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Natural Hybridization between Two Swallowtail Species *Parnassius nomion* and *Parnassius bremeri* (Lepidoptera, Papilionidae) Shown by RAPD–PCR

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Abstract—Genetic evidence for interspecific hybridization between *Parnassius nomion* and *Parnassius bremeri* in nature is presented. To demonstrate hybridization between these species, RAPD analysis was used. By testing 25 decamer primers, three and two diagnostic markers were revealed for *P. nomion* and *P. bremeri*, respectively. Out of 28 animals examined, 4 were shown to be interspecific hybrids. According to the distribution of diagnostic markers, the interspecific hybrids were intermediate with regard to the parental species. Ecological and biological characteristics of two swallowtail species that promote their hybridization in nature are discussed.

INTRODUCTION

As other members of the tribe Parnassiini of the family Papilionidae, *Parnassius bremeri* and *P. nomion* are characterized by extremely high levels of individual and geographic variation. These species inhabit Transbaikalia, Priamur'e, Primorye, Kunashir Island (only *P. bremeri*), Northeast China, North Korea, and Japan (Hokkaido) [1] (Fig. 1). In these regions, the ranges of these swallowtail species substantially overlap. The preferred habitats of *P. bremeri* and *P. nomion* are different but in some cases the species may come into contact.

Putative hybrids between *P. bremeri* and *P. nomion* were reported as early as in the beginning of the 20th century [2, 3]. However, these studies did not present facts confirming the hybrid origin of individuals that could not be unambiguously assigned either to *P. bremeri* or to *P. nomion* on the basis of their phenotypes. Later, Glushchenko and Martynenko [4] made an attempt to demonstrate hybridization between these species by means of discriminant analysis. Based on their results, these authors statistically proved the intermediate character of the morphological trait distribution in the putative hybrids but could not unambiguously determine whether hybridization between *P. bremeri* and *P. nomion* occurred in nature. Descimon and Geiger [5] successfully used allozyme analysis and methods of molecular genetics to show hybridization in other Lepidoptera species.

The aim of the present study was demonstrating natural hybridization between *P. bremeri* and *P. nomion* by means of RAPD–PCR, which was successfully used by several authors to detect interspecific hybrids in butterflies [6–8]. This aim required resolving the following tasks: isolation and purification of total cell DNA, optimization of the amplification conditions, screening for species-specific molecular markers and their use for determining the taxonomic status of the individuals that were tentatively identified as hybrids between *P. bremeri* and *P. nomion*.

MATERIALS AND METHODS

Experimental material. I used entomological material collected by Glushchenko and Martynenko in the vicinity of the settlement of Shumnyi, the village of Sadovoe, and in the middle flow of the Krasnaya River (Dal'negorskii raion). In addition, I collected some material in the vicinity of the settlement of Luk'yanovka (Shkotovskii raion) (Fig. 2).

The entomological material used in this study is listed in Table 1. The thorax of each butterfly was used for isolation of DNA, and wings, abdomen, head, and legs were stored as collection material.

Isolation of the total cell DNA and the polymerase chain reaction were conducted as described in [9]. Analysis of the PCR products was carried out in 2% agarose gel containing 0.5 μg of ethidium bromide and in the 1 × TBE buffer. To determine size of the amplified fragments, *Pst*I and *Eco*RI + *Hin*dIII restricts of the phage lambda DNA were used as molecular markers. The PCR products were termed according to the accepted nomenclature [10].

To detect homologous amplified fragments with similar electrophoretic mobilities, the RAPD patterns of all individuals examined were visually compared. The comparisons yielded character matrices reflecting the presence (1) or absence (0) of the corresponding band on the electrophoregram. From these matrices, Nei's genetic distances were calculated as

