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First comparative phenetic studies of the polymorphic species of *Coccinella undecimpunctata* Linnaeus, using morphometric and RAPD approaches in Egypt

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ABSTRACT

In this study, numerical phenetic method and RAPD-PCR technique were carried out on the species *Coccinella undecimpunctata* L. and its 13 aberrations which were collected from 10 localities in Egypt.

The morphometric analysis and phenotypic features were determined for the aberrations to reveal the phenetic relationships among them. The resulted phenogram showed high degree of variations and affinities (Similarity coeffeciant ranged from 44.7 to 85.7%). RAPD fingerprint profiles were generated by using 5 random primers on genomic DNA to evaluate their phenetic relationships and to investigate the molecular markers among the aberrations genotypes. The similarity coefficient of the produced DNA fragments ranged from 40% to 93.75%.

Cluster analysis based on both morphometric and RAPD data showed that the 14 morphs are grouped into 9 clusters against 8 clusters respectively. In addition, PCA plot allowed differentiating three groups from morphometric data against four groups from RAPD data.

Keywords: Phenetic taxonomy, Morphometric measures, RAPD-PCR, DNA polymorphism, Molecular markers, Aberrations, *Coccinella undecimpunctata*, Egypt.

INTRODUCTION

Coccinella undecimpunctata Linnaeus is one of the common species in Egypt; it is considered as an important predator of the eggs and the newly hatched larvae of cotton leaf worm and aphids. It is characterized with black color, orange elytra spotted with 11 black spots [Dobzhansky, 1933; Ibrahim, 1955 and Brown, 1962]. The race of *C. undecimpunctata* L. is widely distributed all over the country where it occurs all the year round, occasionally assuming migratory habits, 14 aberrations are listed in [Alfieri, 1976] according to the absence of some spots or fused with the others on the elytra. The interpretation of the taxonomic positions of these aberrations and the relationships among them is equivocal, and no clear taxonomic or molecular treatments made among them.

Although the external morphology of adult Coccinellidae is fairly simple, their identification can -in some cases- be surprisingly difficult because of the variability within many species, especially in coloring [Harde, 1981].

Traditionally, identification of the aberrations of *C. undecimpunctata* L. is based on morphological traits which are very difficult among closely related taxa. Recently, the numerical phenetic taxonomy and the (RAPD) molecular studies have added powerful tools for studying and evaluating the variation and the genetic structure of the different taxa of most organisms.

Numerical phenetic taxonomy has been used in classification of insects by the phenetic method such as mosquitoes, honey-bees, chewing lice, sand flies and soldier flies [Belkin *et al.*, 1980; Rinderer, 1988; Zlotorzycka *et al.*, 1989; Bermudez *et al.*, 1993 and Badrawy *et al.*, 2006].

The molecular markers were applied to identify different insects, such as aphids [Black *et al.*, 1992], *Aedes* spp. [Kambhampati *et al.*, 1992], strains of Mediterranean fruit fly [Haymer & Me Innis, 1994], termite casts [El-Gohary *et al.*, 2000] and *Coccinella septumpunctata* [Haubruge, 2002] as well as to identify different plants such *Acacia* (Fabaceae) [Casiva *et al.*, 2002]. The advantages of RAPD analysis are its simplicity and rapidity, the sample requirement is only for small quantity of DNA, the ability to generate numerous polymorphisms [Cheng *et al.*, 1997]. Therefore, it becomes a good technique for genetic analysis [Wight *et al.*, 1993 and Tsai *et al.*, 2002].

The present study is the first attempt for applying the two techniques (numerical phenetic method and RAPD molecular analysis) on the aberrations to compare between them and to show the advantages in the combination of two taxonomic studies against one-dimensional taxonomy.

