RESEARCH ARTICLE

Multivariate morphometrics of elytral colour polymorphism in seven-spotted ladybird beetle, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae)

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Coccinella septempunctata **Linnaeus exhibits three kinds of elytral coloration in cereal agroecosystems, especially in wheat cultivated in the northern plains of India. A multivariate morphometric study was conducted to see if any difference exists in the morphological make-up of the three elytral colour morphs, using multivariate statistical analyses. The results showed that the observed elytral colour variation has a strong morphological basis. The major sources of morphological variation are mainly concentrated on the legs. Our study reveals existence of stable elytral colour polymorphic populations in *C. septempunctata*.**

Keywords: *Coccinella septempunctata*, elytra colour, polymorphism, morphometrics, multivariate analysis.

THE seven-spotted ladybird beetle, *Coccinella septempunctata* Linnaeus is a potential aphid predator found abundantly in cereal agroecosystems, especially in the wheat-cultivated belts of the North Indian plains[1]. The general assumption is that there is little variation in the elytral pattern of large coccinellids (e.g. *Coccinella septempunctata*). However, there is a potential scope for variation in this large coccinellid species, but it is rarely expressed[2]. Contrary to this general assumption, there were some observations[3,4] with records of the presence of variation in elytral colour patterns in *C. septempunctata*. In our study, we found three sympatric populations with distinct stable elytral colour patterns, viz. red, orange and yellow within this species in wheat-cultivated ecosystems in and around Delhi region. The population dynamics of these three-colour polymorphic populations showed a clear trend in abundance over years, hinting a strong presence of variability among them[4]. Differences within or among populations, whether geographically isolated or not, are commonly considered indicative of taxonomic distinctness[5]. Since these three colour variants of *C. septempunctata* are present in large numbers in the same area and at the same time, the differences could result from differential resource utilization as in other animals[6,7]. The variability can be intimately connected with fitness[8,9] and may indicate the fitness of a character in populations sharing the same niche. Therefore, comparison of variations within and among different populations indicates to what extent differences among individuals are moulded into the differences that separate races and species[8,10,11]. Further, the comparison of differences in variability of the same organs and structures in individuals of different populations will yield a key to determine the nature of influence of natural selection[10]. The morphometric variability is of fundamental importance in such variability studies[12,13]. External differences other than sexual dimorphism[14] among members of the same species are most commonly quantitative rather than qualitative, as in geographic variation[15], host forms[16], polymorphism[17] and social castes[18–21]. Insects are good subjects for studies on morphological variation[14,22,23] because the exoskeleton is easily measurable and largely free from physical distortion, and also the insect mode of life is reflected in the dimensions and shape of its exoskeleton[14].

All these points prompted us to study the morphometrics of *C. septempunctata*, which shows three kinds of elytral coloration. Since morphometrics is the measurement and analysis of form[14], the measured external characters need to be analysed with appropriate statistical tools. We used univariate and multivariate statistical methods for this purpose. Univariate ANOVA has been used to test for intergroup differences in insects[24–26]. However, univariate analyses are inadequate in the assessment of variation in natural populations[5], as they do not consider the possible correlations among characters in an individual in the populations[5,27,28]. Multivariate analyses were successfully used in the morphometric variation studies of insects such as springtails[29,30], mayflies[31],

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aphids[32–35], hymenopterans[36–38], beetles[28,39,40] and butterflies[41].

In this study we examine the morphological basis of the different elytral colorations in *C. septempunctata*. We discriminate the three elytral colour polymorphs into morphometrically distinct populations, through multivariate analyses. The study also assesses the fidelity of the characters which distinguish the three populations.