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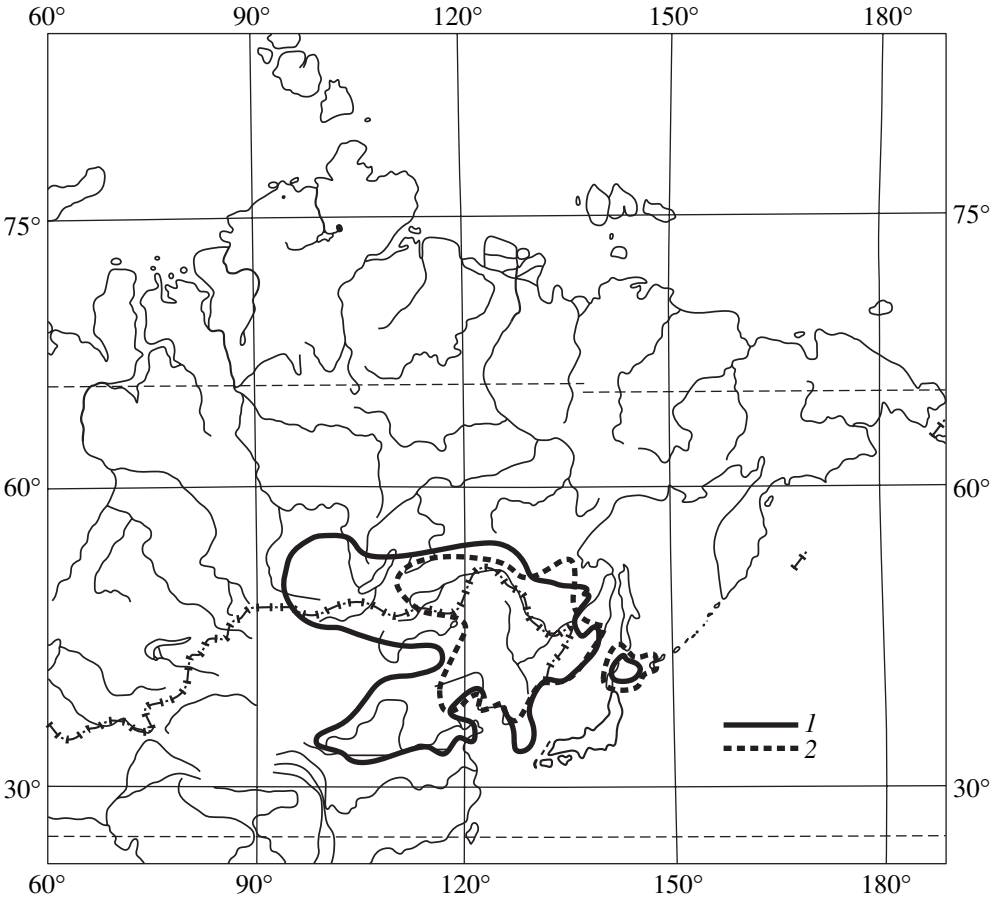

Fig. 1. Distribution of *P. nomion* and *P. bremeri*.

$$D = -\ln \frac{\sum_m \sum_i a_{mi} b_{mi}}{\left(\sum_m \sum_i a_{mi}^2 \sum_m \sum_i b_{mi}^2 \right)^{1/2}},$$

where a_{mi} and b_{mi} are the frequencies of the ith allele of the mth locus in the compared individuals a and b.

On the basis of the genetic distances, dendrograms were constructed using the pair-group method with arithmetic averages (UPGMA) and minimum spanning trees (MSTs) were obtained.

The calculation of Nei's genetic distances and the dendrogram construction were carried out using the NTSYS-pc (version 1.7) program package [12].

RESULTS

Twenty-five randomly chosen decamer primers (Operon Technologies, Alameda, United States) were tested on two DNA samples from *P. nomion* and *P. bremeri* (Table 2). Out of these, 22 primers initiated synthesis of PCR products whose size ranged from 250 to 2000 bp.

Further screening of primers was carried out using DNA from four individuals of the two species collected in four isolated populations. Based on the screening results, I selected primers that initiated amplification predominantly of major fragments readily identifiable on electrophoregrams. Sixteen of these selected primers amplified major fragments that were regarded as possible markers. These primers were tested on 16 DNA samples isolated from the following material: one individual of *P. bremeri* and five individuals of *P. nomion* from the vicinity of Luk'yanovka; five individuals of *P. nomion* collected by the middle flow of the Krasnaya River, and five individuals of *P. bremeri* from the vicinity of Shumnyi. Ten primers were selected on the basis of their effectiveness in identification of individual populations. The primers were tested using animals from isolated populations of the two species and animals collected in the mixed population in the vicinity of Sadovoe. In total, 28 individuals were examined.

