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ELECTROPHORETIC COMPARISONS OF VICARIANT *VANESSA*: GENETIC DIFFERENTIATION BETWEEN *V. ANNABELLA* AND *V. CARYE* (NYMPHALIDAE) SINCE THE GREAT AMERICAN INTERCHANGE

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ABSTRACT. *Vanessa carye* and *V. annabella* are very similar species found in South America and North + Central America, respectively; they probably differentiated in the three million years since the Great American Interchange. Electrophoretically they are differentiated at a level typical of animal morphospecies (Nei's I = 0.855, D = 0.157) and are much more unlike than small samples of *V. cardui* from California vs. France. Using the Sarich method of estimating time of divergence, we date their speciation at roughly 2.97 million years ago, suggesting that *Vanessa* was an early crosser of the Panama land bridge. Our results support continued recognition of *V. carye* and *V. annabella* at the species, rather than the subspecies, level.

Additional key words: systematics, biogeography.

Since the emergence of protein electrophoresis as a technique in population genetics, it has been applied widely in systematics as well (Burns 1975, Ayala 1983). Several attempts have been made to compare levels of electrophoretic differentiation to conventional (morphologically-based) taxonomic judgment (Avise 1974, Ayala 1983, Ayala & Powell 1972, Ayala et al. 1974, Nevo et al. 1974, Mickevich & Johnson 1976, Thorpe 1982). Burns and Johnson (1967) first suggested that enzyme variation might offer a powerful tool for recognizing sibling species; Webster and Burns (1973) demonstrated its value in a pioneering study with lizards. Despite the widespread use of electrophoresis in systematic investigations of other taxonomic groups, it has seldom been brought to bear on butterflies (Geiger & Scholl 1985). The present study attempts to resolve the status of two putative vicariant mor-

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phospecies of the genus *Vanessa* Fabricius (Nymphalidae) by electrophoretic means, and interprets the data in historical-biogeographical terms to estimate the antiquity of their speciation event.

Hamadryas carye was described by Hübner in 1812 with no specific type locality. The name was subsequently applied to both North and South American, superficially similar populations later placed in the genus *Vanessa*. As early as 1951, W. D. Field had noted color and pattern differences between North and South American specimens, which he communicated personally to his Chilean collaborator J. Herrera. Herrera et al. (1958) then asserted that "after studying the genitalia of the examples which we possess from the United States (Oregon and California), Mexico, Argentina and Chile we are able to affirm that we are dealing with two quite different species." Once Field was able to establish from Hübner's figure that his (lost) type must have been South American, it was now possible to fix that usage; Field (1971) named the (newly-nameless) North American entity *Cynthia annabella*. Although his generic judgment has not been generally accepted, the specific epithet *annabella* continues in use for material from Central America northward.

Vanessa carye, sens. str., and *V. annabella* have sufficient phenotypic differences (in both habitus and genitalia) that if they co-occurred without intergrading there would be no hesitation in calling them different, though very closely related, species. However, they are apparently completely allopatric; *carye* ranges from southern Patagonia to Colombia, *annabella* from British Columbia to Guatemala. Neither species is recorded from montane or lowland Costa Rica (DeVries 1986). Such allopatric sister-species were called "vicars" by Udvardy (1969) and are commonly known as "vicariants" or "vicariant species" in the literature; they are often considered to be relatively recently-differentiated. In the absence of genetic data, and sometimes in the *presence* of such data, taxonomists' judgments as to how to rank such entities are often controversial. Thus, the suggestion by Higgins and Riley (1970) that several Palearctic-Nearctic pairs of pierid taxa were conspecific has remained unresolved despite laboratory hybridizations and electrophoretic studies (Shapiro 1980, Shapiro 1983, Shapiro & Geiger 1986 for *Pontia*, in which compatibility studies were done between populations far-removed from one another on the alleged Holarctic cline). In *Vanessa carye* and *annabella* there is near-unanimity in usage; doubts as to the validity of a species-level distinction have remained largely unpublished, appearing only in one major work (Scott 1986:283–284 treats them as subspecies). Such doubts are sure, however, to be exacerbated by the recent demonstration by Herrera (1987) of wing-pattern overlap between the taxa, and his forthcoming publication of laboratory

hybridization data (Herrera pers. comm.). Although we had only limited local samples available, we considered it worthwhile to attempt to assess the level of electrophoretic differentiation between the taxa. Since there are no published taxic comparisons within the Nymphalini, we attempted to place the data in context by comparing these two entities to members of different species-groups in *Vanessa,* to European vs. American *V. cardui* L., and to a few other nymphalines to which we had access at the time the study was done. Once in hand, the data permit a very crude estimate of the time since gene flow was interrupted, that is, the time of speciation—an estimate which is particularly interesting in cases such as this one, in which very different models of the history and biogeography of the situation may be advanced.

