Original Research Article

Phenotypic Variation And Genetic Similarities Of Polymorphic Butterfly *Papilio polytes* (Papilionidae: Lepidoptera) From Eastern Himalaya Of Arunachal Pradesh, India.

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Abstract: Present investigation was emphasized to establish the morphological and genetic distances and relationship among the female polymorphic forms (Stichius & Cyrus) and male *Papilio polytes* butterfly of eastern Himalayan region of India. Part of eastern Himalayan region belonging to Arunachal Pradesh is recognized as a biodiversity hotspot in India. *Papilio polytes* monomorphic male with two polymorphic female is available in this eastern Himalayan region. It has been hypothesized that these polymorphic forms has genetic distances and relationship, that may be the eastern Himalayan habitat specific. Specimens were collected from different parts (western, central and eastern) of Arunachal Pradesh invariably of altitude ranges from 1100m, 750m and 185m and climate differences (average temperature 17^{0}C, 22^{0}C and 22.8^{0}C) of the state. Morphometric studies were confined to measurement of total body length and wing span of both hind wing and fore wing. Protein profile has been studied by single dimensional SDS-PAGE. The genomic DNA isolated was studied by random amplified polymorphic DNA (RAPD) analysis using the designed primers of related species of butterflies available in India. Study on morphometry indicated differences in size and shape of both females as well as male. The result of the SDS-PAGE showed differences in the protein profile among females and male. A high degree of genetic polymorphism has been detected in the result produced by the RAPD-PCR using three primers RAPD1, RAPD6, RAPD7. The variability of both polymorphic forms and male *Papilio polytes* was conformed by similarity coefficient.
Keyword: Morphomerty, Protein profile, RAPD-PCR, Dendrogram, Primer

Introduction

Butterflies are one of the most beautiful and highly adapted flying insects in the present living world. Due to their attractiveness and omnipresence people are interested to work with this colourful insect. In present day, butterflies are recognized as a bio-indicator (Rocha *et al.,* 2010), flagship species (Guiney *et al.,* 2008) as well as key for ecological hotspot identification (Werner and Buszko, 2005). Butterflies have been recognized as being highly sensitive to weather and climate (Dennis, 1993). The *Papilio polytes* also known as "Common Mormon" is a mimic swallowtail butterfly having a wide range of distribution in the world (Clarke and Sheppard, 1971) including eastern Himalaya of Arunachal Pradesh, India. Two polymorphic females : Stichius and Cyrus and a monomorphic male available in this eastern Himalaya region are studied in the present investigation. Among these polymorphic forms, many noticeable differences in the wing

size and colour pattern are observed. Mimicry of this beautiful Papilionid is a well known phenomenon among the scientists. Although, the *Papilio polytes* has a wide distribution, a phylogeographic variation has been reported depending upon the climatic landscape for population variation. Polymorphism refers to two or more forms of variation within the same species occur at the same time and in the same area due to the genetic and environmental factors. Species that exhibit colour polymorphism with simultaneous occurrence of two or more discrete phenotypes with a genetic basis are ideal for studying the microevolutionary forces that maintain genetic variation in nature (Gossum *et al.,* 2011). Molecular techniques based on DNA sequence polymorphism are now used in population genetics studies, systematic and molecular taxonomy to get an answer to systematic related problems (Nagaraja and Nagaraju 1995, Sharma *et al.,* 2003). In this work two polymorphic females (Stichius & Cyrus) and male *Papilio polytes* are considered due to their availability in eastern Himalayan region (Arunachal Pradesh) which can be indicator of eastern Himalaya rich biodiversity region. Morphometric variations in terms of total body length and wing span has been studied. Single dimensional SDS-PAGE was used for study of protein profile. RAPD-PCR technique has been used for study of genetic diversity using arbitrary primers (Williams *et al.,* 1990). Colour pattern differences of all three polymorphic forms of *Papilio polytes* butterfly are quite distensible. It has been speculated that the morphological and genetic variability either distances or relationship observed among the polymorphic females and male *Papilio polytes* could be the characteristics of eastern Himalayan ecological conditions. This work showed that the polymorphic *Papilio polytes* butterfly has morphological variation of wing pattern, differences in protein profile and nucleic acid variation when studied using Random Amplified Polymorphic DNA technique.

Materials and methods

Morphometric study

The following morphometric parameters were recorded: total body length, wing (length & breadth), and colour pattern of polymorphic females and male *Papilio polytes* butterfly. During the study, measurement of the forewing and hindwing length (length from basal to apex) and breadth (from mid costa to tornus) have been considered. Wingspan is the distance measured across the wing of *Pieris Brassicae* butterfly (Bhubaneshwari *et al.*, 2012) i.e. distance between the apex of both forewing.

Protein extraction and study of protein profile using SDS-PAGE

The total proteins of adult *Papilio polytes* butterflies have been extracted following standard method (Cilia *et al.*, 2009). The butterflies were dissected and the thorax ground separately using a mortar and pestle to fine powder in liquid nitrogen. The content of each sample was transferred to a 50mL Falcon tube containing 10% TCA with 2% â-Mercaptoethanol (1g tissue: 10ml TCA-acetone w/v) and mixed throughly. Precipitated proteins were kept at -20^{0}C. Then protein was centrifuged at 4°C (eppendorf, 5415R) at 5000rpm for 30 minutes. The supernant was washed three times in 10mL ice-cold acetone, followed by centrifugation at 3000 rpm for 3-5 minutes. The pellets were vigorously disrupted with a glass rod between every wash. The supernatant was discarded and the pellet was dried, the tube was kept inverted on a filter paper. Thereafter the pellet was stored at -20^{0}C till further use.

Sample preparation

The 2x sample buffer containing 12.5ml of 1M Tris-HCl pH 6.8, 4g of SDS, 10ml of b-Mercaptoethanol, 10ml of Glycerol and 4ml of 1% Bromophenol blue were added to the frozen tissue and heated for 5-10 minutes in boiling water and then immediately cooled in ice. The sample was centrifuged in 10,000 rpm for 10 minutes. The supernatant was collected and stored in -20°C until used. Quantitative estimation of the protein was done following the method of Bradford (1976).