The RAPD patterns of the species were markedly different although this difference was masked by high intragenomic heterogeneity and individual variation. In *P. nomion*, most of the amplified fragments were either present in the RAPD patterns of all individuals or occurred at high frequencies (in 80–90% of individu-

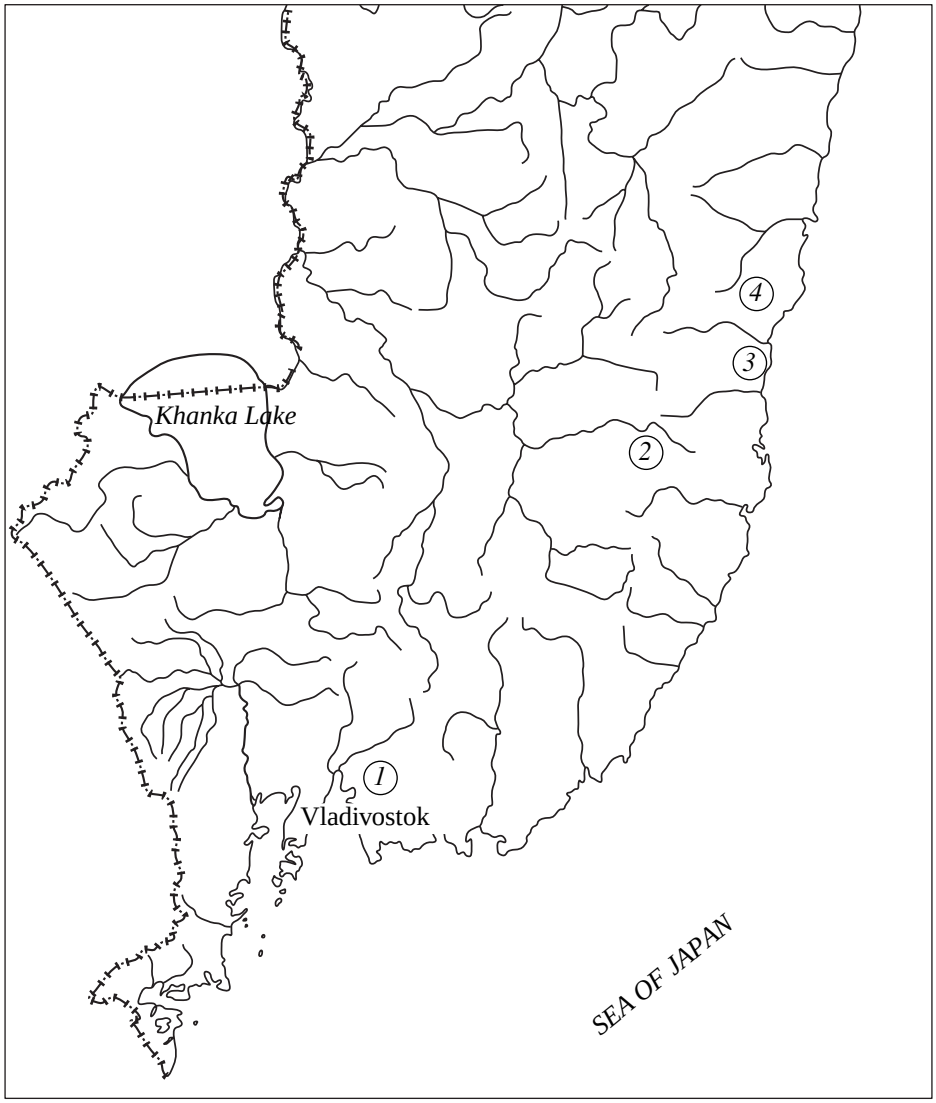

Fig. 2. Collecting localities in Primorye. (*1*) near the village of Luk'yanovka; (*2*) near the settlement of Shumnyi; (*3*) near the village of Sadovoe; (*4*) Middle flow of the Krasnaya River.

als). Some fragments were individually specific; others were species-specific for *P. nomion.* In *P. bremeri*, the PCR products for the most part were unique for each individual but some fragments occurred in all individuals of this species. The presence of such conservative sequences permitted to use them as potential species-specific markers.

Only three primers (OPA-02, OPA-08, and OPB-08) tested on 16 individuals of the two species from isolated populations initiated synthesis of the diagnostic marker fragments (Fig. 3).

Primer OPA-08 (Fig. 3a) amplified a 58-bp fragment in *P. nomion* and 680-bp fragment in *P. bremeri.* Although in all *P. nomion* individuals, a fragment similar in mobility to the *P. bremeri* marker fragment was present, the former markedly differed from the latter in majority. Primer OPA-02 (Fig. 3b) amplified a 300-bp fragment, which occurred at high frequency in the *P. nomion* PCR products. Primer OPB-08 (Fig. 3c) amplified 2000-bp fragments in *P. nomion* and 1300-bp fragments in *P. bremeri.* These fragments ($OPA\text{-}08_{580}$, $OPA\text{-}08_{680}$, $OPA\text{-}02_{300}$, $OPB\text{-}08_{1300}$, and $OPB\text{-}08_{2000}$) were considered diagnostic and used for identification of putative phenotypic hybrids.