MATERIAL AND METHODS

Phenetic taxonomy:

The morphometric study was carried out on the species *Coccinella undecimpunctata* L. and its 13 aberrations. Materials for morphological investigations were collected from 10 localities of Egypt (table $1_{a\&b}$). To determine the phenetic relationships between all specimens available, the phenotypic features were examined as: the presence or absence of spots, the fusion of spots with the others, the distances between the spots and the diameter of spots on the elytra.

The specimens were collected by an aerial net, then by aspirator, and killed by ethyl acetate and preserved in 100 % alcohol. Numbers of specimens were pinned, labeled, provided with date and site of collection and kept in Ain Shams University Collection (ASUC).

Measurements of insect body parts were made with a calibrated ocular lens standardized at 100 units (ocular micrometer) using a stereomicroscope at magnification 100x to 400x. Genitalia were macerated in 10% KOH at room temperature for one day to remove soft tissue, then rinsed and dissected in 75% ethanol with drops of glycerin.

Morphological terminology follows Sharp and Muir, 1912; Wilson, 1930; Stehr, 1930; Dobzhansky, 1933; Ibrahim, 1948; Khnzorian, 1979; El-Akkad, 1979 and Sathe & Bhosale, 2001. The species *C. undecimpunctata* L. and its aberrations were identified and confirmed according to Weise, 1892; Bovie, 1897; Luigioni, 1933; Brown, 1962; Khnzorian, 1979; El-Akkad, 1979 and Blaza, 1984.

Phenetic method by choosing (55) morphological characters, with (110 states) was described in the 14 morphs of *C. undecimpunctata* [Operational Taxonomic Units (OTUs)]. The characters were chosen in male and female adult stages to reveal the phenetic relationship. Phenetic analysis is elaborated by program [PROBIOSYS, version 1.0, (2003)] depending on Single linkage, UPGMA, Complete linkage clustering methods in numerical taxonomy, Cophenetic correlation value. To identify the most important morphological characters to differentiate among the aberrations, the procedure of PCA analysis (three-dimension plot) is applied.

Table (1_a). The different localities of *C. undecimpunctata* L. their morphs surveyed in Egypt and their date of collections.

Morphs	Localities	Date of collections
1- ***Coccinella undecimpunctata*** L. (**sp.**)	El-Fayoum	Mar. & Apr. 2004-2008
	El-Menya	Apr. & May. 2005-2007
	Qena	Apr. 2008
	Banha	Feb., Mar. & Apr. 2007
	Siwa Oasis	Jul. 2007
2- ***C. 11-punctata*** ab. ***aegyptiaca*** Reiche (**ab.1**)	El-Fayoum	Mar. & Apr. 2004-2008
	El-Menya	Apr. & May. 2005-2007
	Qena	Apr. 2006-2008
	Banha	Feb., Mar. & Apr. 2007
	Siwa Oasis	Jul. 2007
	Assiut	Apr. 2006
	Hurghada	Apr. 2001 & May. 2007
	El-Kharga	Aug. 2008
	Alexandria	Jun. 2005 & Jul. 2008
	Ismaileya	Mar. 2007
3- ***C. 11-punctata*** ab. ***maculate*** Walter (**ab.2**)	El-Fayoum	Mar. & Apr. 2005-2007
	El-Menya	Apr. 2005 & Apr. 2007
	Qena	Apr. 2008
	Banha	Aug. 2007
	Siwa Oasis	Jul. 2007
	Assiut	Apr. 2006
	El-Kharga	Aug. 2008
4- ***C. 11-punctata*** ab. ***tamaricus*** Weise (**ab.3**)	El-Fayoum	Mar. & Apr. 2005-2007
	El-Menya	Apr. 2007
	Qena	Apr. 2008
	Banha	Apr. & Aug. 2007
	Assiut	Apr. 2006
5- ***C. 11-punctata*** ab.4 (**ab.4**)	El-Fayoum	Mar. & Apr. 2004-2007
	El-Menya	Apr. 2007
	Qena	Apr. 2008
	Banha	Mar. & Apr. 2007
	Siwa Oasis	Jul. 2007
6- ***C. 11-punctata*** ab. ***brevifasciata*** Weise (**ab.5**)	El-Fayoum	Dec. 2007
	El-Menya	Apr. 2007
	Qena	Apr. 2008
	Banha	Apr. & Aug. 2007
7- ***C. 11-punctata*** ab. ***confluens*** Haworth (**ab.6**)	El-Fayoum	Dec. 2007
	El-Menya	Apr. 2007
	Qena	Apr. 2008
	Banha	Mar. & Apr. 2007
8- *C.* ***11-punctata*** ab. ***novempunctata*** L. (**ab.7**)	El-Fayoum	Dec. 2007
	El-Menya	Apr. 2007
	Qena	Apr. 2008
	Banha	Aug. 2007
	Siwa Oasis	Jul. 2007
9- ***C. 11-punctata*** ab. ***bigara*** Mader (**ab.8**)	El-Fayoum	Dec. 2007
	Siwa Oasis	Jul. 2007
10- ***C. 11-punctata*** ab.9 (**ab.9**)	El-Fayoum	Mar. 2006 & Dec. 2007
11- ***C. 11-punctata*** ab.10 (**ab.10**)	El-Fayoum	Dec. 2007
12- ***C. 11-punctata*** ab.11 (**ab.11**)	Siwa Oasis	Jul. 2007
13- ***C. 11-punctata*** ab. ***oculata*** Thunberg (**ab.12**)	Hurghada	Apr. 2001
14- ***C. 11-punctata*** ab.13 (**ab.13**)	El-Menya	Apr. 2007