Separation of protein by SDS-PAGE

Protein sample was separated by discontinuous single dimensional SDS-PAGE following the method of Ausubel *et al.,* (1999). Samples of 20µl were loaded separately into

each well of 3.9% stacking gel {3.46mL distilled water, 0.50ml 1M Tris-HCl pH 6.8, 0.66ml 22% acrylamide, 40µl10% SDS, 40ul 10% ammonium persulfate (APS) and 4µl tetramethyl ethylene diamine (TEMED)}. The proteins were separated in 15% running gel {3.46ml distilled water, 7.50ml 1M Tris-HCl pH 8.8, 8.36ml 22% acrylamide, 200µl 10%SDS, 100µl10%APS and 10µl TEMED)}. Tris-glycine was used as a tray buffer. Electrophoresis was performed at 50V for 8-10 hrs using a vertical gel electrophoresis unit (Genaxy, SE660). After completion of electrophoresis the location of protein in the gel was determined by Commassie brilliant blue staining. The gel was destained in a mixture of 50ml of 25% methanol and 10ml of 7.5% acetic acid for clear visualization and detection of protein bands. The bands in the gel were documented in a gel doc system (Perkin Elmer, Geliance 200). The molecular weight of the separated proteins was detected comparing with a standard molecular weight marker (Novagene, protein perfect marker 10-225KD, Cat no. #69079-3).

Study on nucleic acid (DNA)

DNA Extraction

DNA isolation was done following the method of Ausubel *et al.,* (1999). To evaluate the genetic distances between two polymorphic female forms as well as male *Papilio polytes* butterfly adults of both sexes have been used. Extraction of DNA was done from preserved specimens and from freshly collected specimen . Preserved samples in alcohol have been washed three times with deionised water and then dried with paper (Gallusser *et al.,* 2004). DNA was extracted from the thorax and abdominal region of the butterflies. The end of abdomen was removed to avoid possible contamination by the spermatophore of male origin in female, as well as the sclerified parts of male abdomen (Aubert *et al.,* 1999). The tissue was homogenized in 500µl of TE buffer. 5% SDS along with 6µl proteinase K (25mg/ml) were added and incubated at 55^{0}C for 30 minutes. 100µl 5M NaCl (chilled) was added again in the sample and incubated in ice for 30 minutes. The sample was centrifuged at 10,000 rpm for 10 minutes at 4^{0}C. At the end of the centrifugation the upper layer of the sample was transferred to a new test tube and added equal volume of phenol and centrifuged at 10,000 rpm for 10 minutes at 4^{0}C. The upper layer of the sample was transferred to another tube and mixed with equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged at 10,000 rpm for 10 minutes at 4^{0}C. The upper aqueous layer was carefully transferred, equal volume of chilled ethanol added and incubated at 4^{0}C overnight. Following incubation, the sample was centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded and the white pellet (DNA) sediment at the bottom of the centrifuge tube was washed with 70% ethanol and dried. DNA was dissolved in 40µl TE buffer and stored in -20^{0}C for further analysis. The concentration of DNA was determined by spectrophometric method using UV visible scanning spectrophotometer.

Table 1. Sequences of the primers tested. Primer RAPD1, RAPD6 and RAPD7 showed results of amplification while, the remaining primers did not show amplification. Results only of the above mentioned primers showing amplification have been presented; remaining RAPD primers' results not shown.

Primer name	Primer seq 5$^/$-3$^/$	MW	Tm	%GC	nM	Pmol/µl
RAPD 1	GGTGCGGGAA	3136	27^{0}C	70	62.1	621
RAPD 2	GTTTCGCTCC	2972	25^{0}C	60	78.4	784
RAPD 3	GTAGACCCGT	3031	25^{0}C	60	58.5	585
RAPD 4	AAGAGCCCGT	3040	25^{0}C	60	29.0	290
RAPD 5	AACGCGCAAC	3009	25^{0}C	60	42.7	427
RAPD 6	CCCGTCAGCA	2975	27^{0}C	70	39.2	392
RAPD 7	CCACAGCAGT	3000	25^{0}C	60	46.0	460
RAPD 8	ACCCCCGAAG	2985	27^{0}C	70	35.4	354
RAPD 9	TGCCGAGCTG	3046	27^{0}C	70	29.8	298
RAPD 10	TGCGCCCTTC	2954.96	34^{0}C	70	55.84	558.4
RAPD 11	TTCCCCCGCT	2914.93	34^{0}C	70	56.61	566.1
RAPD 12	TCCGCTCTGG	2994.99	34^{0}C	70	55.09	550.9
RAPD 13	GTGACGTAGG	3108.09	32^{0}C	60	53.09	530.9
RAPD 14	GTCCACACGG	3013.01	34^{0}C	70	54.76	547.6

DNA Amplification:

Fourteen numbers of RAPD primers as mentioned elsewhere were tested for all butterfly samples (Table-1). Among the 14 primers tested only three primer: Primer RAPD1, Primer RAPD6 and primer RAPD7 showed the cDNA RAPD products in the studied samples.

In RAPD- PCR, 2µl of 10mM mix dNTPs was added in PCR tube for 20µl total volume of PCR reaction. 2 µl of 10x Taq buffer was added, primers were diluted to make the

Table 2. Cycling conditions of Polymerase Chain Reaction

Steps	Temperature	Time
1 Cycle Denaturation	940C	5 minutes
45 Cycle Denaturation	940C	30 Seconds
Annealing	360C	1 Minute
Extension	720C	1 Minute
1 Cycle Final Extension	720C	5 Minutes

specific concentration of 10nmol/µl. 1µl of diluted primer was added to the PCR tube. 1µl sample containing 30ng/µl DNA was added for the reaction. 0.5µl Taq DNA polymerase (DyNazyme TM II DNA Polymerase) having concentration of 2U/µl was added to the PCR tube and make the final volume 20µl with distilled water. A typical PCR reaction was performed in thermal cycler (eppendorf, 5415R) for RAPD. This reaction involved an initial DNA denaturation followed by a number of cycles of denaturation, primer annealing and product extension as presented in Table-2. The PCR products were kept at 4^0C for running in agarose gel electrophoresis.

Data analysis

The morphological data were statistically analyzed using ANOVA to find out significant difference in between male and polymorphic females. The value was considered as statistically significant whenever found differences at 95% level of confidence. All the amplified products RAPD-PCR were calculated using software (AlphaEaseFc) and compared with known DNA ladder ranging from 2000 bp to 75 bp. The similarity coefficient of females and male has been calculated using NTSYSPC version 2.0 software (Murthy *et al*., 2014). The dendrogram was constructed with UPGMA (Unweighted Pair- Group Method with Arithmetic Mean) method, using the software NTSYSPC version 2.0.