MATERIALS AND METHODS

The sources of our samples are listed in Table 1. All animals were collected from the field, transported alive and immediately stored at −70°C until electrophoresis. Only autumn 1985 through 1986 catches were used, except for European *V. cardui*; we had only a handful of old frozen specimens (1979) of these, but they had conserved most of their activity such that the zymograms were completely satisfactory for comparison with recent American material. All wings were retained by HJG. The head and thorax of each butterfly were homogenized in four volumes of Tris-HCl buffer (0.05 M, pH 8.0). We used horizontal starch gel electrophoresis procedures slightly modified from Ayala et al. (1972) (Geiger 1981). Twenty-two enzymes were scored:

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)
pyruvate kinase (PK)

There are no studies known to us of the heredity of any of these loci in Nymphalini, and we made the usual assumption by treating electromorphs as alleles. "Allelic" distributions were generally in good accord with Hardy-Weinberg expectations in samples large enough to warrant such a test. The most frequent "allele" in *carye* was arbitrarily given the standard index 100 in all cases; electromorphs with different mobilities are designated with relation to it, such as an "allele 105" for an enzyme that migrates 5 mm faster than the commonest *carye* allelic product.

TABLE 1. Localities and dates of samples. Altitudes are given only for mountain samples.

Vanessa carye, sens. str.

ARGENTINA: Prov. Salta: Abra Molina, 4000 m, i.2.86 (n = 3); Valle Encantado, 2700 m, i.22.86 (n = 2); Salta, i.22.86 (n = 2). Prov. Tucumán: San Javier, i.18.86 (n = 3); Abra Infiernillo, 3300 m, i.20.86 (n = 1); Tafí del Valle, 2100 m, i.23–27.86 (n = 7); San Miguel de Tucumán, i.29–iii.9.86 (n = 5 + 1 reared ex *Sida*).

V. annabella

CALIFORNIA, USA: Siskiyou Co.: Ball Mt., 2200 m, viii.23.86 (n = 7); Yolo Co.: Davis, ix.7.86 (n = 10); Solano Co.: Suisun City, ii.6.86 (n = 1), Fairfield, ii.6.86 (n = 2); Nevada Co.: Donner Pass, 2100 m, ix.25.85 (n = 9), Lang Crossing, South Yuba River, 1750 m, ix.25.85 (n = 1).

V. cardui

CALIFORNIA, USA: Nevada Co.: Donner Pass, ix.4.86 (n = 5). FRANCE: Dept. Vaucluse: Bollène, vi.4.79 (n = 1); Dept. Bouches du Rhône: Le Grau du Roi, vi.4.79 (n = 1); Dept. Herault: Oppidium d'Enserune, vi.2.79 (n = 1).

V. virginiensis

CALIFORNIA, USA: Nevada Co.: Donner Pass, ix.4.86 (n = 3).

Polygonia zephyrus W. H. Edwards

CALIFORNIA, USA: Nevada Co.: Donner Pass, ix.25.85 (n = 2).

Nymphalis milberti Godart

CALIFORNIA, USA: Nevada Co.: Donner Pass, ix.25.85 (n = 2).

The statistic $\bar{I}$ (Nei 1972) was used to estimate genetic similarity between samples over all loci. Calculated $\bar{I}$ values were then used to construct a dendrogram (Fig. 1) by cluster analysis (UPGMA method, Ferguson 1980). Because the set of loci is very similar and the same statistic has been used, direct comparisons may be made to earlier studies from our laboratories (Geiger & Scholl 1985 for example), while comparisons to others must be made with more caution. For estimating time of divergence of the taxa, a different statistic (D or I, not $\bar{I}$) was employed, as explained below.