According to the RAPD patterns obtained with the primers initiating amplification of the marker fragments, the putative phenotypic hybrids were intermediate to the parental species (Fig. 4).

The OPA-08-initiated RAPD patterns of all of the four putative hybrids had a complete combination of marker fragments $OPA\text{-}08_{580}$ and $OPA\text{-}08_{680}$, which were specific respectively for *P. nomion* and *P. bremeri.* In spite of the presence of the $OPA\text{-}08_{680}$ fragment in PCR products in all individuals of *P. nomion*, the ampli-

Table 1. List of the material used

Species	No. of individual	Sex	Date and place of collection
Parnassius nomion mandschuriae Oberth.	L1	♂	June 30, 1998, near Luk'yanovka, Shkotovskii raion
	L2	♂	
	L3	♂	
	L4	♂	
	L5	♀	
	N1	♂	July 1–4, 1998, near Sadovoe, Dal'negorskii raion
	N2	♂	
	N3	♂	
	R1	♂	July 17–20, 1998, middle flow of the Krasnaya River, Dal'negorskii raion
	R2	♂	
	R3	♂	
	R4	♂	
	R5	♂	
P. bremeri conjucta Stg.	L6	♂	June 30, 1998, near Luk'yanovka, Shkotovskii raion
P. b. orotschonicus Bang-H.	S1	♂	June 30, 1998, Near Shumnyi, Chuguevskii raion
	S2	♂	
	S3	♂	
	S4	♂	
	S5	♀	
	S6	♀	
	B1	♂	June 29, 1998, near Sadovoe, Dal'negorskii raion
	B2	♂	
	B3	♂	
	B4	♂	
P. n. mandschuriae × *P. b. orotschonicus*	H1	?	July 1–4, 1998, near Sadovoe, Dal'negorskii raion
	H2	♂	
	H3	♂	
	H4	♂	

fication intensity of this fragment in this case was far lower than in *P. bremeri* and hybrid animals (Fig. 4a).

In the putative hybrids, primer OPA-02 amplified the OPA-02_{300} fragment, which was specific for *P. nomion.* The RAPD patterns of *P. nomion* and the hybrids initiated by this primer also exhibited significant similarity of other electrophoretically close fragments (Fig. 4b).

Primer OPB-08 revealed a complex distribution of the amplified fragments in the RAPD patterns of hybrids. In addition to the OPB-08_{1300} fragment characteristic for *P. bremeri*, these patterns had minor fragments characteristic for *P. nomion.* The OPB-08_{2000} fragment specific for *P. nomion* was weakly expressed in the RAPD patterns of the hybrids (Fig. 4c).

The remaining primers did not reveal diagnostic marker fragments. However, they showed that in the RAPD patterns of the putative hybrids, fragments amplified in some *P. nomion* individuals may occur together with those recorded in some individuals of *P. bremeri.*

To analyze genetic similarity of the animals examined, I selected five primers (OPA-02, OPA-03, OPA-04, OPA-18, and OPA-20), in whose RAPD patterns major, easily identified fragments prevailed. In total, 195 fragments were recorded (excluding very weak minor PCR products). Mean intra- and interspecies genetic distances for *P. nomion*, *P. bremeri*, and their hybrids (Table 3) were calculated from the matrices of the presence–absence of the character.

Table 2. Primers used for RAPD analysis

Primer	Sequence (5'–3')	Potential markers	Selected for further testing	Used for computing genetic distances	Detected marker fragments
OPA-01	CAGGCCCTTC	+	+	–	–
OPA-02	TGCCGAGCTG	+	+	+	$OPA\text{-}02_{300}$
OPA-03	AGTCAGCCAC	+	+	+	–
OPA-04	AATCGGGCTG	+	+	+	–
OPA-05	AGGGGTCTTG	+	+	–	–
OPA-06	GGTCCCTGAC	–	–	–	–
OPA-07	GAAACGGGTG	–	–	–	–
OPA-08	GTGACGTAGG	+	+	–	$OPA\text{-}08_{580}$ $OPA\text{-}08_{680}$
OPA-13	CAGCACCCAC	–	–	–	–
OPA-16	AGCCAGCGAA	–	–	–	–
OPA-17	GACCGCTTGT	+	–	–	–
OPA-18	AGGTGACCGT	+	+	+	–
OPA-19	CAAACGTCGG	+	+	–	–
OPA-20	GTTGCGATCC	+	+	+	–
OPB-01	GTTTCGCTCC	+	–	–	–
OPB-02	TGATCCCTGG	–	–	–	–
OPB-08	GTCCACACGG	+	+	–	$OPB\text{-}08_{1300}$ $OPB\text{-}08_{2000}$
OPB-13	TTCCCCCGCT	–	–	–	–
OPB-20	GGACCCTTAC	–	–	–	–
OPC-17	TTCCCCCCAG	–	–	–	–
OPC-18	TGAGTGGGTG	–	–	–	–
OPE-16	GGTGACTGTG	+	–	–	–
OPE-17	CTACTGCCGT	+	–	–	–
OPE-20	AACGGTGACC	+	–	–	–
OPF-17	AACCCGGGAA	+	–	–	–