Results

Colour pattern & morphometry of *Papilio polytes*

The morphological study revealed that the Cyrus form of female *Papilio polytes* shows phenotypic similarities with the male butterfly exhibiting red marginal crescents strongly distinct on hindwing (anal angle). The forewing has a series of white spots at the end of outer margin which are decreasing towards the apex. In between the series of central white spots and the outer margin of the hindwing a series of red yellowish spots are present with decreasing size from anal angle to apex. This red-yellowish series of spots are prominent on the under side of hindwing of Cyrus form of *Papilio polytes*. The Stichius form is more colourful and attractive in comparision to Cyrus form. Forewing of dorsal is fuliginous and white black scales prominent on dorsal side of hindwing. A series of large white spots (four) starting with a reddish pinkish colour spot from the inner margin towards the outer margin on the central part of the hindwing is the characteristic feature of Stichius form. From anal angle towards the apex, another series of red-yellowish spots present on the hindwing. The male *Papilio polytes* is fuliginous-velvety black in colour. The outer marginal area of forewing exhibits a series of small white yellowish spots from tornus to apex. It has been observed that the size of the spots present on the outer marginal area decreasing toward the apex. A series of white spots are present on the central region of hindwing. Just bellow the central white line spots and above the end of dorsum a series of yellow curvy spots as well as a series of white spots present on the marginal area under hindwing. Wingspan of the male *Papilio polytes* butterfly is smaller compared with that of the female forms.

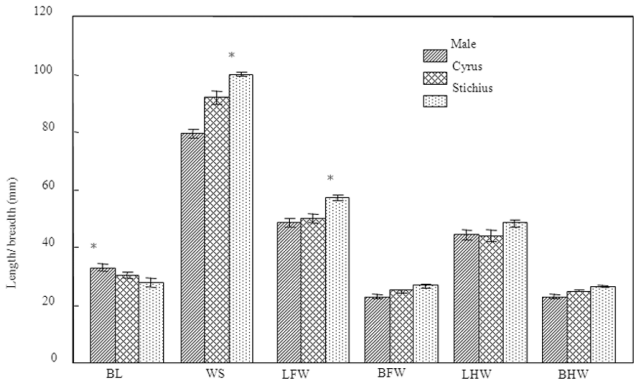

Fig. 1. The morphometry of male and polymorphic females (Cyrus & Stichius) of *Papilio polytes*. Data showed that body length (BL) of male is longer than that of females ($P<0.05$). The wing span (WS) value of Stichius is higher than that of cyrus and males ($P < 0.05$). Similarly, length of fore wing (LFW) of Stichius is more than that of cyrus & males. However, there is no difference in breadth of fore wings among the male and females. Unlike the fore wing length, the length of hind wing (LHW) of male and females and breadth of hind wing (BHW) are approximately similar in male & females without significant difference.

The total body length measured from head to anus has been presented in Fig. 1. The data showed the maximum value in male (33.1 ± 0.11) followed by Cyrus (30.2 ± 0.10) and Stichius (27.9 ± 0.13) forms. Total body length of males is longer than that of females (P<0.05). The wingspan of male *Papilio polytes* (76.4 ± 1.17) is smaller than Cyrus (94.3 ± 1.3) and Stichius (100 ±0.93). The forewing length of male (49.,2 ± 0.12), Cyrus (51.5 ± 0.13) and Stichius (58.2 ± 0.07) showed different values (P<0.05). The maximum length of the forewing has been observed in Stichius and minimum in male. The result showed approximately similar breadth of forewing among the males (23.3 ± 0.07), Cyrus (25.1± 0.06) and Stichius (26.6 ± 0.06), without a significant difference. The length of hindwing of male (41.2 ± 0.05) *Papilio polytes,* Cyrus (41.1 ± 0.06) and Stichius (43.7 ± 0.06) is approximately same. A significant difference of hind wing length among the male and females has not observed. Similar to the hind wing length; the breadth of hindwing of male (24.1 ± 0.05) and Cyrus (24.2 ± 0.05) is approximately same while, the Stichius females showed a higher value (26.1 ± 0.05) without a statistical difference.

Study of Protein Profile by SDS-PAGE

The protein profiles studied by single dimension SDS-PAGE of Stichius & Cyrus and male *Papilio polytes* has been presented in Fig. 2. In present study fifteen protein bands of females (Stichius & Cyrus) and seventeen numbers of protein bands of male *Papilio polytes* have been identified within the range of molecular weight 10 KD to 225 KD. The results showed differential protein expression among male & female. A high molecular weight protein band A (H" 175KD) observed only in Stichius and Cyrus. Certain other proteins (band B H" 135KD, band C H" 107KD and band D H" 90KD) were

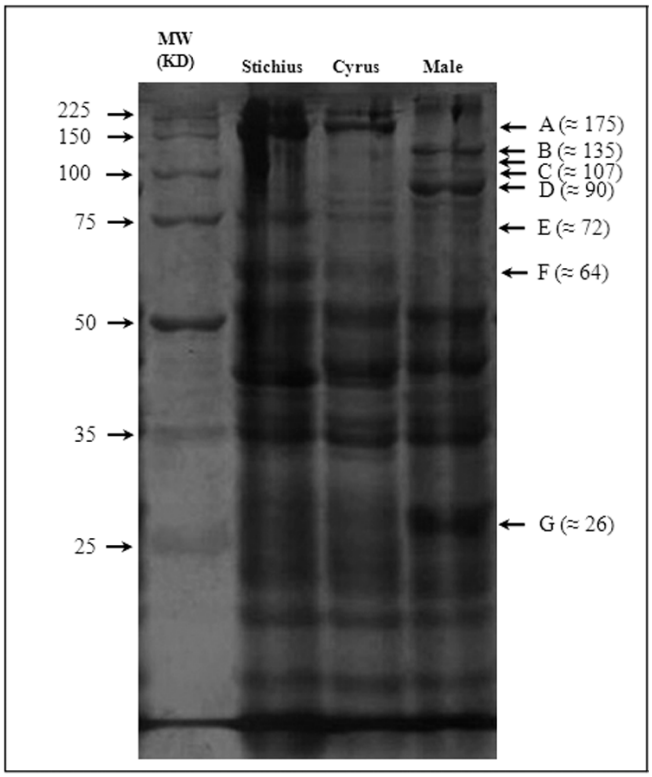

Fig. 2. Protein profile of polymorphic female (Stichius & Cyrus) and male *Papilio polytes* separated in 1D SDS-PAGE (15% gel). Protein bands A (H"175KD) and F (H" 64KD) expressed only Stichius & Cyrus female while, band B (H"135 KD), C (H"107 KD) and D (H" 90KD) expressed in male butterfly. Higher intensity of band B, D and G (H" 26KD) in male than that of females suggests specific role in male. Many protein bands are common is all three (Stichius, Cyrus and male) while, differential intensity of specific bands indicate genetic differentiation among the male and two females.

