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EVIDENCE FOR VARIATIONS IN THE MORPHOMETRIC TRAITS BETWEEN TWO SIBLING SPECIES OF *Drosophila*: *D. ananassae* AND *D. pallidosa*

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Darwinian theory of evolution states that, evolution occurs through the natural selection. Therefore, demonstration of natural selection in nature is the central aim of many evolutionary studies and selection acts primarily at the phenotypic level because it is well known that phenotypic traits are the primary target of natural selection. While keeping this in view, we have studied certain morphometric traits in the sibling species pair, *D. ananassae* and *D. pallidosa* to test intra- and interspecific variations. The traits studied are wing length, thorax length, ratio of wing length and thorax length, sternopleural bristle number, ovariole number and sex-comb tooth number. In females of *D. ananassae*, significant strain differences were found for all the traits except ovariole number. In males, significant strain differences were found for all the traits. On the other hand, in *D. pallidosa*, significant strain differences were found for all the traits in both, males and females. The values of all the morphometric traits were significantly higher in females of both the species in comparison to males. The values of all the morphometric traits were higher in *D. ananassae*. However, the phenotypic variability, expressed in terms of coefficient of variation, was higher in *D. pallidosa*. Except for ratio of wing length and thorax length, CV was higher in the case of females in comparison to males. Size related traits are least variable while bristle numbers and reproductive traits are most variable. Except few, most of the traits are positively correlated with each other in both the species. Intra- and interspecific variations were found with respect to different morphometric traits. Although sibling species have been defined as morphologically identical, our results show that sibling species may show

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variations in certain morphometric traits and these quantitative differences in the morphometric traits act as discriminant marker between these sibling species in the lack of any qualitative differences

Keywords: *D. ananassae*, *D. pallidosa*, geographic strains, morphometric traits, sibling species

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INTRODUCTION

Darwinian theory of evolution states that, evolution occurs through the natural selection. Therefore, detection, demonstration and description of selection in nature are the central aim of many evolutionary studies (BRODIE *et al*., 1995) and selection acts primarily at the phenotypic level because it is well documented that phenotypic traits are the primary target of natural selection (LEWONTIN, 1974). Phenotypic variation is a universal characteristic of living organisms and is observed in a wide variety of traits across the populations and species (BELADE *et al*., 2005). The presence of naturally occurring phenotypic variation is at the core of evolutionary biology whereas, according to the classical typological view, it is considered as a nuisance (DEBAT and DAVID, 2001). Moreover, with the emerging recognition that the expression of phenotypic variations in most of the traits is influenced by both multiple genes and environmental factors, quantitative genetics has become central paradigm for the analysis of phenotypic variation and evolution (LYNCH and WALSH, 1998). Quantitative genetics aims to understand how genes and environment combine to determine phenotypic variation in population and also aims to link phenotypic variation to its underlying genetic basis (FALCONER and MACKAY, 1998). Due to its ubiquitous nature, evolutionary biologists were fretted about how does this variation arise? How do new variants evolve? What kind of molecular changes do they entail? What constraints variation? What are the phenotypic magnitudes and frequencies of origin and pleiotropic effects of mutations generating evolutionarily relevant phenotypic variations? This suggests that yet a century after Darwin; phenotypic variation is an almost unknown subject. The reason behind this is that, modern evolutionary biologists are hardly naïve about phenotypic variation, however, few have been interested (WADDINGTON, 1957) but on the whole, the subject of variation has remained peripheral to study of the mechanisms of evolutionary change at any level of the biological hierarchy (HALLGRIMSSON and HALL, 2005). As a result, understanding the nature of variation would remain a major problem within evolutionary theory from Darwin's time through the creation of the Modern Synthesis of evolution and natural selection and beyond (BOWLER, 2005). Therefore, in order to solve the enigma of phenotypic variation, a detailed study of variation is crucial for understanding the underlying evolutionary processes involved in the origin of variations because only a thorough – going study of variation will lighten our darkness. From many decades *Drosophila* has been employed as a successful model for quantitative genetic studies, most of which are focused on closely related species pairs with varying levels of divergence (COYNE and ORR, 1989; 1997). In the genus *Drosophila*, most of the species complexes (closely related species) contain pairs/groups of sibling species (PATTERSON and STONE, 1952). According to MAYR (1942), sibling species are those species which are morphologically similar or identical natural populations that are reproductively isolated. They are historically important in the study of speciation. Therefore, sibling species pairs that are in the early or incipient stage of speciation, offer a valuable material for quantitative evolutionary analyses (MORAES *et al*., 2004; KOPP and

FRANK, 2005). While keeping this fact in view, in the present study, we have focused on the sibling species pair: *D. ananassae* and *D. pallidosa*. In nature, one finds a number of sibling species pairs, out of which this pair attracted us most. This is because earlier they were light and dark forms of the same species (FUTCH, 1966) but later it was discovered that they are different species (BOCK and WHEELER, 1972; FUTCH, 1973). These two different statements given by Futch himself have goaded us the most. They are unique due to the presence of strong sexual isolation in sympatric situation but absence of post mating barriers such as hybrid inviability or sterility in the interspecific hybrids (OGUMA, 1993; SAWAMURA *et al*., 2008). Both these species belong to the *D. ananassae* species complex of the *ananassae* species subgroup of the *melanogaster* species group (BOCK and WHEELER, 1972). *D. ananassae* is cosmopolitan in nature whereas *D. pallidosa* is endemic to New Caledonia, Samoa, Tonga and Fiji Islands where these two species are sympatric (FUTCH, 1973; TOBARI, 1993). There are few studies involving this pair of sibling species which provided interesting information. However, this pair has not been employed for detailed study. Although *D. ananassae* has been extensively employed for genetical, behavioral and evolutionary studies by several workers particularly by Singh, Moriwaki and others (for references see SINGH, 2015; SINGH and YADAV, 2015), there are few studies in *D. pallidosa*. During the last four decades following studies were done in this sibling species pair: sexual isolation (FUTCH, 1973; YAMADA *et al*., 2002; VISHALAKSHI and SINGH, 2006), behavioral (SAWAMURA *et al*., 2006; 2008), fluctuating asymmetry in hybrids of sibling species (VISHALAKSHI and SINGH, 2009), morphometric traits (VISHALAKSHI and SINGH, 2008) and pattern of sex-combs in two sibling species and their hybrids (SINGH and SINGH, 2014).

