.

The statistic $I$ (Nei, 1972) was used to estimate the genetic similarity between the samples over all loci. The calculated $I$-values for the pooled samples of the two North American taxa plus P. callidice Hübner have been used to construct a dendrogram (Fig. 2) by cluster analysis (UPGMA method, Ferguson, 1980). The $I$-values for the pooled samples are based on only 22 loci (without ACPH) to make the data comparable to an earlier study (Geiger and Scholl, 1985).

Results

At 16 of the 23 loci compared, protodice and occidentalis show only very low polymorphism ($f_{\text{common allele}} \geq 0.98$), and share the same common allele (ALD, AK-1, AK-2, APK, FUM, GADPH, GOT-2, $\alpha$-GPDH, IDH-1, IDH-2, IPO, MDH-2, ME-1, ME-2, 6-PGD, PK). At four other loci (GOT-
Fig. 2. Dendrogram illustrating phenetic relationship, based on Nei's I-statistic (see text.).

1, MDH-1, PGM, PGI) the two taxa have the same common allele in all samples, but are polymorphic (Table 2). For those samples with $n \geq 10$, the frequencies of all alleles are remarkably similar and show no statistically significant interpopulational differences. The situation is different for the three remaining loci (ACPH, HK, GPT) (Table 3). There are two alleles at the GPT locus which are found in both taxa but at very different frequencies: the common allele in *protodice* (GPT 86) is found at very high frequency ($f > 0.96$) in all samples of that taxon but at much lower frequencies ($f < 0.25$) in the *occidentalis* samples. The allele 97 is the common GPT allele in *occidentalis* ($f > 0.75$) and is very rarely recorded in *protodice* ($f < 0.04$). The genetic differences between the taxa are even more pronounced at the HK locus, where only the allele 93 is found in *protodice* ($f = 1.00$); this allele occurs in *occidentalis* only at frequencies $< 0.02$. At the ACPH locus each taxon is monomorphic for a different allele.

It is important to underscore the fact that there were no heterozygous individuals for the ACPH locus at those localities where the taxa are frequently to permanently sympatric (Donner, Sierra Valley—see Table 1). The one possibly ambiguous individual, a female from Sierra Valley, was electrophoretically "pure *protodice,*" which taxon it most closely resembled in wing phenotype. The frequencies of heterozygotes for HK and GPT did not vary significantly between sympatric and allopatric samples.

The very similar allelic frequencies at all loci among population samples result in very high $I$-values for the within-taxon comparisons ($I_{occidentalis} = 1.00$, $I_{protodice} \geq 0.99$) (Table 4). When the two taxa are compared the $I$-value is (as expected) lower, $x_{[1]} = 0.88 \pm 0.01$.

Because HJG had already studied European *P. callidice*, and there has been considerable speculation concerning its relationship to the American taxa (Higgins and Riley, 1970; Shapiro, 1980), we compared it to our results using the 22 loci (omitting ACPH) studied for all three taxa.
Table 2. Allelic frequencies at polymorphic loci.

| Loci         | GOT-1 | MDH-1 | PCM  | GCI  |
|--------------|-------|-------|------|------|
| n animals    | 110   | 115   | 120  | 125  |
| Sierra Valley| .18   | .81   | .02  | .97  |
| Lake Isabella| .05   | .95   | .98  | .16  |
| Mono Lake    | .12   | .83   | .03  | .88  |
| Donner Pass  | .17   | .67   | .17  | .92  |
| Fallon       | .17   | .82   | .02  | 1.0  |
| Florida      | .28   | .72   | .95  | .05  |
| Mexico       | .13   | .87   | .96  | .04  |
| all samples  | 55    | 153   | .83  | .95  |

Table 3. Allelic frequencies at loci with high variability between the taxa.