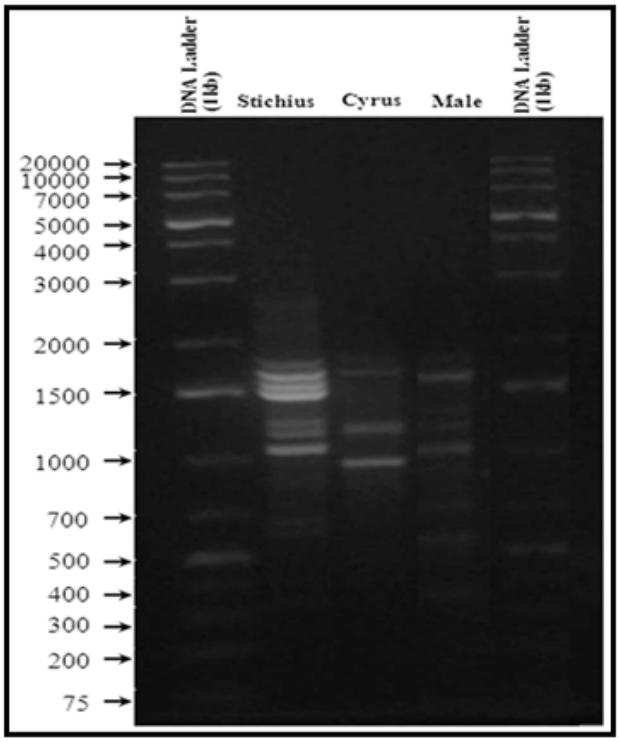

Fig. 3. RAPD analysis of Polymorphic females: Stichius & Cyrus and male *Papilio polytes* using primer RAPD1 (5/ GGTGCGGGAA 3/) on 1.4% agarose gel. Differential amplification of DNA in between Stichius and Cyrus both in number and base pair could be the cause of polymorphism. The male individual showed amplification producing certain fragments present in neither of the females. These bands could be either male specific or cause of other phenotypic characters.

observed only in male butterfly. Similar to the band A mentioned above, protein molecule having molecular weight H" 64KD (band F) has been observed only in female species. Protein band G (H" 26KD) was seen in both female as well as male. However, this particular band of protein appeared in male in higher intensity than that of two polymorphic females (Stichius & Cyrus) of *Papilio polytes*.

Random Amplified Polymorphic DNA (RAPD) analysis

RAPD analysis using primer RAPD1

The product of primer RAPD1 (5′ GGTGCGGGAA 3′) for representative individuals of both females (Stichius & Cyrus) and male showed different banding pattern of nucleic acids (Fig.3). Twenty bands were observed produced by primer RAPD1 from the three samples namely Stichius, Cyrus and male and identified within the range of 2000 bp to 75 bp of DNA ladder. Among these products 9 bands were observed in Stichius, 4 bands in Cyrus and 7 bands in male butterfly. Prominent and well separated amplified RAPD-PCR products (bands) have been selected for distinguishing the differences between the samples.

The amplified products using primer RAPD1 varies from 1815 bp to 575 bp among Stichius; 1815 bp to 935 bp among Cyrus and from 1670 bp to 380 bp among the male and males. The products found to be varied from 1585 bp to 335 bp in Stichius and 1390 bp to 335 bp in Cyrus female while, the male butterfly products were detected from 1075 bp to 335 bp (Table 4). The products sizes 1390 bp, 1075 bp and 335 bp were detected in both females. The band size 1585 bp was found only in Stichius while, 895 bp band detected individuals. The study showed individual specific amplified product indicating the genetic factors of polymorphism (Table 3).

Table 3. RAPD- PCR products of polymorphic female (Cyrus & Stichius) and male with primer RAPD1 (5′ GGTGCGGGAA 3′) . Individual specific products of Stichius (1530 and 1435 bp), Cyrus (935 bp) and male (1670 bp, 1320 bp and 320 bp) signify genetic distances and/or similarities among the polymorphs.

Individuals →Stichius	Cyrus	Male
1815	1815	-
1675	1675	-
-	-	1670
1530	-	-
1435	-	-
-	-	1320
1220	-	-
-	1185	1185
1103	-	-
1015	-	1015
-	935	-
625	-	625
575	-	575
-	-	380

RAPD analysis using primer RAPD6

The results of primer RAPD6 (5′ CCCGTCAGCA 3′) amplification showed different banding pattern of cDNA products of females (Stichius & Cyrus) and male (Fig.4). Based of clarity and separation, 10 numbers of amplified products in the form of bands were considered within the range of 2000 bp to 75 bp of DNA ladder. Among these products, 4 bands were observed in Stichius, and 3 bands in both Cyrus