Materials and methods

Collection and preparation of specimens

Pre-pupal and pupal stages of *C. septempunctata* were collected from the wheat fields in Delhi region during February–March 2009. Pupae were individually kept in glass vials closed with muslin cloth, for adult emergence. The vials were maintained at 24°C, 75% RH and 16L : 8D photoperiod. After 24 h of emergence, the vials containing adult beetles were separated and grouped into red (R), orange (O) and yellow (Y). Only male adults were retained and fed with wheat aphids, through the adult stage. Though females also exhibit elytral colour polymorphism, we used only males for this study because of the following reasons: males emerged more in number from the culture, they have a lower adult longevity, less food requirement and their size make them amenable to microscopic observation. The whole process of adult rearing was carried out to see that the elytral colour was stable. After the death of the adults, the specimens were individually examined under a microscope to evaluate their uniformity and stability in coloration, suitability for morphometrics, especially with regard to their physical conditions like cleanliness and streching, completeness of all morphological parts and amenability for examination under the microscope. Such good specimens of nearly uniform and stable conditions were randomly selected to have 30 for each of the three kinds of elytral coloration, which were then grouped a priori as population R, O and Y. These were individually numbered and stored in microvials before undertaking morphometric analyses. All the specimens used in the study were stored in the National Pusa Collection (NPC), Indian Agricultural Research Institute (IARI), New Delhi.

Selection of characters

A total of 122 characters were selected taking into account the description of the species given by Sasaji[42]. All the taxonomic characters, both diagnostic characters listed as distinguishing key characters and the general descriptive taxonomic characters were considered while selecting them. The list of characters detailed part-wise is given in Table 1.

Measurement of characters

The linear measurements of 122 characters were taken using a Nikon SMZ10 stereozoom microscope fitted with an ocular micrometer. While measuring, a ruler was positioned next to the pinned specimen to ensure that it was always in a level plane or set uniformly every time at the same angle. Thus, care was taken to maintain uniformity and concordance of values. Measurements of the taxonomic characters were tabulated separately for these populations. The table of character matrices for each of the characters for the sample from the populations was then prepared.

Statistical analysis

All the values in the table of character matrices were $\log_{10}$ transformed before the statistical treatment. Univariate one-way single factor ANOVA was performed individually on all 122 characters to find out significant characters as a conservative mechanism of ascertaining significance before an overall treatment is recognized[43–45]. Coefficient of variation (CV) was calculated for all the characters to test for their stability. Then, the characters which showed significance at $P < 0.01$ were subjected to principal component analysis (PCA), multivariate analysis of variance (MANOVA) and discriminant function analysis (DFA). PCA is specifically designed to analyse sets of correlated variables[46]; it has no prior assumption of multiple groups and thus allows for their discovery[47]. In the present study, PCA was used as a dimension-reducing technique[48] and to investigate morphological variation[41,49]. MANOVA on the first seven principal components (PCs) was used to test for significant differences between groups[41,49], as it utilizes rather than ignores correlations among characters and it is the correct statistical test for evaluating overall group differences[5]. Since DFA maximizes variation among groups[28,41,50], we used it for separating the groups and assessing the utility of the characters used. Statistical analyses were carried out using MS Excel (version 12) for univariate ANOVA and CV; SPSS (version 11.5) for PCA and DFA; and SAS (version 9.2) for MANOVA.

Results and discussion

Univariate single factor one-way ANOVA results showed significant differences in 25 ($P < 0.01$) out of 122 characters analysed. CV values for these characters indicated their high stability. If we consider these 25 characters, it is evident that the maximum number of these is on the legs, followed by elytra, with nine and six characters respectively. The result indicates that to distinguish the populations, importance must be given to the study of characters on the legs and elytra.

All the 25 variables (significant characters) were subjected to PCA to reduce the number of dimensions and to