RESULTS

There are genetic differences at several loci between *Vanessa carye* and *V. annabella* (Tables 2, 3). At most loci they consist of moderately to strongly divergent allelic frequencies; at only one locus (HK) is there an apparent fixed difference. In sympatry these data would be unequivocal evidence for speciation. In allopatry they must be compared to similar data for entities in other groups, using the same statistic and more or less similar procedures, to determine what constitutes a "species-level" difference. It is now well-established that some groups are much more conservative electrophoretically than others, and that the relation

TABLE 2. Common alleles for all taxa investigated.[a]

	APK	AK-1	AK-2	IDH1	IDH2	PGI	PGM	MDH1	MDH2	GOT1	GOT2	ALD	ME-1	ME-2	GPT	6PGD	GPDH	FUM	GAPD	PK-1	HK	IPO
annabella	100	100	100	100	100	100	103	100	100	100	100	100	100	100	104	107	100	100	100	100	96	100
carye	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
cardui France	100	100	100	101	110	100	112	100	100	94	90	100	102	100	93	103	100	100	120	110	95	100
cardui USA	100	100	100	101	110	100	105	100	100	94	90	100	102	100	93	103	100	100	120	110	95	100
virginiensis	100	100	100	96	110	100	109	100	100	94	98	100	103	100	90	107	100	100	120	110	100	100
zephyrus	100	100	100	88	100	100	102	89	100	94	90	100	95	96	85	105	100	100	120	110	104	110
milberti	100	100	100	84	90	100	102	89	100	105	90	100	107	92	102	105	100	100	100	110	110	110

[a] Common allele of *carye* always designated "100."

TABLE 3. Allelic frequencies at loci with high variability between *Vanessa annabella* and *V. carye*.

	N	PGM				GPT			6PGD			HK	
Sample		95	100	103	107	60	100	104	100	107	110	96	100
Davis	10			1.0				1.0		1.0		1.0	
Donner Pass	9			1.0				1.0	0.06	0.94		1.0	
Ball Mt.	7			1.0				1.0		0.86	0.14	1.0	
Σ *annabella*	30	0.02		0.98				1.0	0.02	0.95	0.03	1.0	
Tucumán	5		0.70		0.30	0.10	0.70	0.20	1.0				1.0
Tafí	7	0.17	0.50		0.33		0.21	0.79	1.0				1.0
Σ *carye*	24	0.04	0.65		0.30	0.02	0.70	0.28	0.96	0.04			1.0

between rates of morphological and electrophoretic differentiation can be extrapolated among taxa only with great caution. The classic study of the *Drosophila willistoni* (Diptera: Drosophilidae) complex by Ayala et al. (1974) established $\bar{I}$ values of 0.970 for conspecific, geographic populations; 0.795 for subspecies; 0.873 for semispecies; 0.517 for sibling species and 0.352 for morphospecies (recalculated from the original figures, which were given for Nei's I). The corresponding values for the same taxonomic levels are considerably higher in pierid butterflies, a very conservative group at the level of electrophoretic genetics (Geiger 1981, Geiger & Scholl 1985, Shapiro & Geiger in prep.). Thus, within the genus *Pieris, sens. lat.*, the European and Japanese subspecies of *Pieris rapae* L. cluster at 0.989; these with the morphospecies *P. mannii* Mayer at 0.902; these three with *P. canidia* L. at 0.874; the European and North American groups of "*napi*"-taxa with each other at 0.748 and the *napi* and *rapae* species-groups *in toto* at 0.546 (23 loci). The $\bar{I}$ value for *V. carye* and *V. annabella*, being in the mid-0.8 range, would indicate very well-differentiated species in *Pieris* and in Pieridae generally, but only infraspecific status in the *D. willistoni* group.

By Field's (1971) classification, the other two *Vanessa* used in this study (*cardui* and *virginiensis* Drury) belong to different species-groups (or splitter's genera). Thus the degree of differentiation in the dendrogram (Fig. 1) is not surprising. The lack of differentiation between Californian and French *V. cardui* mirrors their phenotypic similarity but is still somewhat surprising, especially given the small samples which would tend to amplify any differences purely probabilistically. *Vanessa cardui* is migratory in both Europe and America, with a huge summer breeding range (whence come our samples from both continents) but a much smaller overwintering one. This situation would tend to swamp out any tendency to local population differentiation, as in the Monarch, *Danaus plexippus* L. (Danaidae) (Eanes & Koehn 1979, Kitching 1985). But migration between Europe and America is neither known nor suspected for *Vanessa cardui*; nor is it a recent introduction in North America—at least Boisduval (1868) and Scudder (1889) treat it as native on the Pacific and Atlantic coasts, respectively. The possible stability of gene frequencies over its vast range deserves further study.