The mean within-species distance was significantly higher in *P. bremeri* (0.537) than in *P. nomion* (0.374).The mean genetic distance between these species was 1.350. For the hybrids, the mean genetic distance was 0.552. The mean distances between the hybrids and the parental species were 0.689 (*P. nomion*) and 0.838 (*P. bremeri*), which is significantly higher than the mean distances within species.

The results of the UPGMA cluster analysis are presented in Fig. 5. The similarity dendrogram constructed for the parental species individuals from isolated populations shows that all of them fall into two distinct clusters according to the species-specific characters. At the same time, populations did not form subclusters within the species. The branch lengths was greater in *P. bremeri* than in most *P. nomion* individuals.

A comparison of the parental species with hybrids revealed three distinct clusters. The cluster of the hybrids occupies an intermediate position with regard to those of the parental species being closer to *P. nomion.* In branch length and mean within-species genetic distances (Table 3), this cluster is only slightly different from the cluster of *P. bremeri.*

The minimum spanning tree also demonstrates genetic independence of the parental species (Fig. 6a). At the same time, the hybrids occupy an intermediate position with regard to the parental species (Fig. 6b).

DISCUSSION

A comparison of the RAPD patterns of the parental species and their hybrids and analysis of their genetic distances showed that *P. nomion* and *P. bremeri* are genetically well-diverged species. At the same time, as seen from the values of mean genetic distances (Table 3) and grouping of individuals on the dendrograms (Fig. 5), polymorphism is significantly higher in *P. bremeri* than in *P. nomion.* Moreover, the value of the mean within-