- Both sexes (♂♂ & ♀♀) are collected from the 12 morphs, while the ab.10 which collected ♀♀ only and ab.13 which collected ♂♂ only.

Table (1_b). The localities and abbreviations.

Localities	Abbreviations
1- El-Fayoum	(Fa.)
2- El-Menya	(Me.)
3- Qena	(Qe.)
4- Banha	(Ba.)
5- Siwa Oasis	(Si.)
6- Assiut	(As.)
7- Hurghada	(Hu.)
8- El-Kharga	(Kh.)
9- Alexandria	(Al.)
10- Ismaileya	(Is.)

RAPD-PCR technique:

Samples preparation and DNA extraction:

Specimens of different aberrations of *C. undecimpunctata* L. were obtained from 10 localities. Starved beetles were preserved by freezing at -20 ºC until used. The method of prepared samples was described by Haymer and Me Innis (1994). The genomic DNA was extracted from different morphs and the whole body tissues of insects were used for DNA extraction according to Hunt & Page (1995). The DNA pellet was dissolved in 30-50 µl of TE buffer and storage at -20 ºC until used. The concentration of DNA was determined by spectrophotometric method using UV visible scanning spectrophotometer (UNICAM UV/Vis spectrometer).

Amplification of DNA by PCR:

For DNA amplification, 5 random decanucleotides [Operon Technologies (Kit C and Kit K)] (table 5) were used for screening genomic DNA from the polymorphic species, *C. undecimpunctata* L.

RAPD-PCR amplifications were performed in a total volume of 25 µl containing 10mM Tris-HC1 pH 8.3, 50 mM KC1, 1,5mM $MgC1_2$, 100 µM dNTP, 10 pM primer, 1.5 U *Taq* polymerase and 25 ng genomic DNA. Amplifications were carried out in a thermocycler (Primus-Germany): first cycle 94°C for 5 min, 36°C for 2 min and 72°C for 3 min; then 39 cycles at 94°C for 1 min, 36°C for 1.5 min and 72°C for 2 min. PCR products were separated in 1.5% TAE agarose gels. Gels were run at 5 V/cm for 3 hours along with the 1 kb ladder DNA size marker (ABgene) [ranged from 0.25 to 10.0 kb.], stained with ethidium bromide and photographed under UV light by using digital camera (Canon, Power Shot A460, 5.0 Mega Pixels).