Both *Vanessa carye* and *V. annabella* are highly vagile, though neither is documented as a seasonal mass-migrant as is *V. cardui*. Our samples are drawn in both cases from more or less contiguous lowland and montane sites. There are hints in both species (Shapiro unpubl.) of a disorganized, individual altitudinal migration in mountainous terrain, tracking the seasonal availability of hosts. The virtual identity between nearby high- and low-elevation populations is not surprising. The biology of *V. carye* in Argentina is largely unpublished, but like *V.*

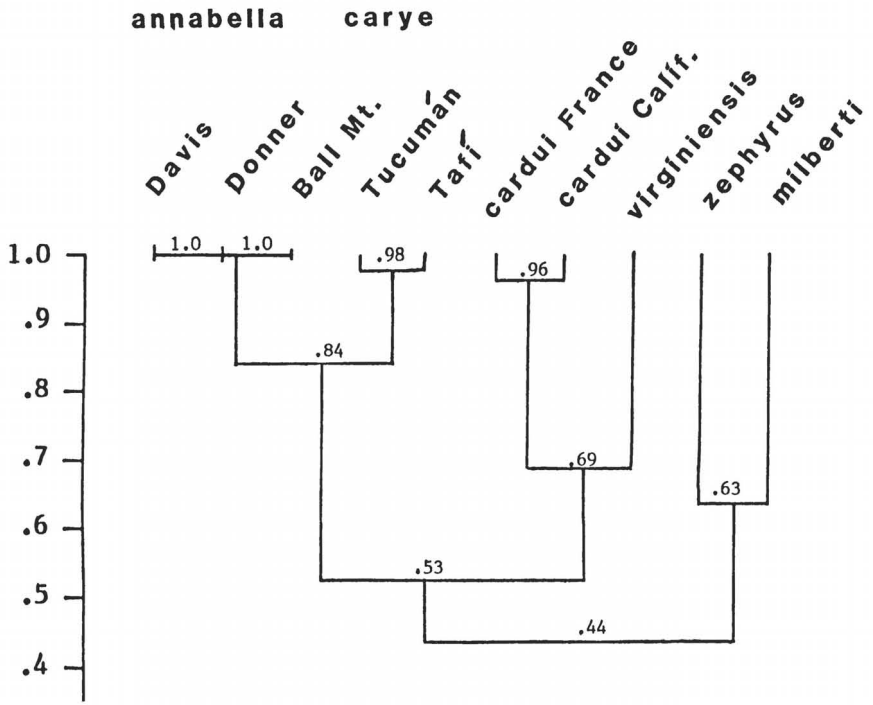

FIG. 1. Dendrogram illustrating clustering among three populations of *Vanessa annabella*, two of *V. carye*, and small samples of *V. cardui*, *V. virginiensis*, *Polygonia zephyrus* and *Nymphalis milberti*, using Nei's statistic Ī.

annabella it is a "weedy," often urban species, and its behavior is nearly identical to *V. annabella*. There is very pronounced rainfall seasonality at Tafí del Valle, while hosts are available all year at San Miguel de Tucumán. Other butterflies—several Pierini and Coliadini at least—appear to undergo regular seasonal up- and downslope movements in the Province of Tucumán (Shapiro & R. Eisele pers. obs.).

DISCUSSION

Instances in which speciation can be associated with a specific geohistorical event afford the opportunity to time-calibrate rates of biochemical evolution in particular groups, which is intrinsically superior to existing procedures for estimating time of divergence from genetic similarity or distance data (Nei 1971, Sarich 1977, Carlson et al. 1978, Thorpe 1982, Menken 1982). When biogeography suggests a specific time of speciation, this can be cross-checked using these procedures; agreement does not necessarily validate the scenario, nor disagreement falsify it, but such results are always suggestive.