Table 1. List of characters used in morphometric studies

Ch. no.	Character
	A. Whole body
C1	Length of whole body on ventral side
C2	Breadth of body at middle across Elytra
C3	Length of body along elytral suture
	B. Head
C4	Length of head
C5	Distance between compound eyes
C6	Length of labrum
C7	Breadth of labrum
C8	Length of clypeus
C9	Breadth of clypeus
C10	Length of frons
C11	Breadth of head between antennae
C12	Breadth of frons
	C. Compound eye
C13	Area of compound eye
C14	Length of compound eye (horizontal)
C15	Breadth of compound eye (vertical)
	D. Antenna
C16-26	Length of first–eleventh antennal segment
C27-37	Breadth of first–eleventh antennal segment
	E. Maxillary palp
C38-42	Length of first–fifth segment of maxillary palp
43-47	Breadth of first–fifth segment of maxillary palp
	F. Thorax
C48	Length of pronotum at middle
C49	Breadth of pronotum at middle
C50	Length of pronotum at lateral margin
C51	Breadth of pronotum at anterior margin
C52	Length of scutellum
C53	Breadth of scutellum at base
C54	Distance between forecoxal margin and prosternal anterior margin
C55	Distance between mesocoxal margin and mesosternal anterior margin
C56	Distance between metacoxal margin and metasternal anterior margin
C57	Breadth of prosternum
C58	Breadth of mesosternum
C59	Breadth of metasternum
	G. Elytra
C60	Distance of first spot from lateral margin
C61	Distance of second spot from apex
C62	Distance of third spot from apex
C63	Distance of third spot from apex
C64	Distance of fourth spot from apex
C65	Area of first spot
C66	Area of second spot
C67	Area of third spot
C68	Area of fourth spot
C69	Breadth of elytra at base
C70	Breadth of elytra at middle
C71	Length of elytra at apex
C72	Length of elytra at middle
C73	Breadth of elytral epipleuron at base
C74	Breadth of elytral epipleuron at middle
	H. Leg
C75-77	Length of trochater, femur and tibia of foreleg
C78-80	Length of first–third tarsal segment of foreleg
C81-83	Breadth of trochater, femur and tibia of foreleg
C84-86	Breadth of first–third tarsal segment of foreleg
C87-89	Length of trochater, femur and tibia of middle leg
C90-92	Length of first–third tarsal segment of middle leg
C93-95	Breadth of trochater, femur and tibia of middle leg
C96-98	Breadth of first–third tarsal segment of middle leg
C99-101	Length of trochater, femur and tibia of hind leg
C102-104	Length of first–third tarsal segment of hind leg
C105-107	Breadth of trochater, femur and tibia of hind leg
C108-110	Breadth of first–third tarsal segment of hind leg
	I. Abdomen
C111-116	Length of first–sixth ventrite
C117-122	Breadth of first–sixth ventrite

find out major sources of variation. PCA indicated that the first seven PCs with eigenvalues more than one accounted for 66.3% variation (Table 2). Adequacy of data for the analysis was well supported by KMO measure (0.779) and Bartlett's test of sphericity ($\chi^2 = 820.556$; d$f = 300$; sig.0.000), showing that the extracted components accounted for a substantially large amount of variation. Out of the seven PCs, only PC1 and PC2 had the loadings (≥ 0.5) for more than one variable and the remaining five PCs had loadings for only one or none (Table 2). Therefore, PC1 and PC2 are the best components having been loaded with maximum information and would provide more meaningful interpretations for the present study. The unloaded characters, namely distance of first spot from lateral margin (C60), breadth of elytral epipleuron at base (C73), breadth of elytral epipleuron at middle (C74), length of femur of foreleg (76), breadth of femur of foreleg (82), breadth of sixth ventrite (122) are of lesser importance in explaining the morphological variation among the three populations of *C. septempunctata*.

Among the seven PCs, PC1 and PC2 had the highest contribution with 41.2% cumulative variance described. PC1 explains 24.0% of the total variation and has loadings for 12 characters, namely breadth of body at middle across elytra (C2), breadth of head between antennae (C11), breadth of third antennal segment (C29), length of elytra at apex (C71), length of trochanter of foreleg (C75), length of first tarsal segment of foreleg (C78), length of second tarsal segment of foreleg (C79), breadth of tibia of foreleg (C83), length of femur of middle leg (C88), length of tibia of middle leg (C89), length of second ventrite (C112) and breadth of first ventrite (C117). The second PC2 explains 17.2% of the total variation. PC2 has loadings for six characters, namely length of head (C4), breadth of anterior extension of pronotum (C51), breadth of scutellum at base (C53), breadth of