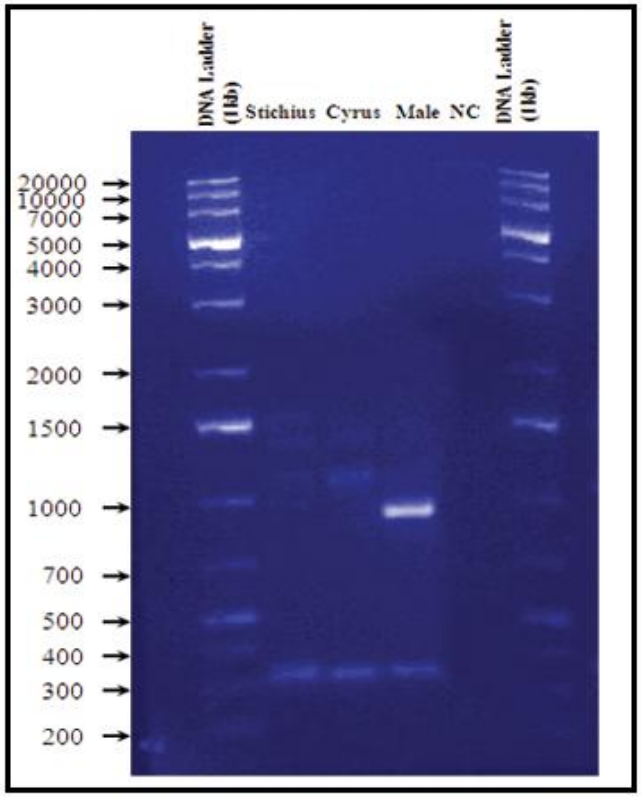

Fig. 4. RAPD result of Polymorphic females: Stichius & Cyrus and male of *Papilio polytes* using primer RAPD6 (5′ CCCGTCAGCA 3′) on 1.4% agarose gel showed only a few fragments. The band size having molecular weight 895bp observed in high intensity only male while, others are lower in intensity. The two females showed differences in banding pattern with lesser number in Cyrus form than that of Stichius. The band size lowest in bp (335) among all amplified fragments could be the *Papilio polytes* species specific. NC: negative control.

Table 4. RAPD- PCR products of polymorphic female (Cyrus & Stichius) and male *Papilio polytes* with primer RAPD6 (5/ CCCGTCAGCA 3/) showed maximum number of bands (four) in Stichius with a specific band (1585bp) not present in other two individuals. Cyrus and male showed three numbers of amplified products each , among which product having size of 895 bp was only in male individuals.

Individuals→Stichius		Cyrus	Male
↑	1585	-	-
Molecular	1390	1390	-
weight	1075	1075	1075
of band size (bp)	-	-	895
↓	335	335	335

in the amplified product of male individuals. Two bands having the size 1075 bp and 335 bp have been appeared common in both female (Stichius & Cyrus) and male butterfly.

RAPD analysis using primer RAPD7

This primer showed different banding patterns of cDNA products among three different individual samples: females (Stichius & Cyrus) and male (Fig. 5). Nine numbers of bands

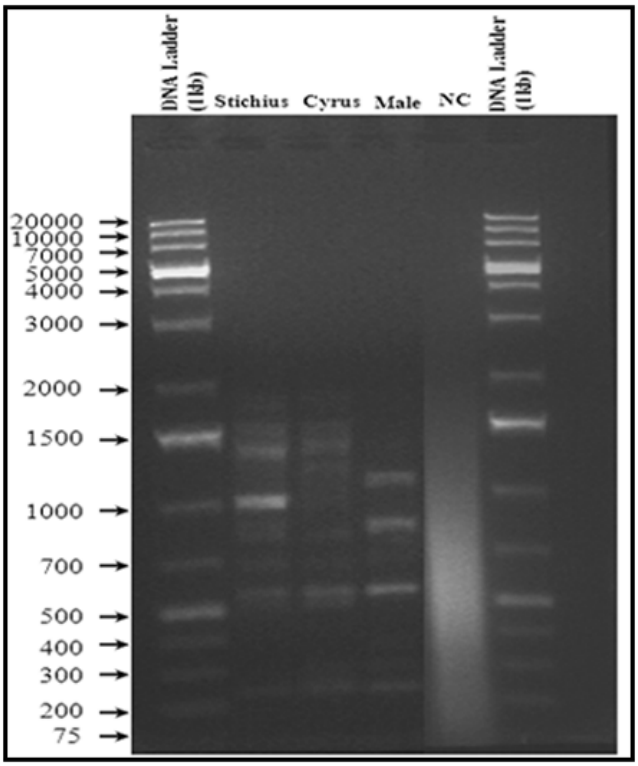

Fig. 5. RAPD analysis of Polymorphic females: Stichius & Cyrus and male *Papilio polytes* using primer RAPD7 (5/ CCACAGCAGT 3/) on 1.4% agarose gel showed higher number of amplified products in females than that of male. The fragment having the size 1020 bp amplified in appeared in string intensity only in Stichius. Minimum number of amplified products was observed in male; among which two fragments (1175 bp & 855bp) were not produced in female individuals. NC: negative control.

Table 5. RAPD- PCR products of polymorphic female (Cyrus & Stichius) and male *Papilio polytes* with primer RAPD7 (5/ CCACAGCAGT 3/) amplified maximum number of fragments (7) in Stichius folloed by Cyrus (6) and male (4). The fragment having size (1020bp) could be the cause of polymorphic character in Stichius, while two products in male (1175bp and 855bp) induces either polymorphic character of male specific.

Individuals→Stichius	Cyrus	Male	
	1645	1645	-
	1410	1410	-
↑	-	-	1175
Molecular	1020	-	-
weight	-	-	855
of band size (bp)	760	760	-
↓	525	525	525
	495	495	-
	260	260	260

Table 6. Similarity coefficient values calculated based on RAPD- PCR using three RAPD primers: primer RAPD1 showed the minimum value of similarity coefficient (0.286); primer RAPD 6 showed minimum value 0.400 while, the primer RAPD 7 showed the value as 0.222.

RAPD primer 1			
	Stichius	Cyrus	Male
Stichius	1		
Cyrus	0.357	1	
Male	0.286	0.357	1
RAPD primer 6			
	Stichius	Cyrus	Male
Stichius	1		
Cyrus	0.800	1	
Male	0.400	0.600	1
RAPD primer 7			
	Stichius	Cyrus	Male
Stichius	1		
Cyrus	0.833	1	
Male	0.222	0.333	1

produced by primer RAPD7 were calculated within the range of 2000 bp to 75 bp of DNA ladder. Among these cDNA products (bands) 7 bands were observed in the sample of Stichius, 6 bands in Cyrus and 4 bands in male butterfly. The cDNA products size varied from 1645 bp to 260 bp in females while, the male butterfly's cDNA products were detected from 1175 bp to 260 bp (Table 5). The data showed certain common amplified products of Stichius and Cyrus where in, one band (1020 bp) was specific for Stichius and the two others (1175 bp and 855bp) amplified only in male.