Morphological characters have been used historically in evolutionary and taxonomic studies. Therefore, we have focused on the morphometric traits of both the sibling species by employing different geographic strains. Although morphometric traits of two sibling species were compared by VISHALAKSHI and SINGH (2008), this was only a preliminary study by taking one strain of each of the two species. Therefore, in the present study we have measured six morphometric traits in five strains of *D. ananassae* and three strains of *D. pallidosa*. Being cosmopolitan, it is already known that *D. ananassae* is heterogeneous but it will be worthwhile to know whether there are differences among different populations of *D. pallidosa* as they are endemic. Also, what is the level of differentiation between the sibling species? Differentiation has been investigated in certain sibling species pairs such as *D. persimilis* and *D. psueudoobscura* and *D. melanogaster* and *simulans* (see DAVID *et al*., 1983). But in these cases, the main interest was focused on the geographic variability of the mean values of various traits (between population variability) than on within population variability. So, main aim of our study is to compare the variability of laboratory populations of *D. ananassae* and *D. pallidosa* to resolve the following questions: How much variability is found in these two species? Are there variations among the different populations of *D. ananassae* as well as *D. pallidosa*.

To answer these questions, the variability between species and within species was investigated for six morphological traits: thorax length (TL), wing length (WL), wing/thorax ratio (W/T), sternopleural bristle number (SBN), sex-comb tooth number (SCTN) and ovariole number (ON) (Fig. 1). Results of these investigations are described in this communication.

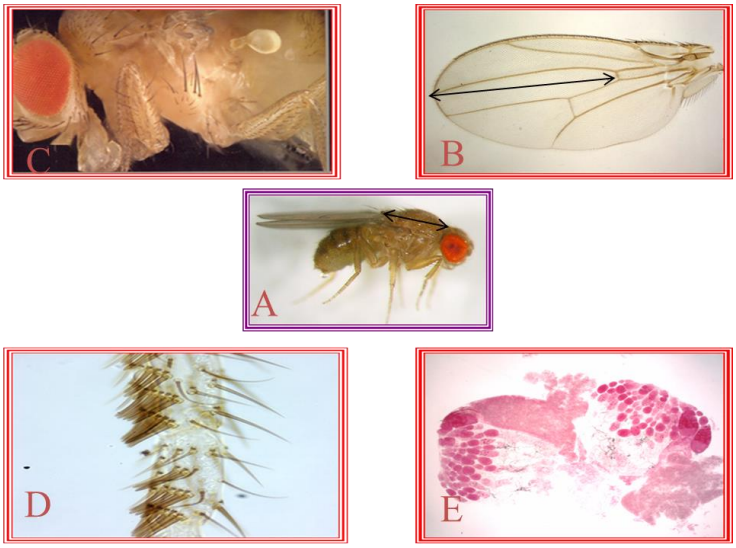

Figure 1. Different morphometric traits of *Drosophila* used in the present study. A-thorax length; B-wing length, C-sternopleural bristle number; D-sex-comb tooth number; E- ovariole number.

MATERIALS AND METHODS

Drosophila stocks

Five mass culture stocks of *D. ananassae*, established from flies collected from different geographic localities in India were used. These mass culture stocks are JU, MY, PC, DL and BR. Three wild type strains of *D. pallidosa* used are: NOU 88, NAN 66 and TBU 155 which were kindly provided by Prof. M. Matsuda of Kyorin University, Japan. Details of all these stocks of *D. ananassae* and *D. pallidosa* are given in Table 1. These stocks are being maintained in the laboratory on the simple yeast agar culture medium at approximately 24ºC following 12 hours cycle of light and darkness.

Table 1. Details of strains of D. ananassae and D. pallidosa used in the present study

Species	Strain	Place of origin	Time of Collection
D. ananassae	MY	Mysore	2000
	BR	Baripada	1987
	PC	Pondicherry	1999
	JU	Jammu	2006
	DL	New Delhi	2011
D. pallidosa	NOU 88	Noumea	NI
	NAN 66	(NewCaledonia)	NI
	TBU 155	Lautoka (Fiji)	NI
		Tongatapu	

NI=No information

Experimental design

To study the morphometric traits, 20 pairs of 7 days old females and males from each strain were transferred to culture bottles. From these bottles virgin females and males were collected and aged in vials which were further used to set the culture in food bottles for the experiments. Flies were kept for 3 days to allow them to oviposit and were then discarded. Virgin females and males from all the strains of *D. ananassae* and *D. pallidosa* were separated under anaesthesia and aged in vials. Different morphological traits (thorax length, wing length, W/T ratio, sternopleural bristle number, sex-comb tooth number and ovariole number) were scored in 100 individuals (50 males and 50 females) of 4-5 days old flies in all the five strains of *D. ananassae* and three strains of *D. pallidosa*.

Measurement of morphometric traits

Except thorax length, all the morphometric traits were measured on both left and right sides of an individual. Thorax length was measured from anterior end of the thorax to the posterior end of the scutellum at 50X magnification using ocular micrometer (1 unit =16.67μ). Thorax length of male and female was measured separately. Wing length was measured as the absolute length between the anterior cross vein to the distal tip of the third longitudinal vein, under a microscope at 50X magnification using ocular micrometer (1 unit = 16.67μ). Wing to thorax (W/T) ratio were calculated from the data of wing and thorax lengths. On the sternopleuron of males and females, two sets of bristles are present. Anterior bristles occur in an oblique row from the forecoxa towards the midline whereas the transverse bristles run in a thin line towards the centre of the fly just anterior to middle leg. The anterior and transverse sternopleural bristles were counted under stereo binocular at 25X magnification. The total number of sternopleural bristles was taken as the sum of anterior and transverse bristles. Females were etherized and then kept on a glass slide containing a drop of insect's saline (0.67% NaCl) and ovaries were dissected out with the help of needles under a zoom binocular microscope, stained with a drop of 2% acetocarmine stain for 2 minutes then washed and mounted in 45% acetic acid for proper visualization of ovarioles. Ovariole number was counted under a microscope at 50X magnification. Sex-comb in males of *D. ananassae* and *D. pallidosa* is characterized by several transverse rows of stout blackish bristles on the ventral surface of first, second, and third tarsal segments of prothoracic legs. Forelegs of males were dissected and mounted in insect's saline and number of the teeth on the first and second tarsal segments was counted under a microscope at 40X magnification.