Table 2. Principal component loadings for 25 characters

Character no.	Component						
	1	2	3	4	5	6	7
C2	0.6340	–0.0680	0.0910	–0.1050	0.3690	–0.1020	0.2580
C4	0.4640	0.5130	–0.1830	–0.2010	–0.1270	0.0240	0.3210
C11	0.6080	–0.1530	0.4090	–0.0130	0.1020	0.0280	0.2660
C17	0.4760	–0.3140	0.0280	0.3380	–0.4070	0.1980	–0.2210
C29	–0.6860	0.2010	0.1210	0.1970	–0.1180	–0.1760	0.0250
C51	0.1290	0.6340	–0.3850	0.1650	–0.1190	0.0210	–0.2470
C53	0.3420	0.5420	0.4610	0.0760	–0.1800	–0.1480	–0.0860
C57	–0.0660	0.5730	–0.0450	0.3650	0.0660	–0.3530	0.2030
C60	–0.2290	0.4830	0.2710	0.0960	–0.0430	0.5220	–0.1850
C61	–0.1280	0.6860	–0.0130	–0.2370	–0.0070	0.2620	–0.0670
C71	0.5060	0.1630	0.4130	0.2660	0.1340	0.1950	–0.0740
C73	0.4670	0.3280	–0.2160	–0.2880	–0.2490	0.2520	0.2170
C74	–0.0890	0.4850	0.3510	–0.4840	0.0700	–0.3520	–0.1840
C75	0.6390	–0.1820	–0.0870	0.2030	0.2640	–0.2090	–0.3350
C76	0.1500	–0.3690	0.3210	0.2740	–0.3490	0.0460	0.4640
C78	0.6370	–0.2430	–0.0360	–0.2110	0.1080	0.0910	0.1320
C79	0.7200	–0.1570	–0.1480	0.0960	0.0040	0.2010	–0.1420
C82	–0.4160	–0.3070	0.0370	0.0100	0.5730	0.4860	0.0140
C83	0.5430	–0.3250	–0.2600	–0.2680	–0.0480	–0.0990	0.0010
C84	–0.0170	0.7980	–0.2230	0.1160	–0.0210	0.1700	0.1580
C88	0.6950	0.3070	–0.1390	0.2090	0.2670	–0.1160	–0.1010
C89	0.5510	0.4790	0.0990	0.1750	0.1880	–0.0680	–0.0940
C112	0.7520	0.3740	0.0170	–0.0220	0.0150	0.0920	0.1360
C117	–0.5420	0.4720	0.1530	–0.0950	0.2380	0.0620	0.1270
C122	0.4050	–0.0960	0.3040	–0.4070	–0.2350	0.0400	–0.3680
Eigen value	6.00	4.31	1.40	1.33	1.23	1.20	1.10
Percentage of variance described	24.01	17.26	5.59	5.31	4.94	4.79	4.41
Cumulative (%)	24.01	41.27	46.86	52.18	57.12	61.91	66.32

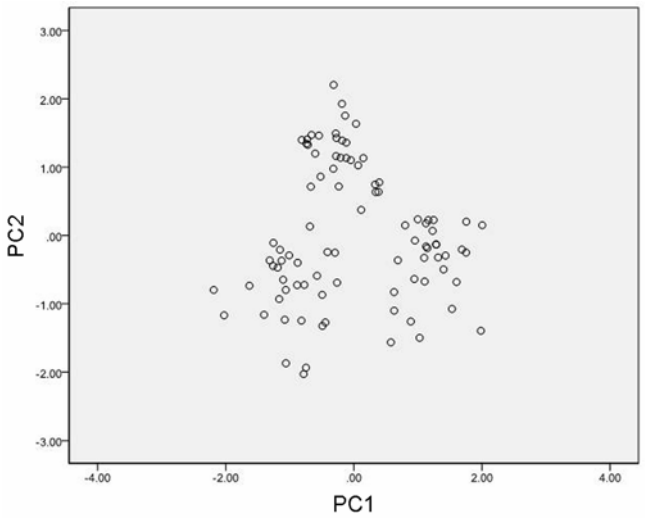

Figure 1. Scatter plot of the first two principal components (PCs) showing three distinct groups or clusters.

prosternum (C57), distance of second spot from apex of elytra (C61) and breadth of first tarsal segment of foreleg (C84). The other components, namely PC3, PC4, PC5, PC6 and PC7 explain 5.5%, 5.3%, 4.9%, 4.7% and 4.1% of the total variation respectively. These components have no significant factor loadings and have less contribution in explaining the variation in the population.