| Loci         | ACPH | GPT | HK |
|--------------|------|-----|----|
| n animals    | 88   | 95  | 86 | 97 |
| Sierra Valley| 55   | 1.0 | .99 | .01 |
| Lake Isabella| 19   | 1.0 | 1.0 | 1.0 |
| Mono Lake    | 17   | 1.0 | .97 | .03 |
| Donner Pass  | 6    | 1.0 | 1.0 | 1.0 |
| Fallon       | 30   | 1.0 | 1.0 | 1.0 |
| Florida      | 9    | 1.0 | 1.0 | 1.0 |
| Mexico       | 12   | 1.0 | .96 | .04 |
| all samples  | 153  | 1.0 | .99 | .01 |

| Loci         | ACPH | GPT | HK |
|--------------|------|-----|----|
| Ball Mtn     | 28   | 1.0 | .23 | .77 |
| Adin         | 24   | 1.0 | .23 | .77 |
| Sierra Valley| 46   | 1.0 | .13 | .87 |
| Castle Peak  | 26   | 1.0 | .25 | .75 |
| Leviathan    | 17   | 1.0 | .06 | .94 |
| all samples  | 147  | 1.0 | .18 | .82 |
Occidentalis and callidice cluster at a slightly higher level than protodice. The relationships with other species of the genus Pontia remain unchanged.

Discussion

We had predicted a high level of genetic similarity over large distances in these species because both are highly vagile, colonizing or “weedy” species and because P. occidentalis is a facultative “hilltopper,” a mating strategy which would tend to promote gene flow and prevent local ecotypic differentiation. (For discussion of the population dynamics of P. protodice, see Shapiro, 1979; for dispersal ability of P. protodice, Shapiro, 1982 and of P. occidentalis, Shapiro, 1977; an explicit prediction was made in Shapiro, 1984, p. 181). We were nonetheless surprised at the extreme homogeneity of protodice over a continent-wide range (Florida, Mexico, Nevada, California). We know that these populations are not so homogeneous for such adaptive traits as the photoperiodic thresholds for induction of pupal diapause, the programming and control of diapause, hostplant adaptation and disease resistance (AMS, unpublished data). Mexican protodice also lay smaller eggs than other populations, even under standardized rearing conditions and on a standard diet (Shapiro, in press). All protodice populations tested to date have been fully reproductively compatible with one another, even in such wide crosses as New York

Table 4. I-values for the comparison of all populations samples (n >6) based on the data of 23 enzyme loci.

| protodice      | occidentalis |
|----------------|--------------|
| Lake Isabella | Ball Mtn     |
| Mono Lake     | Adin         |
| Donner Pass   | Sierra Valley|
| Fallon        | Castle Peak  |
| Florida       | Leviathan    |

| Sierra Valley | 1.00 1.00 1.00 1.00 1.00 1.00 | .89 .89 .88 .89 .87 |
| Lake Isabella | 1.00 1.00 1.00 1.00 1.00       | .89 .88 .88 .89 .87 |
| Mono Lake     | 1.00 1.00 1.00 1.00 1.00       | .89 .89 .88 .89 .87 |
| Donner Pass   | 1.00 .99 1.00                   | .88 .89 .88 .88 .87 |
| Fallon        | 1.00 1.00                     | .89 .89 .88 .89 .87 |
| Florida       | 1.00                      | .89 .89 .88 .88 .87 |
| Mexico        |                          | .89 .89 .88 .89 .87 |
| Ball Mtn      | 1.00 1.00 1.00 1.00 1.00       |               |
| Adin          | 1.00 1.00 1.00               |               |
| Sierra Valley | 1.00 1.00                    |               |
| Castle Peak   | 1.00                        | 1.00           |
X California or Texas, or Mexico X California; but the control of diapause is routinely disrupted in such wide crosses, usually resulting in failure to diapause or very rapid spontaneous termination, and much less often in extended, lethal diapause. We have less extensive experience crossing occidentalis populations but have found complete compatibility among California and Colorado ones and between California and the Alaskan subspecies nelsoni Edwards (Shapiro, 1975) which is highly incompatible with European callidice (Shapiro, 1980). Diapause is largely unstudied in these cases.