Statistical analyses

To test whether there are significant intraspecific differences in each morphometric trait, in females and males of different strains of *D. ananassae* and *D. pallidosa*, comparisons were made by applying one–way ANOVA followed by post hoc analysis with Bonferroni t-tests for pair wise comparisons. Two-way ANOVA was applied to test sex and strain interactions in each morphometric trait of both the species. Student's t-test was applied to compare each morphometric trait in females and males of both the sibling species. Student's t-test was also applied to compare each morphometric trait between females and males of *D. ananassae* and *D. pallidosa* separately. The variability of each morphometric trait in females and males of *D. ananassae* and *D. pallidosa* was estimated by calculating coefficient of variation (CV). To

evaluate the correlation between different morphometric traits, correlation coefficients were calculated in females and males of *D. ananassae* and *D. pallidosa*.

RESULTS

Mean ± S.E values of different morphometric traits of *D. ananassae* and *D. pallidosa* females and males are given in Tables 2 and 3 respectively.

Table 2. Mean±S.E for five morphometric traits in different strains of D. ananassae and D. pallidosa females

Species	Strains	TL	WL	W/T	SBN	ON
D. ananassae	MYS	66.40±0.30	92.58±0.40	1.40±0.004	8.66±0.10	11.01±0.20
	BR	69.56±0.39	101.64±0.55	1.46±0.005	8.52±0.07	11.52±0.19
	PC	64.68±0.64	94.04±0.73	1.46±0.006	8.62±0.09	10.78±0.25
	JU	65.74±0.53	92.09±0.69	1.40±0.006	8.19±0.09	10.84±0.25
	DL	68.50±0.33	98.84±0.31	1.44±0.005	7.95±0.07	11.17±0.25
D. pallidosa	NOU 88	66.16±0.25	92.00±0.24	1.39±0.004	7.80±0.07	10.00±0.22
	NAN 66	61.48±0.30	81.16±0.29	1.32±0.004	6.82±0.06	9.4±0.20
	TBU 155	64.68±0.47	93.45±0.55	1.45±0.006	7.43±0.09	10.89±0.22

TL=thorax length, WL=wing length, W/T=ratio of wing length and thorax length, SBN=sternopleural bristle number and ON=ovariole number

Table 3. Mean±S.E for five morphometric traits in different strains of D. ananassae and D. pallidosa males

Species	Strains	TL	WL	W/T	SBN	SCTN
D. ananassae	MYS	57.18±0.47	79.88±0.46	1.40±0.007	8.18±0.12	36.02±0.37
	BR	61.80±0.48	89.11±0.61	1.44±0.005	8.45±0.09	29.84±0.34
	PC	59.80±0.47	87.68±0.52	1.47±0.006	8.23±0.08	33.41±0.46
	JU	59.34±0.40	83.09±0.46	1.40±0.005	7.79±0.08	35.84±0.45
	DL	60.58±0.20	86.54±0.30	1.43±0.004	7.48±0.06	35.74±0.39
D. pallidosa	NOU 88	60.78±0.23	82.64±0.23	1.36±0.005	7.36±0.07	30.33±0.36
	NAN 66	56.92±0.26	73.70±0.26	1.29±0.004	6.89±0.07	21.37±0.20
	TBU 155	59.20±0.34	83.86±0.43	1.42±0.006	7.25±0.08	32.97±0.45

TL=thorax length, WL=wing length, W/T=ratio of wing length and thorax length, SBN=sternopleural bristle number and SCTN=sex-comb tooth number

In females of *D. ananassae*, significant strain (intraspecific) variations were found in WL ($F=55.94$, d.f.=4, $P<0.001$), TL ($F=19.20$, d.f.=4, $P<0.001$), W/T ($F=37.46$, d.f.=4, $P<0.001$), SBN ($F=13.39$, d.f.=4, $P<0.001$) but not ON ($F=1.64$, d.f.=4, $P>0.05$) (Table 4). In

males of *D. ananassae*, significant differences were found in WL (F=60.11, d.f.=4, P<0.001), TL (F=16.82, d.f.=4, P<0.001), W/T (F=29.19, d.f.=4, P<0.001), SBN (F=18.69, d.f.=4, P<0.001) and SCTN (F=43.05, d.f.=4, P<0.001) (Table 4).

Table 4. Analysis of Variance for different morphometric traits among different strains of D. ananassae

Sex-Trait	Source of variation	SS	df	MS	F
Female-TL	Total	3379.86	249	-	-
	Between strains	806.54	4	201.63	19.20***
	Within strains	2573.32	245	10.50	
Female-WL	Total	7391.69	249	-	-
	Between strains	3528.50	4	882.13	55.94***
	Within strains	3863.19	245	15.77	
Female-W/T	Total	0.51	249	-	-
	Between strains	0.19	4	0.05	37.46***
	Within strains	0.32	245	0.00	
Female-SBN	Total	104.86	249	-	-
	Between strains	18.81	4	4.70	13.39***
	Within strains	86.05	245	0.35	
Female-ON	Total	677.98	249	-	
	Between strains	17.65	4	4.41	1.64^{NS}
	Within strains	660.33	245	2.70	
Male-TL	Total	2708.10	249	-	-
	Between strains	583.32	4	145.83	16.82***
	Within strains	2124.78	245	8.67	
Male-WL	Total	5644.16	249	-	-
	Between strains	2797.45	4	699.36	60.19***
	Within strains	2846.70	245	11.62	
Male-W/T	Total	0.54	249	-	-
	Between strains	0.17	4	0.04	29.19***
	Within strains	0.37	245	0.00	
Male-SBN	Total	128.08	249	-	-
	Between strains	29.95	4	7.49	18.69***
	Within strains	98.14	245	0.40	
Male-SCTN	Total	3392.03	249	-	-
	Between strains	1400.14	4	350.04	43.05***
	Within strains	1991.88	245	8.13	

***P<0.001, NS=Not significant

In females of *D. pallidosa*, there were significant variations in WL (F=309.04, d.f.=2, P<0.001), TL (F=45.71, d.f.=2, P<0.001), W/T (F=159.17, d.f.=2, P<0.001), SBN (F=39.66, d.f.=2, P<0.001) and ON (F=13.00, d.f.=2, P<0.001) (Table 5).