Data analysis:

Amplified RAPD markers were scored as either present (+) or absent (-) for each aberrations. Ambiguous bands that could not be easily distinguished were not scored. The similarity of samples was calculated as follows: similarity = $2N_{AB}/(N_A+N_B)$, where N_{AB} is the number of bands shared by individuals A and B, and N_A & N_B are the number of bands in individuals A and B, respectively (Nei and Li, 1979). By using the computational program "PROBIOSYS" [version, 1.0, (2003], a dendrogram was constructed based on the data of similarity matrix, using the unweighted pair-group method analysis (UPGMA) [Sneath and Sokal, 1973]. Genetic distances were calculated by the following formula: (genetic distance = 1- similarity coefficient) according to (Nei and Li, 1979). Relationships among the morphs were also evaluated by a PCA analysis.

RESULTS

Phenetic taxonomy:

The aberrations of *C. undecimpunctata* L. are numerous in Egypt according to the states of the elytral spots or the color of pronotum and scutellum. These aberrations are widely distributed allover the country where they occur all the year round, occasionally assuming migratory habits.

The aberrations can be listed as follows:

All the aberrations differ from the *C. undecimpunctata* L. by the posterior extension of the two whitish yellow (cream white) patches on the posterior angles of the pronotum.

[Figure (1) Elytral color patterns, Figure (2) Male genitalia and Figure (3) Female receptaculum seminis (=spermatheca)].

(ab.1) *C. undecimpunctata* ab. *aegyptiaca* Reiche
Body width from shoulders shorter than half body length, elytral width longer than 2 times length of pronotum.

(ab.2) *C. undecimpunctata* ab. *maculata* Walter
The humeral spot absent, the marginal and apical spots fused.

(ab.3) *C. undecimpunctata* ab. *tamaricis* Weise
The marginal and apical spots fused.

(ab.4) *C. undecimpunctata* ab.4
All spots surrounding with the yellowish circles except the humeral spot.

(ab.5) *C. undecimpunctata* ab. *brevifasciata* Weise
The lateral and discal spots fused, the marginal and apical spots fused.

(ab.6) *C. undecimpunctata* ab. *confluens* Haworth
The lateral and discal spots fused.
(ab.7) *C. undecimpunctata* ab. *novempunctata* Linnaeus
The humeral spot absent.
(ab.8) *C. undecimpunctata* ab. *bigara* Mader
Elytra with large discal spot and very small other spots.
(ab.9) *C. undecimpunctata* ab.9
Elytra with small spot beside the lateral spot.
(ab.10) *C. undecimpunctata* ab.10
The humeral spot absent, the lateral and discal spots fused, the marginal and apical spots fused.
(ab.11) *C. undecimpunctata* ab.11
It differs from the typical *undecimpunctata* by the brown pronotum and scutellum.
(ab.12) *C. undecimpunctata* ab. *oculata* Thunberg
The apical and scutellar spots absent.
(ab.13) *C. undecimpunctata* ab.13 The discal spot absent.