Vawter and Brussard (1984) found similar uniformity in the weedy, introduced species Pieris rapae L. in eastern North America, but more genetic diversity in the west. Populations of P. rapae in the west are discontinuous, separated (except in the Central Valley of California) by broad expanses of inhospitable terrain. The ability of P. rapae, as an obligatorily multivoltine species, to accommodate to western climates by altitudinal migration seems very limited in comparison to P. protodice; indeed, rapae is largely confined to local “mesic” pockets created by irrigation within arid or semiarid regions, while protodice is able to colonize throughout. This is most dramatically illustrated in central Mexico: protodice, a native species, is quite generally distributed, but the introduced rapae is ecologically “trapped” in the floating gardens of Xochimilco, near Mexico City, where continuous breeding is possible. It is hardly surprising that the homogenizing effects of gene flow are more evident in P. protodice than in P. rapae.

Vawter and Brussard argue that gene flow should be countered in colonizing or fugitive species by genetic drift and founder effect, which would tend to cause stochastic differences among populations. We are examining the genetic structure of truly ephemeral populations of P. protodice (presumably resulting from colonizations by single females) in the hope of addressing this question.

The clear genetic differences between protodice and occidentalis at three loci are in sharp contrast to the low variation within the taxa. The most important samples are those from Sierra Valley, where both taxa are very abundant and apparently in stable coexistence (over 10 years, AMS observations) and where occasional (<3%) ambiguous phenotypes are encountered. There is absolutely no evidence for gene flow in the sympatric Sierra Valley samples; the lack of ACPH heterozygotes shows that there were no F₁ hybrids in our collections. There may indeed be occasional, very rare spontaneous hybridization (AMS has collected one mixed pair in copula in Donner Pass), but the electrophoretic data provide clear confirmation that protodice and occidentalis represent separate gene pools, corresponding to biological species. There is no evidence of introgression (that is, the two taxa are not more similar genetically in sympatry than in allopatry).

It is exceedingly difficult to hybridize these two taxa spontaneously, and
pairings can normally only be secured with a pre-excited male and a substituted *teneral* allospecific female. Hand-pairings are easily achieved, but to date the level of developmental incompatibility has been high, resulting in dwarfing, high mortality, malformation, deficiency of the heterogametic sex (female), and hybrid sterility. Further information on experimental hybridization will be published elsewhere; it suffices to note that it is fully in accord with the electrophoretic results.

Genetic differences within the group of three taxa (*protodice, occidentalis, callidice*) are relatively low but within the range previously reported for closely-related species in Pieridae (Geiger, 1981; Geiger and Scholl, 1985). This observation can be interpreted as evidence for the recency of speciation in that group. Shapiro (1980) interpreted the group as derived by fragmentation of the range of a widespread circumglacial steppe-tundra entity more or less resembling the climatic adaptation of some contemporary *occidentalis* populations. The phenotypic characteristics of *protodice* are clearly derivative reductions from the full *occidentalis* pattern, which in the western United States is polyphenic and shows some reduction from the *nelsoni-callidice* pattern. Larval and pupal characters vary concordantly (Shapiro, unpublished data). The dendrogram thus further supports the proposed phylogeny, which would derive *occidentalis* from circumpolar *proto-callidice* and *protodice* in turn from *occidentalis*, without specifying time scales. Certain central Asian taxa assigned as subspecies to *callidice* (*orientalis* Alph., *kalora* Moore, etc.) are extremely close phenotypically to western North American *occidentalis*. This may represent parallel evolution in similar climates—but then again, it may not. Nominate *callidice* from the Alps and Pyrenees seems to represent the extreme end of a long cline, physiologically as well as geographically.

We note in closing that the extreme genetic homogeneity shown by *protodice* and *occidentalis* over a large range suggests the reality of a “general purpose genotype” associated with weediness and physiological adaptability (Baker, 1965) and the utility of these common animals as vehicles to get a closer look at its structure.

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