As the first two PCs accounted for 41.2% of variability in the populations, the characters which are loaded in these components can be considered as major sources of variation which differentiate the populations of *C. septempunctata*. PC1 has high degrees of correlation with length characters, whereas PC2 is correlated with breadth dimensions of the beetles. The PCA results show a trend of length versus breadth in differentiating the populations of *C. septempunctata* and this can further be explained by plotting the two components as in Figure 1, which clearly brings out three distinct groupings. It may be noted that PCA has no prior assumption of multiple groups as the data were not analysed by a priori grouping. PCA explores the data to bring out such groups, if they exist. Thus, there are three distinct group identified in the population by PCA (Figure 1).

The seven PCs were further analysed to test the significant differences between the three populations of *C. septempunctata*, by subjecting them to MANOVA. For this, the PCs were treated as a vector of dependent variables and the three populations taken as treatments. These analyses revealed that out of the seven PCs, only PC1 and PC2 (Table 3) showed significant effect (PC1: $F = 290.34$; d$f = 2, 72$; $P < 0.0001$; PC2: $F = 144.41$; d$f = 2, 72$; $P < 0.0001$), while the remaining five showed non-significant variations. MANOVA performed

Table 3. MANOVA results for dependent variable: PC-1 and PC-2

Source	DF	Sum of squares	Mean square	*F* value	Pr > *F*
PC-1					
Population	2	463.56	231.78	290.34	< 0.0001
Error	72	57.47	0.79		
Corrected total	74	521.047			
PC-2					
Population	2	246.97	123.48	144.41	< 0.0001
Error	72	61.56	0.85		
Corrected total	74	308.54			

Table 4. Results of combined MANOVA of seven PCs

Statistic	Value	*F* value	Num DF	Den DF	Pr > *F*
Wilks' λ	0.01	70.27	14	132	< 0.0001
Pillai's trace	1.75	69.82	14	134	< 0.0001
Hotelling–Lawley trace	15.22	71.00	14	102.29	< 0.0001
Roy's greatest root	9.27	88.75	7	67	< 0.0001

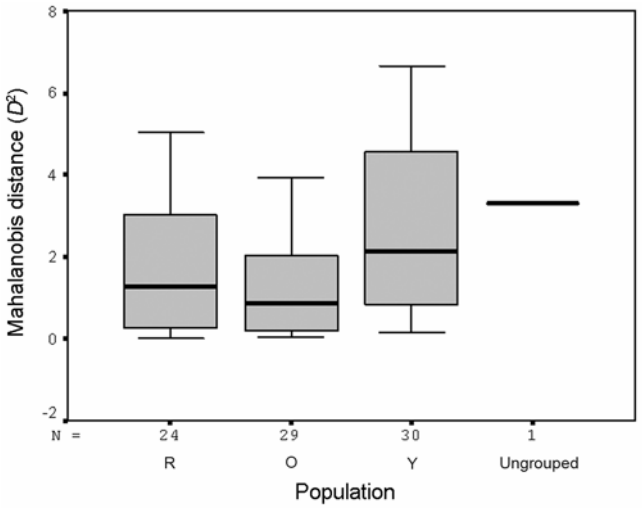

Figure 2. Mahalanobis distance (D^2) range and mean for three populations.

on all the seven PCs combinedly (Table 4) showed that the statistics, namely Wilks' λ, Pillai's trace, Hotelling–Lawley Trace and Roy's greatest root were significant at $P < 0.0001$. The statistics clearly depicts that the tested effects contribute significantly more to the model with very low Wilks' λ (0.01), which is closer to zero. This is also true with other statistics as Hotelling–Lawley trace value (15.22) is larger than Pillai's value (1.72) and Roy's value (9.27).