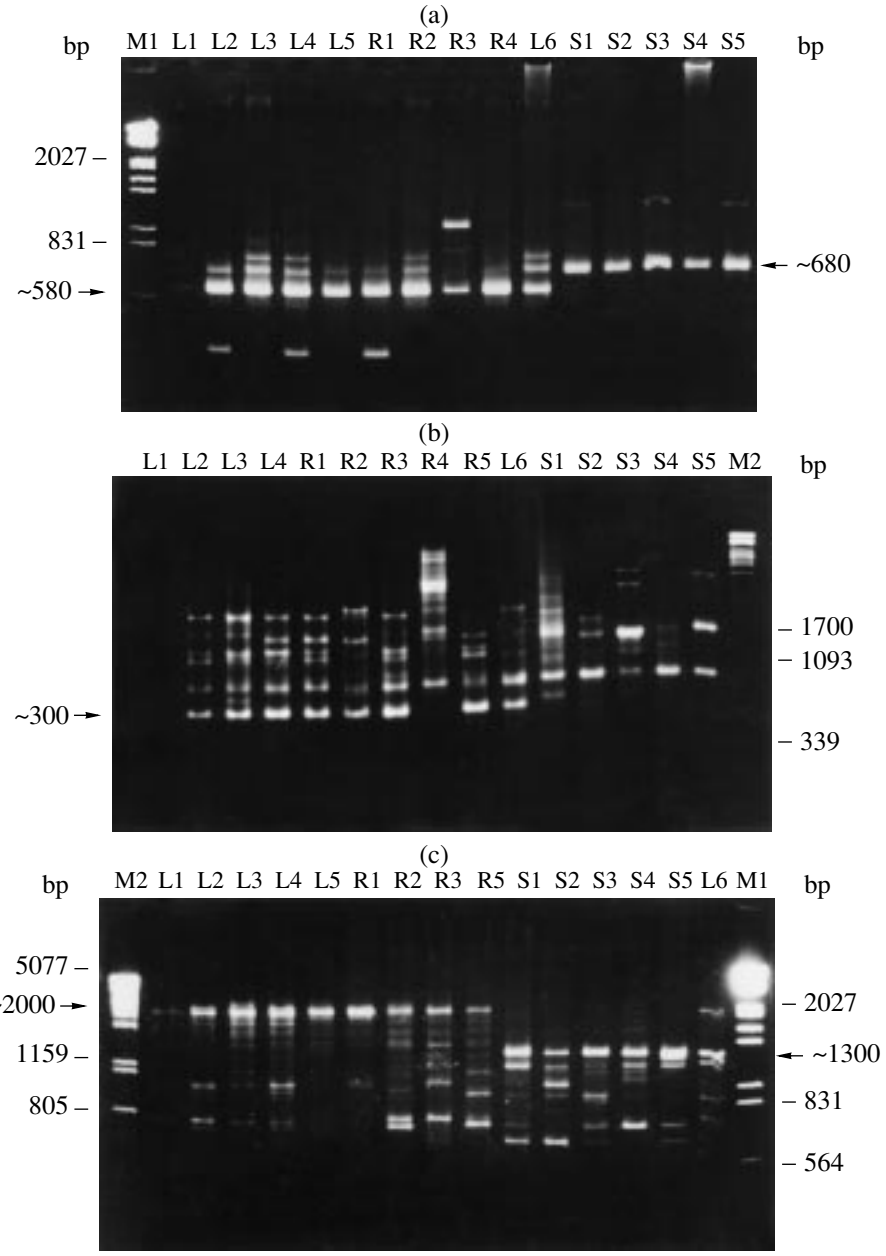

Fig. 3. RAPD markers amplified by primers OPA-08 (a), OPA-02 (b), OPB-08 (c) in *P. nomion* collected near Luk'yanovka (L1–L5) and in the middle flow of the Krasnaya River (R1–R5) and *P. bremeri* collected near Luk'yanovka (L6) and Shumnyi (S1–S5). M1, *Eco*RI + *Hin*dIII restricts of the phage lambda DNA; M2, *Pst*I restricts of the phage lambda DNA. Marker fragments are indicated by arrows.

species genetic distance in *P. bremeri* (0.537) is comparable to that in the interspecific hybrids (0.552). This may indirectly support a hypothesis of Kurentsov on the hybrid origin of the subspecies *P. b. orotschonicus* [13]. The *P. bremeri* individuals examined in the present study are assigned to this subspecies.

The results obtained showed that the putative phenotypic hybrids occupy an intermediate position with regard to *P. bremeri* and *P. nomion* since these hybrids are characterized by the presence of the diagnostic markers of the both species.

RAPD markers are generally regarded as dominant and having Mendelian inheritance [14]. Consequently, identification of intraspecific hybrids is most conclusive in the case when one of the parental species carries a marker absent in the other species. In the present study, I describe three diagnostic fragments (OPA-08_{580}, OPA-03_{300}, and OPB-08_{2000}) for *P. nomion* and two diagnostic fragments (OPA-08_{680} and OPA-08_{1300}) for *P. bremeri*. Each fragment was considered an independent locus having complete (OPA-08_{580}, OPA-03_{300}, and OPB-08_{2000}) or incomplete (OPA-08_{680} and OPA-08_{1300}) dominance.