Similarity Coefficient

The similarity coefficient values were calculated in between the females and male (Table 6). The primer RAPD1 showed a minimum similarity coefficient value of 0.286. A coefficient

value 0.357 was calculated in between Stichius & male and Cyrus & male. The primer RAPD6 indicated a minimum similarity coefficient value of 0.400 among Stichius and male and maximum 0.800 in between two females (Stichius & Cyrus). The primer RAPD7 showed the minimum value (0.222) in between Stichius and male while, the maximum coefficient value (0.833) was calculated in between females (Stichius & Cyrus).

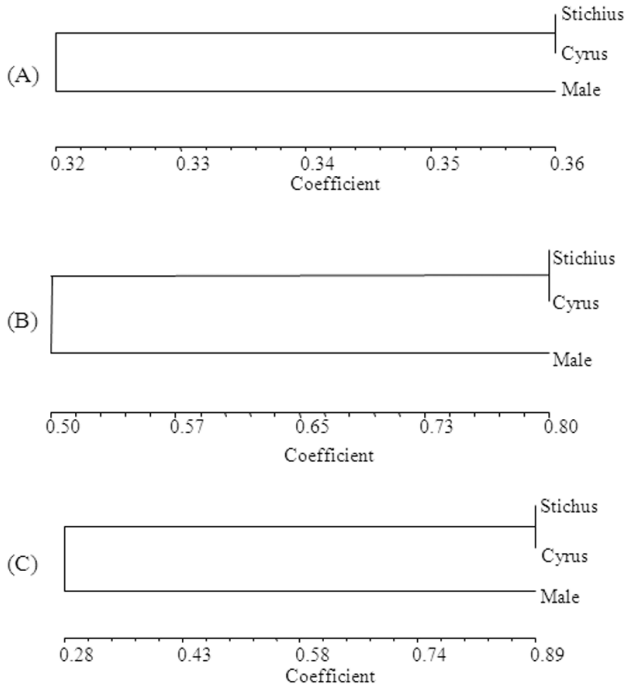

Fig. 6 (A, B & C). Dendrogram showing the relationship among polymorphic female (Stichius & Cyrus) and male *Papilio polytes* based on RAPD analysis using three primers {(A) RAPD1, (B) RAPD6 & (C) RAPD7}. The females have closer genetic similarity than that of monomorphic male and females.

Based on the similarity coefficient values of RAPD- PCR profile a dendrogram was drawn to determine the genetic similarity and/or distances among the individuals (Fig. 6). It was observed that two polymorphic females are closer genetically than that in between females and monomorphic male.

Efficiency of Primer

The efficiency of tested primers was calculated based of the percentage of polymorphism in terms of monomorphic and polymorphic bands (Table 7). The primer RAPD6 showed total number of 5 bands among which 2 bands were monomorphic and other 3 bands appeared as polymorphic.

Table 7. Efficiency of RAPD primer s tested based on number of bands, % of polymorphism The RAPD primer1 showed highest (100 %) polymorphism in terms of monomorphic and polymorphic bands The primer RAPD7 showed 77.78 % polymorphism while, the primer RAPD6 showed the least percentage of polymorphism (60%) considering all the cDNA products. This result suggests that RAPD1 primer is the most efficient in determining the genetic closeness among two polymorphic females (Stichius & Cyrus) and monomorphic male.

Primer	Sequencce (5/ - 3/)	Total bands	Monomorphic bands	Polymorphic bands	Polymorphism (%)
RAPD1	GGTGCGGGAA	14	0	14	100
RAPD6	CCCGTCAGCA	5	2	3	60
RAPD7	CCACAGCAGT	9	2	7	77.78
Total		28	5	24	79.26

The primer RAPD7 produced total 9 bands; among which only 2 bands were monomorphic while, other 7 bands appeared as polymorphic.

Discussion

Butterflies are very sensitive to the climate change and have complex interaction with environment (Dempster and McLean, 1999), as well as specificity to the habitat (Swaay *et al.*, 2012). Due to these specific qualities, butterflies are popularly considered to assess the factors viz., abiotic and biotic which influence the climate change. Due to their high level of sensitivity, these organisms are recognized as an indicator species (Rocha *et al.*, 2010) as well as an agent for monitoring the biodiversity. The abundance and richness of butterfly in a particular habitat or an ecosystem indicate the species diversity of both animals and plants. It has already been established that the species-area & endemic-area relationship of butterfly provide sufficient information regarding the identification of biological hotspot (Myers *et al.*, 2000). The present investigation was carried out to establish the morphological/ phenotypic variation of *Papilio polytes* could be reflected in protein profile encoded in genetic setup which, in turn regulated by environmental factors and habitat ecology. The *Papilio polytes* have three female polymorphic forms namely Stichius, Cyrus, Romulus and monomorphic male. During the survey period it has been observed that the abundance of Romulus form of female butterfly is not available in the study area. Due to the unavailability of Romulus, the Stichius & Cyrus and male butterfly have been examined in the present study.

Morphometric study of wing pattern and shape played a major role in taxonomic and evolutionary studies on Lepidoptera (Bookstein *et al*., 1999). It gives the information regarding independent characters of that particular species that shows the inter-intra specific variation (Monteiro *et al*., 1997). Detail morphology of the species is the key to working out eumaeine phylogeny (Balint and Moser, 2007). In this present investigation the external morphology (measurement & colouration of wings) of both polymorphic females (Stichius & Cyrus) and male *Papilio polytes* have been studied. The results showed morphological variation of polymorphic females (Stichius & Cyrus) and male *Papilio polytes*. The measurement of total wingspan of *Papilio polytes* showed the maximum wingspan in Stichius followed by Cyrus and finally the male. The morphometric variation of the wings is accompanied by its colour pattern among females and male. It has been speculated that the habitat and the environmental factors of eastern Himalaya of Arunachal Pradesh is the cause but not the reason of phenotypic variation as well as genetic distance and /or similarities observed in *Papilio polytes.*

Study of polymorphism in insect have played pivotal role in advancing our understanding of population dynamics, life history, evolution and the physiological basis of adaptation (Zera and Denno, 1997). The Color polymorphism of insects possesses considerable ecological significance (Forsman *et al*., 2008). In current era, the ecological geneticists emphasize genetic polymorphism as the most important mechanism of phenotype determination (Leimar, 2007). It is common that phenotypic variants can be introduced either by environmental or by allelic variation. In support of this content, the present study determines the genetic variation of polymorphic females and male *Papilio polytes* of Arunachal Pradesh and found corroborative with earlier research work.