Table 5. Analysis of Variance for different morphometric traits among different strains of D. pallidosa

Sex-Trait	Source of variation	SS	df	MS	F
Female-TL	Total	1492.29	149	-	-
	Between strains	572.21	2	286.11	45.71***
	Within strains	920.08	147	6.26	
Female-WL	Total	5583.72	149	-	-
	Between strains	4510.87	2	2255.44	309.04***
	Within strains	1072.85	147	7.30	
Female-W/T	Total	0.591	149	-	-
	Between strains	0.404	2	0.20	159.17***
	Within strains	0.187	147	0.00	
Female-SBN	Total	69.88	149	-	-
	Between strains	24.49	2	12.25	39.66***
	Within strains	45.39	147	0.31	
Female-ON	Total	398.65	149	-	-
	Between strains	54.82	2	27.41	13.00***
	Within strains	206.65	147	2.11	
Male-TL	Total	956.83	149	-	-
	Between strains	376.57	2	188.29	47.70***
	Within strains	580.26	147	3.95	
Male-WL	Total	3830.33	149	-	-
	Between strains	3077.29	2	1538.65	300.36***
	Within strains	753.04	147	5.12	
Male-W/T	Total	0.59	149	-	-
	Between strains	0.40	2	0.20	161.05***
	Within strains	0.18	147	0.00	
Male-SBN	Total	46.33	149	-	-
	Between strains	6.04	2	3.02	11.02***
	Within strains	40.29	147	0.27	
Male-SCTN	Total	4590.77	149	-	-
	Between strains	3696.85	2	1848.43	303.97***
	Within strains	893.92	147	6.08	

***P<0.001

In males of *D. pallidosa*, there were significant differences in WL (F=300.36, d.f.=2, P<0.001), TL (F=47.70, d.f.=2, P<0.001), W/T (F=161.05, d.f.=2, P<0.001), SBN (F=11.02, d.f.=2, P<0.001) and SCTN (F=303.97, d.f.=2, P<0.001) (Table 5). Results of two-way ANOVA (Table 6) in *D. ananassae* revealed that there is sex and strain variations in mean values of different morphometric traits except W/T (sex variations being more pronounced than strain variations for all morphometric traits except W/T) and these variations are dependent on each other for all traits except SBN. The interaction between sexes and strains was found to be statistically significant for all the morphological traits except SBN. Whereas, in *D. pallidosa* sex wise variations were more pronounced than strain wise variations for TL and WL but for W/T and SBN it was just reverse (Table 7).

Table 6. Mixed model two- way Analysis of Variance to test the interaction between sex and strain for different morphological traits (TL, WL, W/T, and SBN) in D. ananassae

Traits	Source of variation	SS	df	MS	F
TL	Total	12699.88	499	-	-
	Sex (S)	6472.80	1	6472.80	662.19***
	Strain (ST)	1139.80	4	284.95	29.15***
	S x ST	297.57	4	74.39	7.61***
	Error	4789.70	490	9.77	-
WL	Total	27021.55	499	-	-
	Sex (S)	13985.70	1	13985.70	1021.25***
	Strains(ST)	5537.00	4	1384.25	101.07***
	S x ST	788.46	4	197.12	14.39***
	Error	6710.38	490	13.69	-
W/T	Total	1.04	499	-	-
	Sex (S)	0.00	1	0.00	1.84^{NS}
	Strain (ST)	0.35	4	0.08	63.21***
	S x ST	0.01	4	0.00	2.81*
	Error	0.68	490	0.00	-
SBN	Total	249.33	499	-	-
	Sex (S)	16.38	1	16.38	43.58***
	Strains(ST)	45.93	4	11.48	30.55***
	S x ST	2.83	4	0.71	1.88^{NS}
	Error	184.18	490	0.37	-

*P<0.05, ***P<0.001, NS=Not significant

The interaction between sexes and strains was found to be statistically significant for WL and SBN but it was statistically insignificant for TL and W/T. Further, to test the degree of variability we calculated the coefficients of variation (CV) for different morphometric traits in males and females of both the species. In *D. ananassae* females, CV ranged from 3.19 (W/T) to 14.91 (ON) whereas in males, CV ranged from 3.28 (W/T) to 10.80 (SCTN). In *D. pallidosa* females, CV ranged from 4.53 (W/T) to 16.23 (ON) whereas in males CV ranged from 4.29 (TL) to 19.66 (SCTN). In *D. ananassae* females (Fig. 2) CV for WL was greater in comparison to males whereas for W/T and SBN it was greater in males but CV for TL was equal in both males and females. In *D. pallidosa* females (Fig. 3), CV for TL, WL and SBN was greater in comparison to males. Whereas, for W/T it was greater in males. We found clear cut difference in CV values between the two sibling species (Fig. 4 and 5). Except TL, it was greater for all the traits in *D. pallidosa* females in comparison to *D. ananassae* whereas CV values were greater in *D. pallidosa* male for all the traits except TL and SBN. Except for W/T mean values of all the morphometric traits were significantly higher in females in comparison to males of *D. pallidosa* whereas in *D. ananassae* mean values were significantly lower for all the traits in females in comparison to males except WL. There were significant differences in various morphological traits between the two sibling species in both males and females (Table 8).

Table 7. Mixed model two-way Analysis of Variance to test the interaction between sex and strain for different morphological traits (TL, WL, W/T, and SBN) in D. pallidosa

Traits	Source of variation	SS	df	MS	F
TL	Total	4430.60	299	-	-
	Sex (S)	1981.47	1	1981.47	388.28***
	Strain (ST)	936.05	2	468.02	91.71***
	S x ST	12.74	2	6.37	1.25^{NS}
	Error	1500.34	294	5.10	-
WL	Total	15226.45	299	-	-
	Sex (S)	5812.40	1	5812.40	935.90***
	Strain (ST)	7519.83	2	3759.92	605.41***
	S x ST	68.33	2	34.17	5.50**
	Error	1825.89	294	6.21	-
W/T	Total	1.24	299	-	-
	Sex (S)	0.06	1	0.06	52.70***
	Strain (ST)	0.81	2	0.40	320.17***
	S x ST	0.00	2	5.23	0.04^{NS}
	Error	0.37	294	0.00	-
SBN	Total	117.21	299	-	-
	Sex (S)	2.34	1	2.34	8.11**
	Strain (ST)	26.48	2	13.24	45.87***
	S x ST	3.51	2	1.76	6.08**
	Error	84.88	294	0.28	-