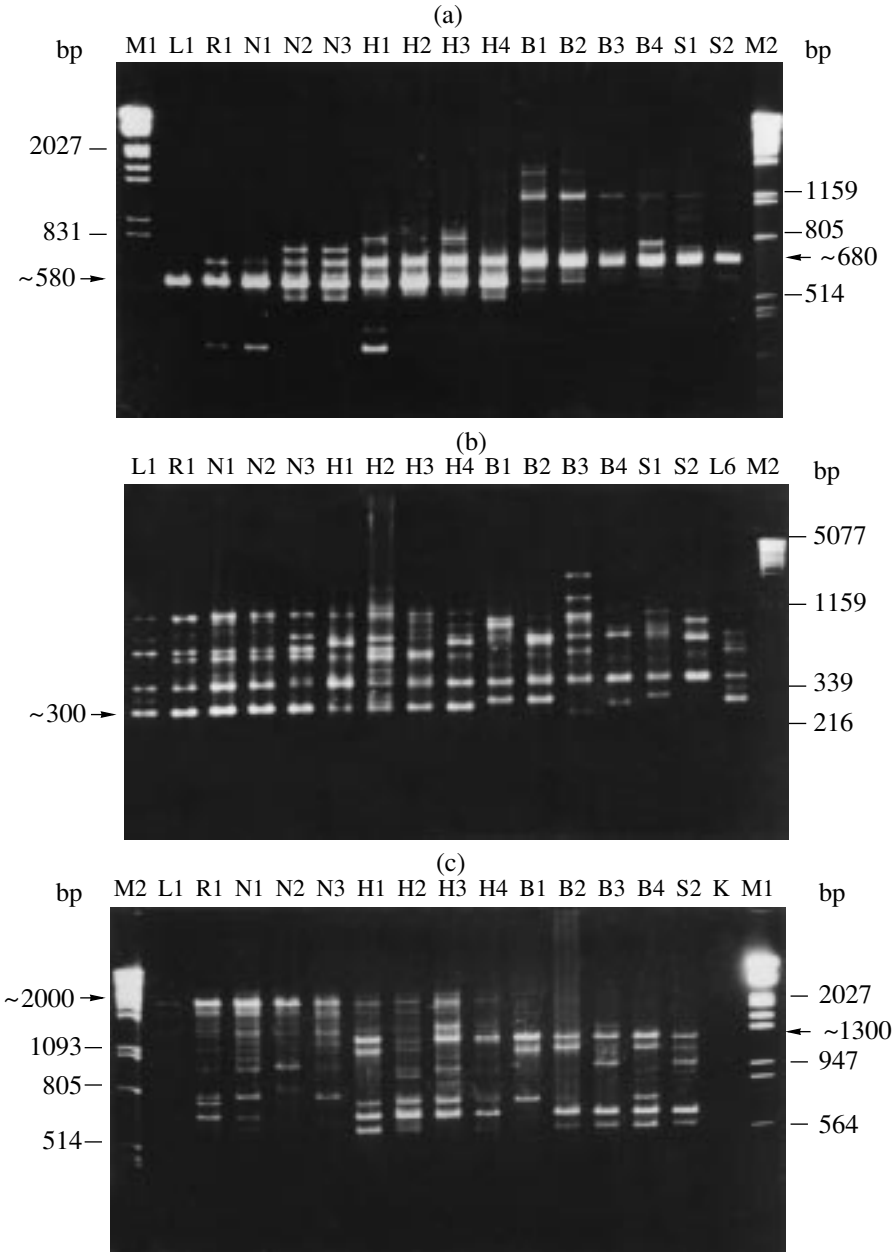

Fig. 4. The evidence of interspecific hybridization obtained using RAPD with primers OPA-08 (a), OPA-02 (b), and OPB-08 (c). L1, R1, N1–N3: *P. nomion*; H1–H4: *P. nomion* × *P. bremeri*; B1–B4, S1, S2, L6: *P. bremeri*; K, control without DNA; M1, *Eco*RI + *Hin*dIII restricts of the phage lambda DNA; M2, *Pst*I restricts of the phage lambda DNA. Marker fragments are indicated by arrows.

If all these markers exhibit Mendelian inheritance, then the *P. nomion* × *P. bremeri* hybrids must have species-specific marker fragments characteristic for both parents. In the present study, only primer OPA-08 detected markers of both parental species in all of the four putative hybrids. In the other cases, marker fragments showed different distribution in the hybrids.

The distribution of marker fragments in the putative phenotypic hybrids unambiguously indicates the hybrid origin of the individuals, which are phenotypically different from *P. nomion* and *P. bremeri.* However, as I detected only one primer (OPA-08) that initiated synthesis of completely additive RAPD patterns in the hybrids, I cannot unambiguously state whether these individuals are first-generation hybrids or some of them was produced by backcrosses.

The comparison of the mean genetic distances as well as the distribution of the hybrids and the parental species on the dendrograms show that in spite of the intermediate position of the hybrids with regard to the parental species, they are closer to *P. nomion.* This may be explained by some ecological features promoting hybridization between *P. nomion* and *P. bremeri.*

Table 3. Mean values of between-species and within-species genetic distances

Species and population	*P. nomion* Luk'yanovka	*P. nomion* Krasnaya River	*P. nomion* Sadovoe	*P. nomion*	*P. nomion* × *P. bremeri*	*P. bremeri*	*P. bremeri* Sadovoe	*P. bremeri* Shumnyi
P. nomion Luk'yanovka	0.302 ± 0.087							
P. nomion Krasnaya River		0.515 ± 0.254						
P. nomion Sadovoe			0.305 ± 0.203					
P. nomion				**0.374 ± 0.085**				
P. nomion × *P. bremeri*			0.668 ± 0.070	0.689 ± 0.072	**0.552 ± 0.087**			
P. bremeri				**1.350 ± 0.235**	0.838 ± 0.163	**0.537 ± 0.098**		
P. bremeri Sadovoe					0.827 ± 0.179		0.468 ± 0.098	
P. bremeri Shumnyi								0.606 ± 0.136