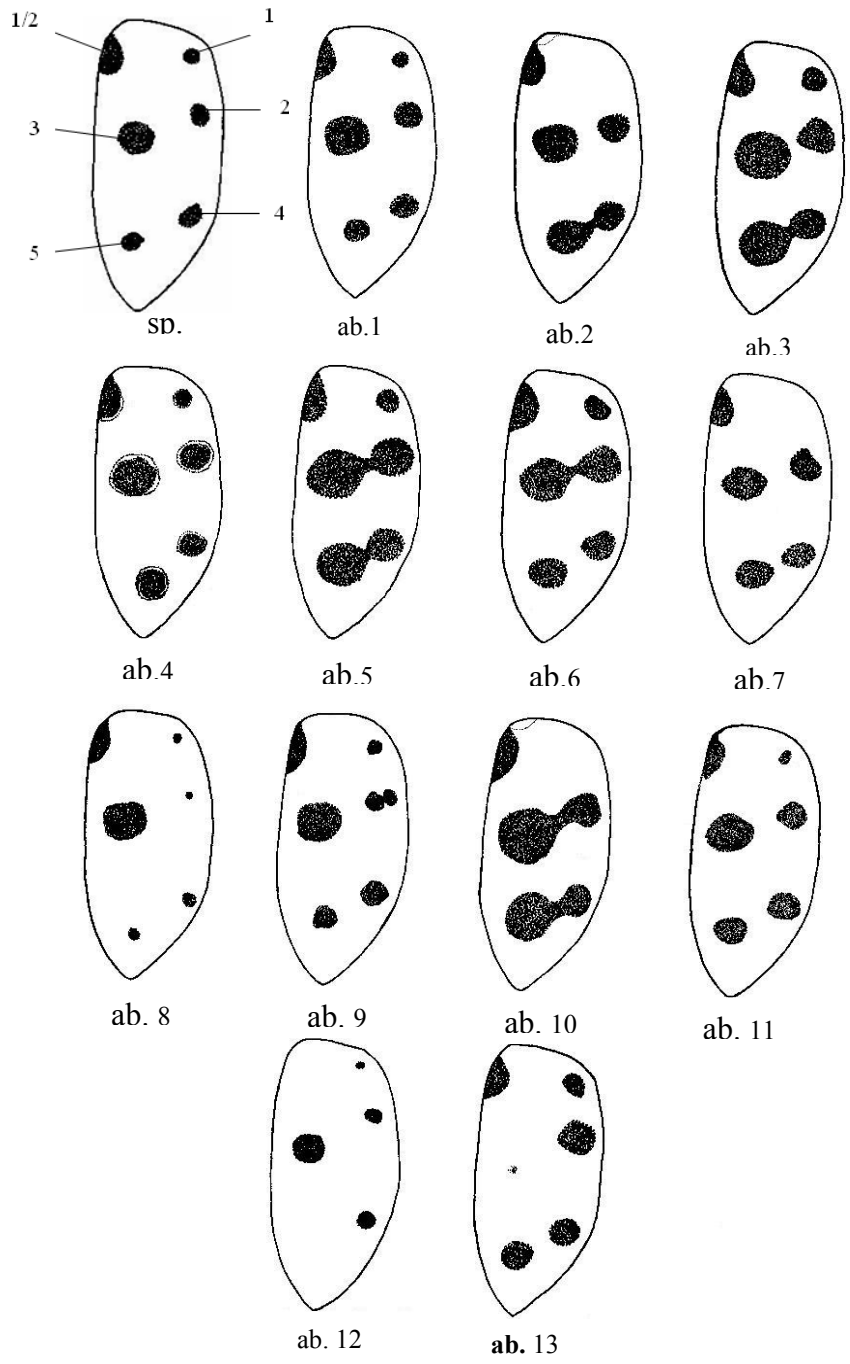


Fig. (1). Elytral color patterns of *C. undecimpunctata* L. and its 13 aberrations, right elytron. Abbrevitions: 1/2. spot 1/2 or scutellar spot, 1. the first or humeral spot, 2. the second or lateral spot, 3. the third or discal spot, 4. the fourth or marginal spot, 5. the fifth or apical spot, ep. epipleuron.