**P<0.01, ***P<0.001, NS=Not significant

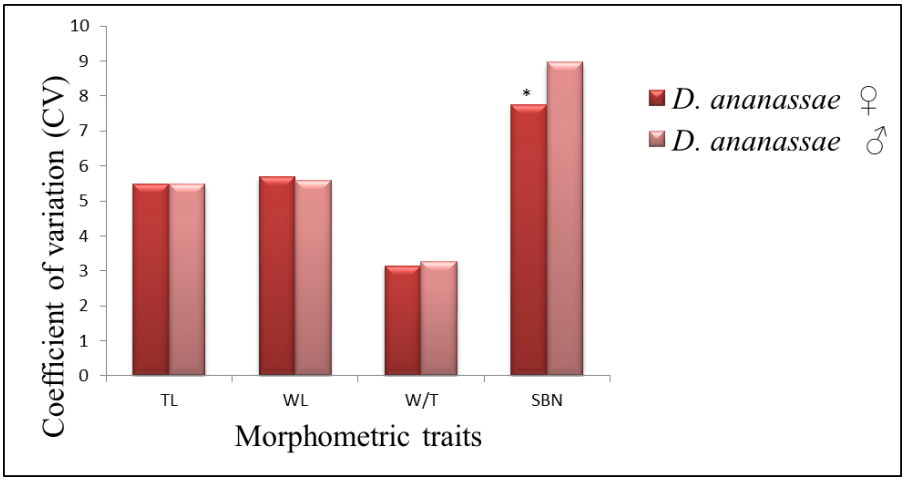

Figure 2. Phenotypic coefficient of variation (CV) for morphological traits in males and females of *D. ananassae*. TL-thorax length; WL-wing length; W/T-ratio of wing length and thorax length; SBN-sternopleural bristle. *P<0.001

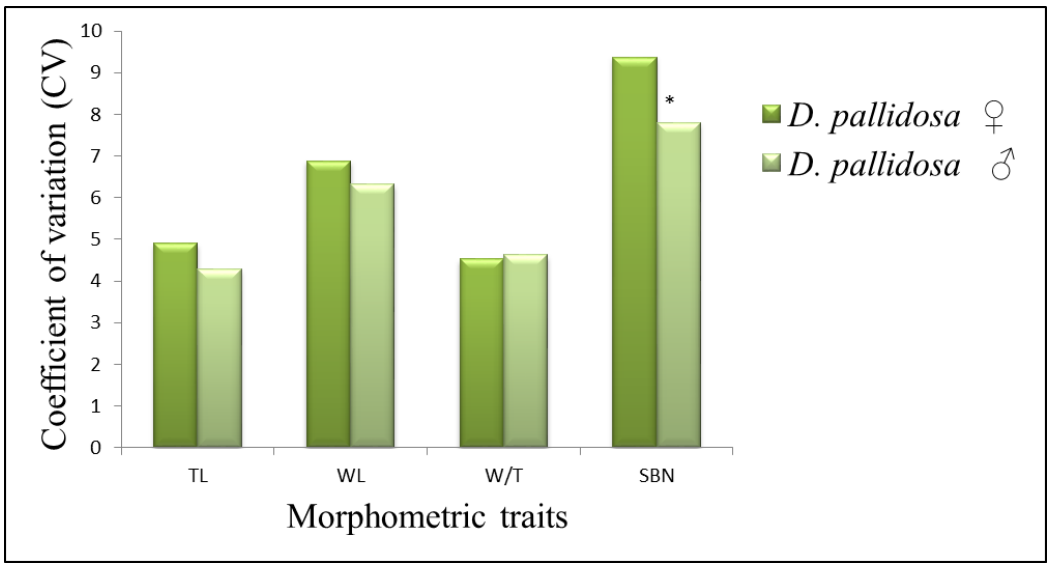

Figure 3. Phenotypic coefficient of variation (CV) for morphological traits in males and females of *D. pallidosa*. TL-thorax length; WL-wing length; W/T-ratio of wing length and thorax length; SBN-sternopleural bristle. *P<0.001

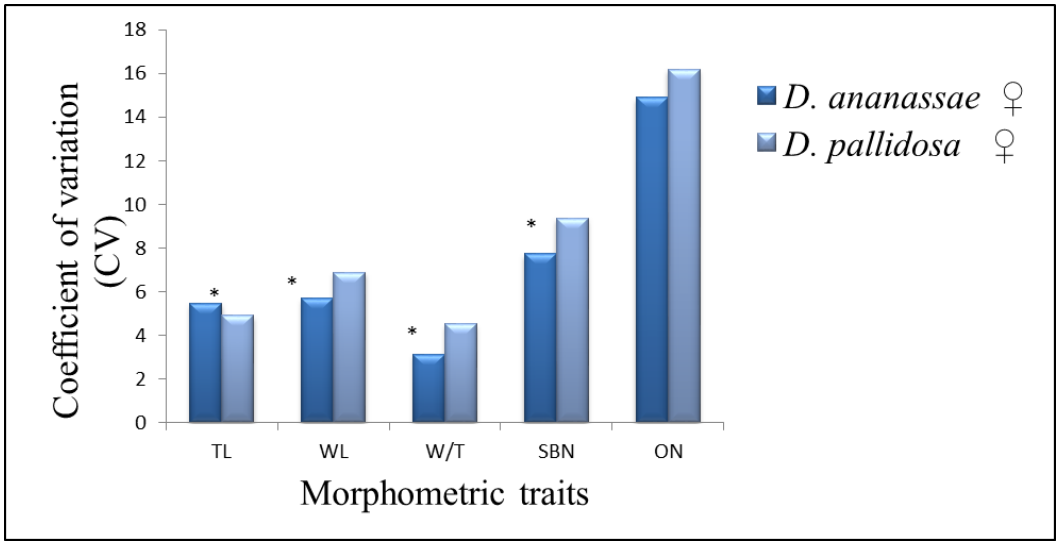

Figure 4. Phenotypic coefficient of variation (CV) for morphological traits in females of *D. ananassae* and *D. pallidosa*. TL-thorax length; WL-wing length; W/T-ratio of wing length and thorax length; SBN-sternopleural bristle; ON-overiole number. *P<0.001