The ranges of *V. carye* and *V. annabella* can be viewed as products of strict vicariance (a formerly continuous range divided by the appearance of a barrier) or of dispersal followed by differentiation (the classic geographic-speciation model). Herrera (1987) takes a vicariance position and attributes this case to continental drift, the "vicariance event" leading to speciation being the breakup of Pangaea. Such a scenario makes the last common ancestor of *carye* and *annabella* as old as the Triassic (some 200 million years ago), which seems unlikely for many reasons. However, the geography of this case strongly suggests dispersal across the Isthmus of Panama during the Great American Interchange which commenced roughly three million years ago when that corridor emerged, and which is very thoroughly documented for mammals (Marshall 1988, Stehli & Webb 1985). It resulted in colonization of each continent by faunal elements from the other, with a higher percentage of successful colonizations from North into South America than the reverse. Until fairly recently there was a tendency to attribute virtually all High Andean occurrences of otherwise Holarctic groups to this event (compare Mani 1968), regardless of the amount of evolutionary differentiation observed in the Andean biota (which by this view must have occurred since the Interchange). A consensus is now emerging to the effect that insects have evolved more slowly than mammals, at least in the Quaternary (Brown 1982, Coope 1978, 1979; D. W. Jenkins & L. D. Miller pers. comm.)—such that evolutionary origin of taxa above the species level in the Quaternary seems unlikely in Lepidoptera. Indeed, most butterfly evolution in the Quaternary seems to have been at the subspecies level, despite great geoclimatic dynamism. We suspect that the level of differentiation shown by *V. carye* and *V. annabella,* if fairly represented here, lies near the high end of the range to be expected once many candidates for Quaternary trans-Isthmian differentiation have been investigated.

The Panama land bridge was not only a corridor for migration and colonization by terrestrial organisms; it also formed a barrier to marine ones at the same time (Woodring 1966), and several speciation events have been attributed to it as a result. The genetic differentiation of sister species of marine organisms in the tropical eastern Pacific vs. the Caribbean has been quantified and cross-checked using dating estimates from electrophoretic data (Lessios 1979, 1981, Vawter et al. 1980). There is no reason in principle why the same should not be possible for terrestrial species. Like Vawter et al., we used Sarich's (1977) procedure, as modified by Carlson et al. (1978), to convert Nei's distance measure for *V. carye* and *V. annabella* (I = 0.855, D = 0.157) to an estimate of time of divergence, which is 2.97 million years (discussion of significant figures below). Such estimates entail many assumptions

and should not be taken unduly seriously, even when they give remarkably close agreement to estimates derived from biogeography. [Sarich's method was developed using vertebrate data, and we are aware of the dangers in extrapolating among taxonomic groups—as were Vawter et al. (1978)]. But this number is in fact very consistent with speciation consequent on the Great American Interchange and with an early dispersal across Panama, perhaps even before a continuous land corridor was available. What is most important is that it is wildly inconsistent with Herrera's (1987) invocation of the breakup of Pangaea, 250 to 100 million years ago depending on how far the animals could still disperse over water.

It is premature to state the direction of dispersal before a careful phylogenetic analysis of *Vanessa* is completed. There are more species-groups represented in North than South America, but more species on the latter continent. Herrera (1987) provides no explicit rationale for his claim that "The origin of *carye* is indubitably in Gondwanaland."

We have successfully resisted the temptation to generate scenarios for the history of *V. carye* and *annabella,* such as the proximate cause of the interruption of gene flow after invasion of one continent from the other, or for their failure to re-establish contact in montane Central America. Such exercises of the imagination are not in any sense testable with the tools used in this investigation.

Summing over many studies, Thorpe (1982) concludes that in general, "If allopatric populations of dubious status have genetic identities below about 0.85 it is improbable that they should be considered conspecific, while nominate species with I values above 0.85 should be considered doubtful if there is no other evidence of their specific status." He goes on to chide geneticists for violating common sense and the rules of significant figures by treating three-digit decimal I values as givens. Thorpe's rule of thumb for species status is inappropriate for Pierini but may be appropriate in Nymphalini and various other butterflies; time (and more studies) will tell. Nymphalini seem to undergo very slow morphological differentiation: Nearctic and Palearctic populations of *Nymphalis* and *Vanessa* species do not differ phenotypically; the genera are so uniform morphologically that generic splitting and lumping are a chronic problem in the group; even different genera show homologous responses to temperature shock during development (Shapiro 1984); and an Oligocene fossil attributed to *Vanessa* by Miller and Brown (1988) demonstrates morphological near-stasis over geologic time. On the other hand, Nymphalini seem to be more normal animals electrophoretically than Pierini are, that is, more labile, at least to judge by our work.

We agree with Thorpe's comments on significant figures; slight dif-

ferences in electrophoretic data must be interpreted reasonably in the context of overall patterns of variation in the group, and calculations such as Sarich's estimator—one more manipulation removed from reality—must be used with still more caution. When one is primarily interested in orders of magnitude, as we are here, they are still quite valuable. Although our findings are very preliminary and we are cognizant of the limitations of our study, including small sample sizes and the use of samples from arbitrary locations within very large ranges, we are pleased with the outcome—and still comfortable with Field's decision to treat *Vanessa annabella* as a species distinct from *V. carye*.

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