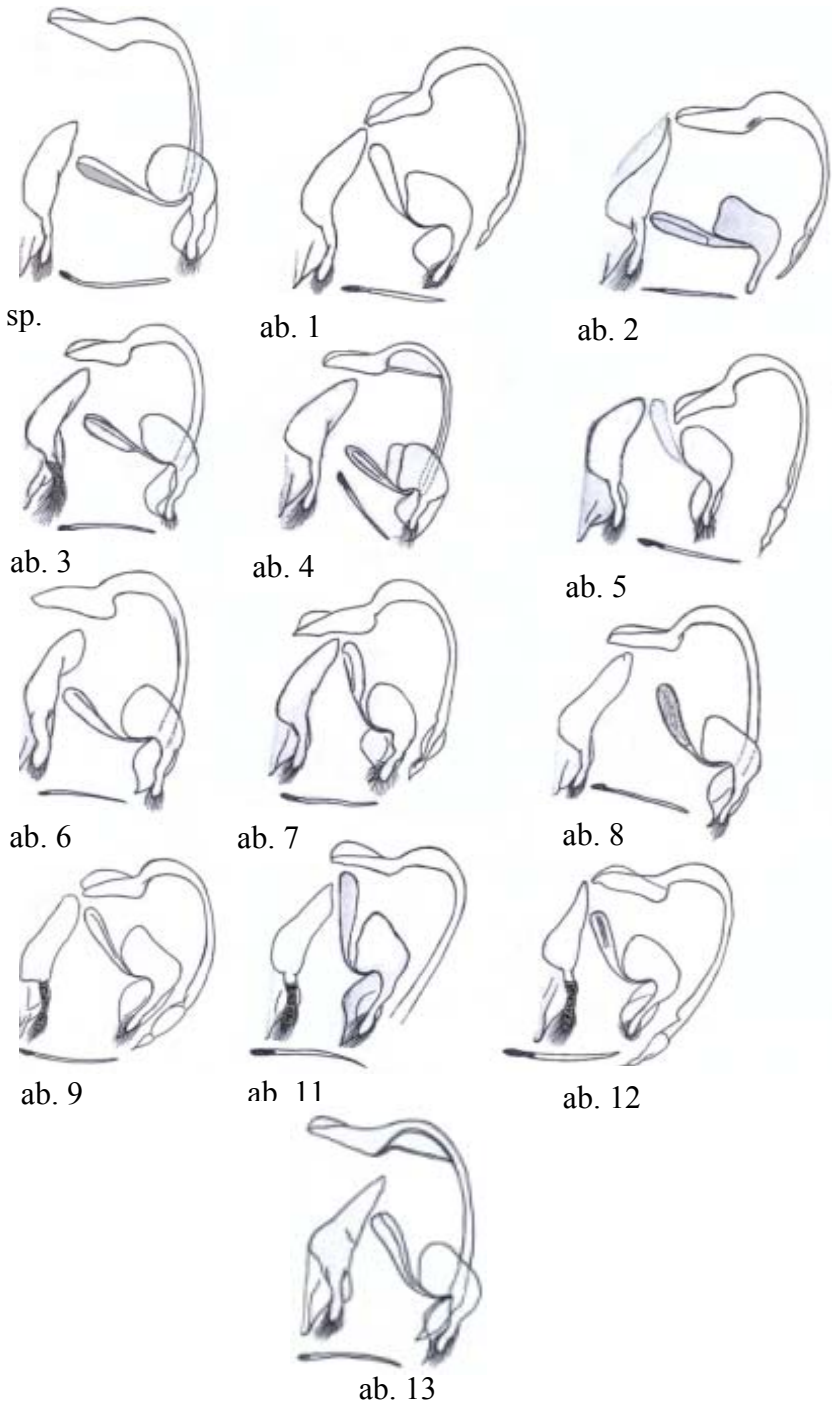


Fig. (2). Male genitalia of *C. undecimpunctata* L. and its aberrations; right lateral view of sipho (= aedeagus) with phallobase in right side, ventral view of right half part of phallobase in left side, apophysis in below.