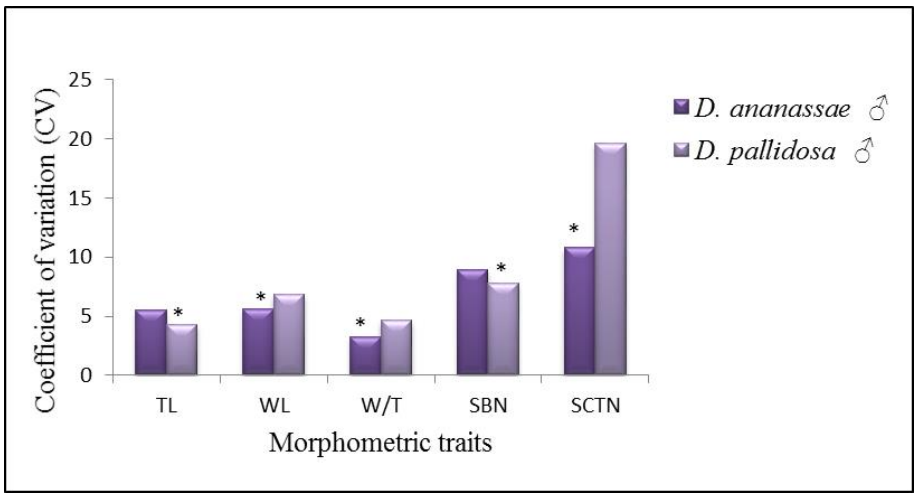

Figure 5. Phenotypic coefficient of variation (CV) for morphological traits in males of *D. ananassae* and *D. pallidosa*. TL-thorax length, W-wing length, W/T-ratio of wing length and thorax length, SBN-sternopleural bristle; SCTN-sex-comb tooth number. *P<0.001

Table 8. Results of student's t-test for comparing different morphometric trait: Females vs Males of D. ananassae and D. pallidosa both, Females vs Females between D. ananassae and D. pallidosa, Males vs Males between D. ananassae and D. pallidosa

Traits	Group	T	Df	*p*
TL	Females vs Males (*D. ananassae*)	23.08	498	<0.001***
	Females vs Males (*D. pallidosa*)	15.52	298	<0.001***
	Females vs Females	7.94	398	<0.001***
	Males vs Males	2.47	398	0.014*
WL	Females vs Males (*D. ananassae*)	23.12	498	<0.001***
	Females vs Males (*D. pallidosa*)	13.46	298	<0.001***
	Females vs Females	11.82	398	<0.001***
	Males vs Males	20.10	398	<0.001***
W/T	Females vs Males (*D. ananassae*)	1.10	498	0.271^{NS}
	Females vs Males (*D. pallidosa*)	4.10	298	<0.001***
	Females vs Females	8.43	398	<0.001***
	Males vs Males	12.93	398	<0.001***
SBN	Females vs Males (*D. ananassae*)	5.92	498	<0.001***
	Females vs Males (*D. pallidosa*)	2.54	298	0.012*
	Females vs Females	15.17	398	<0.001***
	Males vs Males	12.57	398	<0.001***
ON	*D. ananasssae* vs *D. pallidosa*	5.66	398	<0.001***
SCTN	*D. ananasssae* vs *D. pallidosa*	12.86	398	<0.001***

*P<0.05, ***P<0.001, NS=Not significant

Further, we tested the correlation of five different morphometric traits with each other (Table 9). In *D. ananassae*, TL was positively correlated with WL, SBN, ON, SCTN and negatively with the W/T; WL was positively correlated with W/T, SBN, ON and SCTN; W/T was negatively correlated with SBN and ON in females whereas in males it was negatively correlated with SCTN but positively correlated with SBN; SBN was positively correlated with ON but negatively correlated with SCTN. Similarly in *D. pallidosa*, TL was positively correlated with WL, W/T, SBN, ON and SCTN; WL was positively correlated with W/T, SBN, ON and SCTN; W/T was positively correlated with SBN, ON and SCTN; SBN was positively correlated with ON and SCTN.

Table 9. Correlation among different morphological traits in females and male of D. ananassae and D. pallidosa

Species	Traits	TL	WL	W/T	SBN	ON	SCTN
D. ananassae	Females						
	TL	1	0.85***	-0.26***	0.22***	0.45***	
	WL		1	0.29***	0.15**	0.42***	
	W/T			1	-0.12^{NS}	-0.03^{NS}	
	SBN				1	0.15*	
	ON					1	-
	Males						
	TL	1	0.84***	-0.29***	0.25***		0.09^{NS}
	WL		1	0.27***	0.25***		0.08^{NS}
	W/T			1	0.22***		-0.31***
	SBN				1		-0.11^{NS}
	SCTN					-	1
D. pallidosa	Females						
	TL	1	0.77***	0.08^{NS}	0.34***	0.28***	
	WL		1	0.69***	0.44***	0.38***	
	W/T			1	0.32***	0.19*	
	SBN				1	0.15^{NS}	
	ON					1	-
	Males						
	TL	1	0.72***	0.06^{NS}	0.26**		0.52***
	WL		1	0.74***	0.29***		0.84***
	W/T			1	0.18*		0.71***
	SBN				1		0.29***
	SCTN					-	1

*P<0.05, **P<0.01, ***P<0.001, NS=Not significant

DISCUSSION

Morphometric traits such as wing length, thorax length, sex-comb tooth number, ovariole number and sternopleural bristle number have been used as an index of body size, variations in morphometric traits have been the subject of many evolutionary studies, since it affects numerous life history traits (fecundity, mating success etc.) and may be a target of different evolutionary forces. This study has shown interspecific variations between two sibling

species, *D. ananassae* and *D. pallidosa*, with a recent origin of divergence (BOCK and WHEELER, 1972) as well as intraspecific variations among different strains of each of the two sibling species. Similar to this study, numerous quantitative characters have been investigated in geographical populations of *D. melanogaster* and its sibling species *D. simulans* (DAVID *et al*., 2004; GILBERT *et al*., 2004). It is evident from Tables 3 and 4 that all morphometric traits exhibit intra- and inter specific differences. In females of *D. ananassae*, significant strain wise differences were found for WL, TL, W/T and SBN but not for ON. In *D. ananassae* males, significant differences were found for all the traits studied (WL, TL, W/T, SBN and SCTN). In *D. pallidosa*, both females and males showed significant differences for all the traits studied (WL, TL, W/T, SBN, ON and SCTN).