The genetic effect is mainly reflected by qualitative variation such as protein polymorphism (Torbica and Mastilovic, 2008). In insects, the sex specific and developmental stage specific protein bands have been detected respectively in *Bombyx mori* (Ananthanarayana, 1980) and *Antherea mylitta* (Kumar *et al*., 2011). The haemolymph proteins of silkworms were analysed on PAGE and polymorphic variation of electrophoretic mobilities of storage proteins has been reported earlier (Lokesh and Anantha, 2011). In the present investigation, different proteins bands in SDS-PAGE were recorded in two polymorphic females as well as male *Papilio polytes.* It was observed that difference in number and intensity of protein bands appeared in between two polymorphic females of *Papilio polytes*. Male *Papilio polytes* showed distinctive protein bands which are presenr in neither of the females. Certain other protein bands are common in all three individuals (Stichius, Cyrus and male) but in different intensities. Thus, the present study speculated that band A is female specific wherein, band B, C and D are male specific in *Papilio polytes*. However, it is not clear if some of these proteins are either sex specific or responsible for polymorphic chracters.

Random amplified polymorphic DNA (RAPD) analysis is a technique for rapidly detecting genomic polymorphism. The ability on quick detection of genetic variability among or within the species this technique has become widely use to study the intra-inter specific relationship including silkworm (Ashwath *et al*., 2009), Cattle tick (Velayutham *et al*., 2012), butterfly (Sharma *et al*., 2006). In this present investigation RAPD-PCR has been employed on two polymorphic females (Stichius & Cyrus) and male of *Papilio polytes* butterfly using 14 different RAPD primers. There were no many information of particular suitable primers used in Papilionidae group of butterfly using PCR based techniques, except a few studies which are found (Hoole, *et at*., 1999, Zakharov, 2001) usable in this group. Among the 14 primer as mentioned elsewhere of this study, three primers RAPD1, RAPD6 and RAPD7 produced individual specific bands in polymorphic females (Stichius & Cyrus) and male. This result suggests that different nucleic acid bands could be the cause of phynotypic changes among females and male butterfly. The product of the cDNA synthesis produced by these three primers showed bands with varied molecular weight (bp) as well as different intensities of nucleic acid. Based on the band size the similarity coefficient calculated

exhibited resemblance among or within the male and female *Papilio polytes*. In recent years study of genetic diversity and drawing of phylogenetic tree using the similarity coefficient values among closely related species of butterflies have been carried out (Gallusser, *et al.*, 2004), grasses (Wang, *et al.*, 2006). In our study, it was observed that three specific primers i.e. RAPD1, RAPD6 and RAPD7 possess different similarity coefficient values due to their amplification specificity in male and polymorphic females. Depending upon the similarity coefficient of these primers, dendrogram were drawn elucidating the genetic relatedness between both females as well as male butterfly. The dendrograms revealed that both Stichius and Cyrus form of female were closely related; Cyrus form of female was closer with male than Stichius form of *Papilio polytes* butterfly. It was observed that among the RAPD-PCR bands produced by the three primers, few bands are polymorphic bands while, others are monomorphic. Depending upon the percentage of polymorphism (Table 7) it has been concluded that the primerRAPD1 was the most efficient in determining the genetic relationship among two polymorphic females (Stichius & Cyrus) and monomorphic male papilio polytes.

A number of similar studies have shown a considerable variation among and within the insect population (Beneck, 1998; Ashish, *et al.,* 2010; Chatterjee and Pradeep, 2003). The present study showed the genetic variation in both polymorphic females (Stichius & Cyrus) and male *Papilio polytes* available in the eastrn Himalaya of Arunachal Pradesh. This work is the first molecular analysis of *Papilio polytes* from India especially in the eastern Himalayan region of the subcontinent.

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References

Ali, I. and Basistha, S.K. 2000. Butterfly diversity of Assam state zoo-cum-botanical garden. Zoo's print. 15 (5): 264-265.

Ananthanarayana, S.R. 1980. In: Physiological and Biochemical studies in the silkworm (*Bombyx mori* L). Ph.D thesis, Bangalore University, Bangalore, India. Pp: 275.

Ashish, D.T., Sonali, V.P. and Vishal, P.D. 2010. Molecular characterization of morphologically similar four Pieridae butterflies (Lepidoptera: Insecta) by RAPD-PCR technique. Int J of Pharma and Biosciences. 2: 1-6.

Aubert, J., Barascud, B., Descimon, H. and Michel, F., 1997. Ecology and genetics of interspecific hybridization in the swallowtails, *Papilio hospiton* Gene and *P. machaon* L., in Corsica (Lepidoptera: Papilionidae). Biological Journal Linnean Society. 60: 467-492.

Ausubel, F.M., Brent, R., Kingston, R.R., Moore, D.D., Sideman, J.G., Smith, J.A. and Atruhl, K., 1999. In: Short protocol in molecular biology. John Wiley and Sons, Inc. 4: 15-37.

Balint, Z.S. and Moser, A. 2007. Description of a Denivia species from south and southest Brazil with notes on the genus (Lepidoptera, Lycaenidae: Eumaeine). Folia Entomologia Hungarica. 68: 147-156.

Beneck, M. 1998. Random amplified polymorphic DNA (RAPD) typing of necrophageous insects (Dipteral, Coleopptera) in criminal forensic studies: validation and use in practice. Forensic Science International. 98: 157-168.

Bhubaneshwari, M., Kananbala, A., Joymati, L., Ronikumar, L. and Binarani, A. 2012. Morphometeric measurement of Cabbage butterfly, *Pieris brassicae* Linn. (Lepidoptera: Pieridae) in the Agro ecosystem of Manipur. Int J Basic and App Med Sciences. 2 (3): 31-33.

Bookstein, F.L., Chernoff, B., Elder, R.L., Humphries, J.M., Smith, G.R., and Strauss, R.E., 1985. Morphometrics in Evolutionary Biology: the Geometry of Size and Shape Change. Special Publication No.15, Philadelphia Academy of Natural Sciences. Pp: 277

Bradford, M.M., 1976. A dye binding assay for protein. Animal Biochemistry. 72: 248-254.

Cilia, M., Fish, T., Yang, X., Mclaughlin, M., Thannbauser, T.W. and Gray, S., 2009. A comparison of protein extraction methods suitable for gel-based proteomic studies of Aphid protein. Journal of Bimolecular Techniques. 20: 201-215.