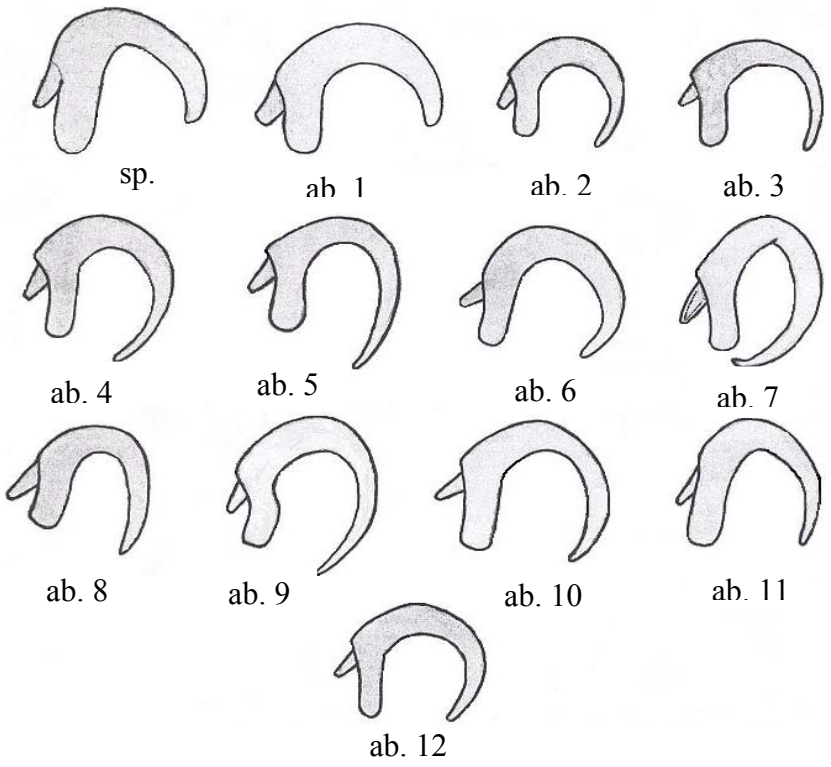


Fig. (3). Female receptaculum seminis (=spermatheca) of *C. undecimpunctata* L. and its aberrations

Phenetic method:

The numerical phenetic taxonomy is elaborated by program [PROBIOSYS, version 1.0, (2003)] depending on Single linkage, UPGMA, Complete linkage clustering methods in numerical taxonomy, Cophenetic correlation value. For creation the data matrix (table 3) based on the morphological characters for each taxon were coded as in being characters (i.e. absent = - & present = +). Phenetic method by choosing (55) morphological characters, with (110 states) was described in the (14) Operational Taxonomic Units (OTUs) of aberrations of *C. undecimpunctata*. The characters were chosen in male and female adult stages to reveal the phenetic relationship.

Characters and their states:

1. Body length.
(-) shorter than 11.7 mm, (+) longer than 11.7 mm.
2. Body width from shoulders.
(-) shorter than half body length, (+) as long as or longer than half body length.
3. Head length.
(-) shorter than 1/3 body width, (+) as long as or longer than 1/3 body width.
4. Head width.
(-) slightly longer than length of pronotum, (+) distinctly longer than length of pronotum.
5. Basal antennal segments.
(-) as long as or shorter than 1/4 head length, (+) longer than 1/4 head length.
6. Length of flagellum.
(-) as long as or shorter than half head width, (+) longer than half head width.
7. Length of pronotum.
(-) as long as or slightly shorter than half body width, (+) distinctly shorter than half body width.
8. Width of pronotum.