In the present study, higher phenotypic variations were obtained for five laboratory populations of *D. ananassae* and three laboratory populations of *D. palidosa*. It is well documented that phenotypic variations of morphometric traits decline in the laboratory as compared to field samples (BRYANT and MEFFERT, 1998) because it is clearly established that bottlenecks depreciate variation within populations but still persistent variations indicate that there is some intrinsic factor, acting against the bottleneck like an action reaction to keep these strains differentiated even after a long periods of time spent in the lab. Since *D. ananassae* has passed longer time in laboratory in comparison to *D. pallidosa*. This shows that there is heterogeneity among different populations of *D. ananassae* as well as *D. pallidosa*. It is well known that *D. ananassae* is polytypic and heterogeneous species due to polymorphisms at the level of inversions and allozymes (SINGH, 2015). However, it is really remarkable and a matter of incredulity that in spite of being an endemic species, *D. pallidosa* exhibits intraspecific variations for all the morphometric traits in both the sexes. It may also be possible that since these three strains have come from three different islands which are widely separated from each other by sea (geographical barrier), they might have accumulated enough genetic differences and evolutionary forces have acted differently in these populations which are reflected in their morphological divergence. It is known that geographical barriers play very important role in allopatric speciation. Therefore, these three populations may be believed to be in incipient stage of allopatric speciation because differentiation among populations of the same species is an important requirement in the process of speciation. Thus, studies involving a variety of populations of both the sibling species, such as *D. ananassae* and *D. pallidosa*, are important in order to quantify the morphological divergence in relation to the genetic divergence because it is well known that chromosome polymorphisms have been associated with morphometric variations (REMIS *et al*., 2000). Since selection operates on phenotypic characters and some of them are modified due to genetic constituent, this interaction is central to the understanding of the parallel evolution of chromosome and phenotype variation. Whether they are still diverging will be unfolded in our future work when we conduct study on reproductive isolation and polytene chromosomes in these two species. Although it is difficult to predict the actual cause of these variations, it is hypothesized that the morphometric variations between and within the species reflect the expression of phenotype resulting from an integrated polygenic control, which is altered during cladogenesis (speciation) and evolution of groups (FALCONER, 1989). Additionally, several epigenetic and environmental factors can affect the formation of a structure (ATCHLEY *et al*., 1992), which makes it difficult to identify the causes of morphological divergence among populations and species. Morphological differences among natural populations are frequently attributed to natural selection but the role of non-genetic

modifications by the environment has been neglected (COYNE and BEECHAM, 1987). A population of one locality might adapt itself to the cyclic climatic changes associated with season, and undergoes morphological change by a rapid type of natural selection (see GURUPRASAD *et al.*, 2011). But according to ANDERSON (1973), morphological variation by a rapid type of natural variations may be simply a phenotypic response to environment, reflecting developmental plasticity or it may be partly or wholly genetic. GRIFFITH *et al*. (2005) predicted that these traits are expected to play important role in adaptation of flies to different environmental conditions. Furthermore, the present findings are contradictory to the findings of KITAGAWA *et al*. (1982) who have demonstrated the lack of genetic divergence of morphometric traits of different populations of *D. nasuta*. On the other hand, TAKANASHI and KITAGAWA (1977) have observed significant differences in the populations of the same species collected from different countries. In the view of the above, we can depict that these differences in morphometric traits might either reflect phenotypic plasticity or genotypic variability or interaction of both.

We have found statistically insignificant difference in ON of *D. ananassae* in contrast to *D. pallidosa* which is very surprising but it may be possible that during the last few decades ON of *D. ananassae* attained equilibrium and change is not taking place in this trait. Therefore, regarding ON in *D. ananassae*, there is optimization in the number of ovarioles favouring maximum egg production. On the other hand, ON in *D. pallidosa* is still diverging to attain optimum phenotype. Also, the *D. ananassae* stocks used in the present study are being maintained in the laboratory for a number of generations and are affected due to strong founder events. Consequently, *D. ananassae* lost its variability in ON or it may be due to lesser geographical distance separating geographic localities from where they were collected, in comparison to *D. pallidosa* because all the three populations of *D. pallidosa* are separated by geographical barrier in the form of sea but out of five strains of *D. ananassae* which were used in the present study, the geographic origins of three (PC, BR and MY) are very close to each other. Indian natural populations of *D. ananassae* are genetically differentiated and there is no correlation between the degree of genetic divergence and geographic distance (SINGH and SINGH, 2007). It is a cosmopolitan species endowed with high degree of genetic variability and adapted to various kinds of ecological conditions showing sub-structuring of populations due to action of different evolutionary forces as compared to its sibling which is endemic to South Pacific Islands (FUTCH, 1966, 1973; SINGH and SINGH, 2010). Recently, we have also reported that age wise variation is more pronounced in comparison to strain wise variation in *D. ananassae* but it is opposite to what is observed in *D. pallidosa*. One of the reasons for this may be less variation in ON of *D. ananassae* (SINGH and SINGH, unpublished)

Results of two-way ANOVA do not reflect definite pattern for the interaction of sexes and strains for different morphometric traits. From this, we can predict that variations in some traits are dependent on sex while in others variations are independent. Thus, we don't have any probable explanation in the support of results of two-way ANOVA.

The phenotypic variability, expressed in terms of the coefficient of variations, was higher in *D. pallidosa* than *D. ananassae*. This is contradictory to the findings of VISHALAKSHI and SINGH (2008). Although *D. ananassae* is a cosmopolitan species but still it exhibits less variability. This may be due to genetic drift as *D. ananassae* stocks used in the present study are being maintained in the laboratory for several generations whereas *D. pallidosa* stocks were provided by Prof. Matsuda in 2013. Except for W/T, CV was higher in case of females in

comparison to males in *D. pallidosa* but in *D. ananassae*, CV for W/T and SBN was higher in males in comparison to females while CV for WL was higher in females. This shows that *D. pallidosa* females are more heterogeneous compared to males but it is opposite to what is found in *D. ananassae*. However, GURUPRASAD *et al.* (2011) found that in *Drosophila*, males are more heterogeneous. On the other hand, MORETEAU *et al.* (2003) reported that in some species of *Drosophila*, CV was higher in females and in other species it was higher in males. Thus, pattern of CV among males and females varies from species to species and there is no uniform pattern of variation. We found that size related traits are least variable while numerical traits are more variable. We can suggest that size related traits are submitted to selection and developmental canalization, thus resulting in a low variability. It may be possible that reproductive traits do not undergo selection easily in comparison to size related traits because it is signature property of a species or strain so it may take longer duration for evolution to undergo selection in comparison to size related traits. Except few, most of the traits are positively correlated with each other in both the species suggesting that all the traits are genetically correlated (MORIN *et al.*, 1997). Such a strong correlation may be considered as a developmental constraint.