Chatterjee, S.N and Pradeep, A. R. 2003. Molecular markers (RAPD) associated with growth, yield and origin of the silkworm, *Bombyx mori* in India. Genetica. 39 (12): 1612-1624.

Clark CA and Sheppard PM. 1972. The genetics of the mimetic butterfly *Papilio polytes* L. Trans R Soc Lond Biol Sci. 263 (855): 431-458.

Dempster, J. and McLean, I., 1999 In: Insect populations: In theory and in Practice. Kluwer Academic Publishers, Boston, Massachusetts. Pp: 454

Dennis, R.L.H., 1993. In: Butterflies and Climate Change. Manchester University press, New York. Pp: 456.

Gallusser, S., Guadagnuolo, R. and Rahier, M., 2004. Genetic (RAPD) diversity between *Oleria onege agarista* and *Oleria onega* ssp. (Ithomiinae, Nymphalidae, Lepidopetra) in north-east Peru. Genetica. 121: 65-74.

Gossum, V.H., Bots, J., Heusden, V.J., Hammers, M., Huyghe, K., and Morehouse, N.I., 2011. Reûectance spectra and mating patterns support intraspecic mimicry in the colour polymorphic damsely *Ischnura elegans*. Evolutionary Ecology. 25: 139-154.

Guiney, M.S. and Oberhauser, K.S., 2008. Insects as flagship conservation species. Terrestrial Arthropod Reviews. 1: 111-123.

Hoole, J.C., Joyee, D.A. and Pullin, S.A. 1999. Estimates of gene flow between populations of the swallowtail butterfly, *Papilio machaon* in Broadland, UK and implications for conservation. Biological Conservation. 89: 293-299.

Kumar, D., Pandey, J.P., Jain, J., Mishra, P.K. and Prasad, B.C. 2011. Qualitative and quantitative changes in protein profile of various tissue of tropical silkworm, *Antheraea mylitta* Drury. Int J Zool Research. 7 (2): 147-155.

Linares, M.C., Soto-Calderon, I.D., Lees, D.C., Anthony, N.M. 2009. High mitochondrial diversity in geographically widespread butterflies of Madagascar: a test of the DNA barcoding approach. Molecular Phylogenetic Evolution. 50: 485-495.

Lokesh, G and Anantha Narayan, S. R. 2011. Changes in the protein profile of silkworm *Bombyx mori* L. (Lepidoptera: Bombycidae) in response to the chemical mutagen. International Journal of Science and Nature. 2 (3): 559-563.

Miesfeld, R.L. 1999. In: Rapid amplification of DNA. In Applied molecular genetics. A John Wiley & Sons Inc. Pp: 275.

Murthy, M.S., Sannaveerappanavar, V.T. and Shankarappa, K.S. 2014. Genetic diversity of Diammondback Moth, *Plutella xylostella* L. (Yeonomeutidae: Lepidoptera) populations in India using RAPD marker. J Entomol. 11 (2): 95-101.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., da Fonseca, G.A.B. and Kent, J. 2000. Biodiversity hotspots for conservation priorities. Nature. 403: 853-858.

Monteiro, A., Brakefield, P.M. and French, V., 1997. The relationship between eyespot shape and wing shape in butterfly *Bicyelus anynana*: A genetic and morphometrical approach. Journal evolutionary Biology. 10: 787-802.

Nagaraja, G.M. and Nagaraju, J., 1995. Genome fingerprinting in Silkworm, *Bombyx mori* using random arbitrary primers. Electrophoresis. 16: 1633-1638.

Rocha da, M.R.J., Almeida, R.J., Lins, A.G. and Durval, A., 2010. Insect as indicators of environmental change and pollution: A review of appropriate species and their monitoring. HOLOS Environment. 10: 250-262.

Schlotterer, C. 2004. The evolution of molecular markers-just a matter of fashion? Nature Reviews Genetics. 5: 63-69.

Sharma, V.L., Kumari, M., Gill, T.K., Sharma, S., Badran, A.A. and Sobti, R.C., 2003. RAPD-PCR in two species of *Catopsilia* (Pieridae: Lepidoptera). Caryologia. 56 (2): 219-222.

Sharma, V.L., Sobti, C.S., Tajinder, K.G., Kumari, S., Badran, A.A. and Kumari, M. 2006. Molecular studies of five species of butterflies (Lepidoptera: Insecta) through RAPD-PCR technique. Caryologia. 59 (3): 226-234.

Swaay, C.A.M. van, Brereton. T, Kirkland, P. and Warren, M.S. 2012. In: Manual of butterfly monitoring, Report VS 2012.10- De Vlinderstichting/ Duch Butterfly Conservation, Butterfly Conservation UK & Butterfly Conservation Europe, Wageningen. Pp: 17.

Torbica, A. and Mastilovic, J. 2008. Influence of different factors on wheat protein. Food Process Qual Safe. 35 (2): 47-52.

Velayutham, V., Shanmugavel, S., Munusamy, A. and Sundaram, J. 2012. Detection of genetic variability in various isolates of cattle tick, *Boophilus microplus* from Tamil Nadu, India using PCR-RAPD analysis. Exp Appl Acarol. 56 (4): 375-383.

Wang, L.J., Gao, B.Y., Zhao, N.X., Ren, A.Z., Ruan, W.B., Chen, L., Liu, J.L. and Li, C.L. 2006. Morphological and RAPD analysis of dominant species *Stipa Krylovii* Roshev in inner Mongolia steppe. Botanical Studies. 47: 23-35.

Werner, U. and Buszko, J., 2005. Detecting biodiversity hotspots using species-area and endemics-area relationships: the case of butterflies. Biodiversity and Conservation. 14: 1977-1988.

Williams, J.G., Kublecik, A.R., Liwak, K.J., Rafaski, J.A., Tinggey, S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful genetic marker. Nucleic Acid Research. 18: 6531-6535.

Zakharov, E.V. 2001. Natural hybridization between two swallowtail species *Parnassius nomion* and *Parnassius bremeri* (Lepidoptera: Papilionidae) shown by RAPD-PCR. Genetika-Moskv. 37 (4): 475-484.

Zera, A.J. and Denno, R.F. 1997. Physiology and Ecology of dispersal polymorphism in Insect. Ann Rev Entomol. 42: 207-230.