As pointed out by Glushchenko and Martynenko [4], in the locality inhabited by the mixed population of *P. nomion* and *P. bremeri* near the settlement of Sadovoe, the seasonal emergence periods of these swallowtail species significantly overlap. The emergence of *P. bremeri* is prolonged and occurs from late May to early August, whereas *P. nomion* emerges from late June to mid-August. In populations of these species occupying geographically overlapping areas, *P. bremeri* females continue to emerge in the period of the mass emergence of *P. nomion* males in spite of the interspecific difference in the beginning of emergence. In this time period, *P. nomion* females are virtually absent whereas most *P. bremeri* males are aged and probably incapable of mating. Thus, at the beginning of *P. nomion* emergence, males of this species are in acute deprivation of virgin females. As at that time, females of *P. bremeri* are virgin and males of this species incapable of mating, this creates favorable conditions for mating of *P. nomion* males and *P. bremeri* females.

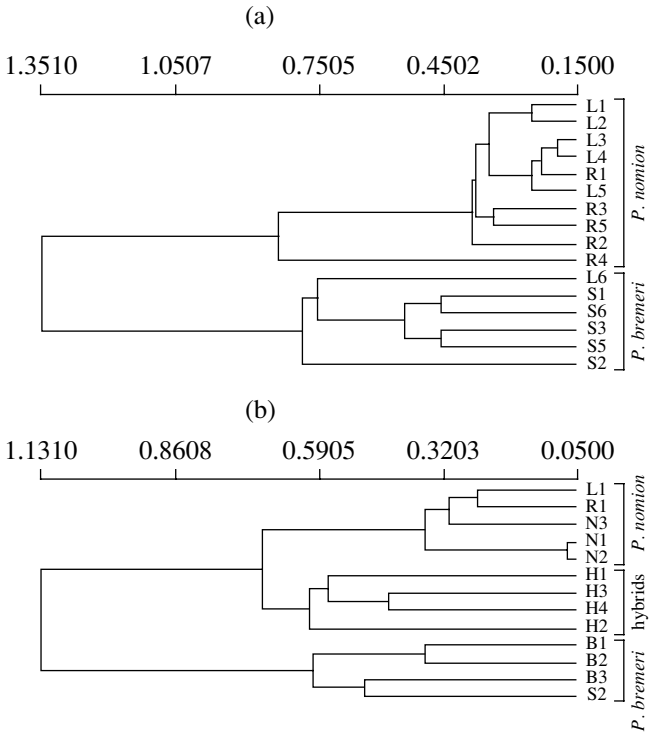

Fig. 5. The UPGMA similarity dendrograms. (a) *P. nomion* and *P. bremeri* from isolated populations; (b) *P. nomion*, *P. bremeri* and *P. nomion* × *P. bremeri*.

Since virtually all individuals that I examined were males, no sex-linked markers were identified. However, the closeness of the hybrids to one of the parental species (*P. nomion* may be explained by unidirectional hybridization between *P. nomion* males and *P. bremeri* females.

Based on the results obtained, I conclude that individuals possessing a set of morphological characters unusual for either *P. nomion* or *P. bremeri* are hybrids between these species, which proves their hybridization in nature. Unfortunately, it was not possible to estimate the frequency of hybrid individuals in the natural population. However, in the case of *P. apollo* and *P. phoebus*, the number of hybrids in mixed populations can reach 10% [5].

The results of the present study, data from the literature, and personal communications of a number of entomologists who recorded similar phenotypic hybrids of swallowtails in the wild suggest that natural interspecific hybridization in this group is rather common.

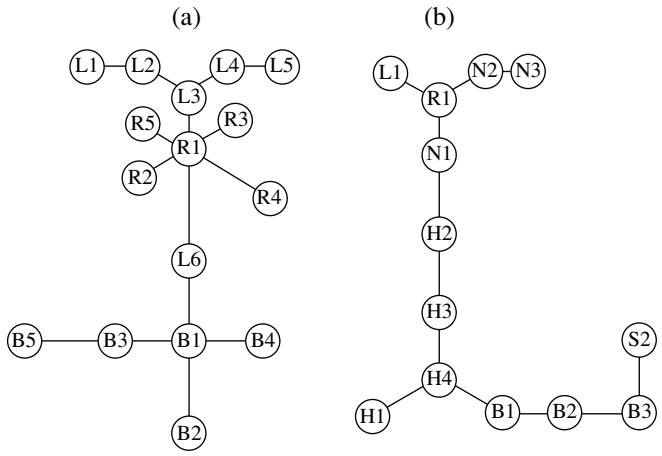

Fig. 6. The minimum spanning tree. (a) *P. nomion* and *P. bremeri* from isolated populations; (b) *P. nomion*, *P. bremeri* and *P. nomion* × *P. bremeri*.

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