Further, the overall values of all the morphometric traits are significantly higher in females in comparison to males in *D. ananassae* as well as *D. palidosa*. Values of all the morphometric traits are also significantly higher in *D. ananassae* in comparison to *D. pallidosa* so we can say that phenotypic differences between males and females must arise from sex-specific differences in developmental programs, and interspecific differences must be the result of developmental alterations arising during evolution. Most of the workers have demonstrated an association between morphology and fitness components (SANTOS *et al*., 1992; NORRY *et al*., 1995). Therefore, flies with higher values of morphological traits show more adaptation. Thus, the present findings support the cosmopolitan occurrence of *D. ananassae*. In *Drosophila*, virility or male mating success shows a positive correlation with size related traits (SANTOS *et al*., 1992; NORRY *et al*., 1995; SISODIA and SINGH, 2004).

Our present findings strengthen the previous finding of VISHALAKSHI and SINGH (2008) but our present study is complete picture of that study because we have extensively used five strains of *D. ananassae* and three strains of *D. pallidosa* and found similar type of differences in all the traits. On the basis of the results of the present study, we can suggest that these differences in morphometric traits between both the species contributed to speciation by developing premating isolation because it is known that morphological divergence can contribute to speciation by promoting premating isolation (MCKINNON *et al*., 2004) and it is also know that this sibling species show premating isolation but absence of post mating isolation. In the other words, we can say that differences in morphometric traits cause premating isolation and because of premating isolation prevention of gene flow occurred and this prevention of gene flow ultimately causes speciation and if any how both this species by pass this obstacle (pre mating isolation which is caused by morphological differences) they will produce fertile and viable hybrids. So, it is evident that differences in morphometric traits play crucial role in evolution indirectly by contributing to speciation via promoting premating isolation. We found only quantitative differences in morphometric traits rather than any qualitative differences between these two sibling species and this is known that the lack of qualitative morphological differences among sibling species makes it difficult to establish the diagnostic morphological characteristics among them, the quantitative variations of morphometric traits are sufficient for discrimination of sibling species (MORETEAU *et al*., 2003). Therefore, we can conclude that these quantitative

differences in morphometric traits act as a discriminant marker between these two sibling species. Considering male genitalia as an important taxonomic trait, reasons behind identical male genitalia of sibling species and their potency of producing fertile and viable hybrids still need to be elucidated. *D. psuedoobscura* and *D. persimilis* are sibling species but still they have variation in male genitalia (see MAYR, 1942). BURLA *et al*. (1949) found variation in male genitalia and lack of hybrid in the *willistoni* group of sibling species. There are no obvious morphological differences in male genitalia of *D. aldrichi* and *D. wheeleri*, suggesting that their status as valid species is questionable (VILELA, 1983). Whereas, reciprocal crosses between *D. aldrichi* and *D. wheeleri* produced sterile males (PATTERSON and ALEXANDER, 1952). Similar to *D. aldrichi* and *D. wheeleri*, *D. ananassae* and *D.pallidosa* also have identical male genitalia but they produced normal viable and fertile hybrids. On the basis of the results of the present study, we can conclude that these two species have recently diversed and are still in the process of evolutionary divergence. These species have taken a step forward by preventing the gene flow by premating reproductive isolation because it is well documented that premating reproductive isolation is an early-acting isolating mechanism that arises due to incompatibility caused by different factors (NANDA and SINGH, 2012) and are expected to diverge in other aspect too (post mating) It may be possible that diversification of that aspect will be completed or appear after many years. Further study involving molecular genetics and ecological aspects may throw light on the mechanism of speciation in this unique pair of sibling species.

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VARIRANJE MORFOMETRIJSKIH OSOBINA IZMEĐU POLUSRODNIKA VRSTA *Drosophila*: *D. ananassae* I *D. pallidosa*

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Izvod

Darvinova teorija evolucije naglašava da se evolucija dešava kroz prirodnu selekciju. Zbog toga je demonstracija prirodne selekcija u prirodi glavni cilj mnogih evolucionih proučavanja, gde se selekcija primarno dešava na fenotipskom nivou, jer su fenotipske osobine primarni cilj prirodne selekcije. Imajući to u vidu, proučavali smo određene morfometrijske osobine kod para polusrodnika *D. ananassae* i *D. pallidosa*, kako bi testirali intra- i inter specifične varijacije. Proučavane su sledeće osobine: dužina krila, dužina toraksa, odnos dužine krila i dužine toraksa, broj sternopleuralnih čekinja, broj ovariola. Kod ženki *D. ananassae* utvrđene su značajne razlike za sve osobine osim za broj ovariola. Kod mužjaka, značajne razlike su utvrđene za sva svojstva. S druge strane, kod *D. pallidosa* utvrđene su značajne razlike za sva svojstva i kod ženki i kod mužjaka. Nivo morfometrijskih osobina je bio značajno viši kod ženki u odnosu na mužjake, kod obe vrste. Nivo morfometrijskih osobina je bio viši kod *D. ananassae*. Međutim, fenotipska varijabilnost, izražena kroz koeficijent varijacije, bila je viša kod *D. pallidosa*. Osim za odnos dužine krila i toraksa, CV je bio viši kod ženki nego kod mužjaka. Osobine vezane za veličinu su imale manju varijabilnost u odnosu na broj čekinja i reproduktivna svojstva, koja su bila najvarijabilnija. Većina osobina je bila u pozitivnoj korelaciji kod obe vrste. Intra- i interspecifične varijacije su utvrđene za različite morfometrijske osobine. Iako su polusrodnici definisani kao morfološki identični, naši rezultati su pokazali da polusrodne vrste mogu pokazati varijabilnost u određenim morfometrijskim osobinama i da ove kvantitativne razlike predstavljaju diskriminantni marker u nedostatku nekih kvalitativnih razlika.

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