The canonical discriminant function analysis was carried out by a priori grouping, unlike PCA. The analysis produced two canonical discriminant functions (CDF1 and CDF2), which showed degrees of correlation with variables similar to PC1 and PC2. The Mahalanobis distance (D^2) of group members from group centroids was calculated to see the cohesiveness of members in a group. Figure 2 shows the range and mean D^2 values for each group. Members of the population O were closer to the group centroid with average D^2 value 1.33, followed by those of population R ($D^2 = 1.73$). Members of population Y were comparatively away from the group centroid ($D^2 = 2.56$). Thus, the members of the populations R and O are morphologically more cohesive within their respective groups, while those of population Y are a morphologically loosely knitted group. Euclidean distance (ED) between cluster centres showed that the three groups are well apart. The populations O and Y were more distinct with ED value 5.750, followed by R and O (ED = 5.597), whereas populations R and Y (ED = 4.289) were less distinct. The territorial map (Figure 3) shows clustering of three populations as distinct groups. This clustering confirms the grouping brought out by PCA (Figure 1). A cross-validation of group membership must be done to assess the utility of the morphological characters used in the analysis. In such a validation, each case is classified by the functions derived from all cases, other than that case. The results of cross-validation (Table 5) correctly identified 100% of populations R and O, and 96.7% of population Y. In the cross-validation, the group cases were classified to 98.7%, which shows the high degree of utility of the characters used in the study. Thus DFA, apart from distinguishing the three groups, proves the worthiness of the characters in classifying the populations. We measured the length and breadth of almost every segment of the body in our study. Therefore, the morphometric grouping along elytral colour has taken into account nearly the whole of the body. Among the three populations, the red-coloured population was recorded as the most abundant among the three polymorphs, followed by orange and yellow[4]. There appears a role of morphological fitness factor in the predominance of these three populations in the field. The two locomotive organs, viz. elytra and legs with maximum number of significant characters provide a clue that the three populations could differ in locomotive fitness. Prey and

Table 5. Classification results of cross-validation of group membership

			Predicted group membership			Total
Original	Count	Population	1	2	3	
		1	24	0	0	24
		2	0	29	0	29
		3	0	0	30	30
		Ungrouped	0	1	0	1
	%	1	100	0	0	100
		2	0	100	0	100
		3	0	0	100	100
		Ungrouped	0	100	0	100
Cross-validated	Count	1	24	0	0	24
		2	0	29	0	29
		3	0	1	29	30
	%	1	100	0	0	100
		2	0	100	0	100
		3	0	3.3	96.7	100

Note: 100% of original grouped cases correctly classified; 98.8% of cross-validated grouped cases correctly classified; 1, Population R; 2, Population O; 3, Population Y.

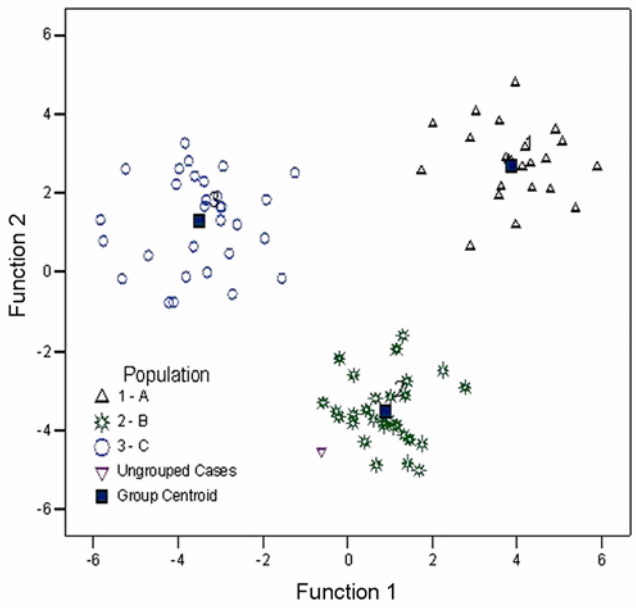

Figure 3. Territorial map showing plots of three populations.

mate-searching abilities are the most essential traits in the success of a predator[51–54]. These abilities are solely dependent upon the locomotive power of the beetle. *C. septempunctata* being a large-sized beetle, competition from other normally occurring co-inhabitant coccinellids is weakened[53]. Though the body size together with other traits gives a competitive edge over heterospecifics, there exists, under such conditions, an increasingly stiff competition among individuals of the large *C. septempunctata* population, especially in cereal ecosystems[4]. Our analysis (ANOVA) suggests that the three elytral colour polymorphic populations of *C. septempunctata* develop variability in locomotive organs to tide over the enormous internal population pressure.

Coccinellids, especially adults walk extensively on plant surfaces in search of prey. Foraging in coccinellids is mainly based on random-walk patterns[55], and prey detection only occurs with physical contact[56]. Intensive and extensive random walking enhances hitting success with aggregated prey[57]. Males appear to be searching mainly for females[58]. Olfactory and other associated cues could be directing the beetle towards the targets, but the physical execution is done by walking to reach the prey. Thus, legs of coccinellids assume an important position in searching and physically probing for prey. This could bring in a sort of selection pressure on the morphology and functioning of the legs. Apart from the leg morphometric dimension, other characters, viz. breadth of body at middle across elytra (C2), length of head (C4), breadth of head between antennae (C11), breadth of third antennal segment (C29), breadth of anterior extension of pronotum (C51), breadth of scutellum at base (C53), breadth of prosternum (C57), distance of second spot from apex of elytra (C61), length of elytra at apex (C71), length of second ventrite (C112), and breadth of first ventrite (C117) contribute to the overall body size variation among the three colour polymorphic populations. Body size has a direct relationship with prey density[59], dietary specialization[60] and foraging success[61] in coccinellids. The tracheal respiratory efficiency is highly dependent on body shape[62]. Thus, morphological variations in body size and shape drive a wide range of biological processes contributing to the fitness of populations. Large geographic intrapopulation variation[63] and variation in the size of the elytral spots[64] pointing to the presence of different populations were already observed in *C. septempunctata*. Hence, contrary to the general belief that *C. septempunctata* being a large coccinellid beetle lacks variation in elytral colour pattern, our study reveals the existence of stable elytral colour polymorphic populations in the species. Seasonal elytral colour polymorphisms were observed in other coccinellids[65], but in *C. septempunctata* all the three polymorphic populations

co-occur in wheat-cultivated ecosystems of Delhi region. The findings of our analysis prove that the elytral colour patterns have the potential to carry morphological variations and associated fitness consequences in the populations[66,67]. Some researchers found that elytral colour patterns in coccinellids were associated with chemical defensiveness to mates or predators[68], maintenance of melanic polymorphism by sexual selection[69] and coloration due to carotenoids of microbial (symbionts) origin, not of plant origin[70]. Thus, the elytral colour patterns in several coccinellids were found to be associated with many biological as well as ecological consequences. In *C. septempunctata*, we have shown that the elytral colour polymorphism is correlated with differences in other morphological traits.

Conclusion

It can be concluded that different elytral colorations in the populations of *C. septempunctata* do have a strong morphological basis, as the three populations get clearly discriminated morphometrically. Out of a total of 122 measured characters, our analyses brought out 25 highly significant characters (through ANOVA) and 18 major sources of variation (through PCA). Maximum morphometric variation is associated with the legs. Validation of utility of the morphometric characters which discriminate the three populations showed a high degree of fidelity. The results of our study provide a base for further investigations into the presence of selection pressure across these three elytral colour polymorphs and the associated fitness traits among populations. Further studies are required mainly on aspects such as cross-mating among the three populations and heritability of elytral colour, molecular studies and prey preference. From the application point of view, it is important to study whether these three populations differ in susceptibility to different pest management efforts, as *C. septempunctata* is a potential aphid predator in many agroecosystems.

Specimens used in the study: India: Delhi: 25-27.ii.2009; 11-24.iii.2009, Coll. A. Kalaisekar, det. V.V. Ramamurthy, on wheat, 30 specimens (R); 30 specimens (O); and 30 specimens (Y); (NPC, IARI, New Delhi).

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