(-) shorter than 2 times length of pronotum, (+) as long as or longer than 2 times length of pronotum.
9. Pronotum with two creamy whitish patches on its side.
(-) absent, (+) present.
10. Color of pronotum.
(-) black, (+) brown.
11. Color of scutellum.
(-) black, (+) brown.
12. Elytral length.
(-) shorter than 2 times elytral width, (+) longer than 2 times elytral width.
13. Elytral width.
(-) shorter than 2 times length of pronotum, (+) longer than 2 times length of pronotum.
14. Hind wing length.
(-) as long as or shorter than 2 times length of epipleuron, (+) longer than 2 times length of epipleuron.
15. Hind wing with pterostigma.
(-) orange in color, (+) black in color.
16. Elytral spots.
(-) one spot or more (not all) absent, (+) all spots presents.
17. Elytra with united spots.
(-) absent, (+) present.
18. Elytra with circle around the spots.
(-) absent, (+) present.
19. Elytra with small spots beside the lateral spots.
(-) absent, (+) present.
20. Elytra with apical-marginal spots fused and discal-lateral spots fused.
(-) absent, (+) present.
21. Scutellar spots.
(-) absent, (+) present.
22. Humeral spots.
(-) absent, (+) present.
23. Discal spots.
(-) absent, (+) present.
24. Apical spots.
(-) absent, (+) present.
25. Apical and marginal spots.
(-) not fused, (+) fused.
26. Discal and lateral spots.
(-) not fused, (+) fused.
27. Epipleuron length.
(-) as long as or shorter than 3/4 elytral length, (+) longer than 3/4 elytral length.
28. Epipleuron width.
(-) as long as or shorter than 1/3 head width, (+) longer than 1/3 head width.
29. The distance between lateral-marginal spots to discal-apical spots.
(-) nearly as long as the distance, (+) distinctly longer than the distance.
30. The distance between lateral-apical spots to discal-marginal spots.
(-) shorter than 2 times the distance, (+) as long as or longer than the distance.
31. The distance between scutellar-marginal spots to scutellar-apical spots.
(-) shorter than the distance, (+) as long as or longer than the distance.
32. The distance between humeral-lateral spots to marginal-apical spots.
(-) shorter than the distance, (+) longer than the distance.
33. The distance between discal-marginal spots to discal-apical spots.
(-) as long as or shorter than the distance, (+) longer than the distance.
34. The distance between discal-apical spots to lateral-discal spots.
(-) as long as or shorter than 2 times the distance, (+) longer than 2 times the distance.

35. The distance between lateral-discal spots to discal-marginal spots.
(-) shorter than 2 times the distance, (+) as long as or longer than 2 times the distance.
36. The distance between humeral-discal spots to lateral-marginal spots.
(-) shorter than the distance, (+) as long as or longer than the distance.
37. The distance between scutellar-lateral spots to both combined distance between humeral-lateral spots and humeral-scutellar spots.
(-) nearly as long as to both combined distance, (+) distinctly shorter than to both combined distance.
38. Proportion of the distance between lateral-discal spots to marginal-apical spots.
(-) as long as or shorter than the distance, (+) longer than the distance.
39. Proportion of the distance between lateral-marginal spots to elytral length.
(-) shorter than 1/4 elytral length, (+) longer than 1/4 elytral length.
40. Proportion of the distance between lateral-marginal spots to elytral width.
(-) shorter than half elytral width, (+) longer than half elytral width.
41. Both combined the distance between lateral-discal spots and discal-marginal spots.
(-) shorter than the distance between lateral-marginal spots, (+) longer than the distance between lateral-marginal spots.
42. The diameter of lateral spots.
(-) shorter than the diameter of marginal spot, (+) as long as or longer than the diameter of marginal spots.
43. The diameter of scutellar spots.
(-) shorter than the diameter of marginal spots, (+) longer than the diameter of marginal spots.
44. The diameter of apical spots.
(-) as long as or shorter than the diameter of lateral spots, (+) longer than the diameter of lateral spots.
45. The diameter of discal spots.
(-) shorter than half elytral width, (+) longer than half elytral width.
46. The distance between apical spot to the margin of elytra.
(-) as long as or shorter than half elytral width, (+) longer than half elytral width.
47. The distance between lateral spot to the margin of elytra.
(-) equal the distance between marginal spot to the margin of elytra, (+) not equal (larger than) to the distance between marginal spot to the margin of elytra.
48. Both combined the diameters of lateral and marginal spots.
(-) as long as or shorter than the length of pronotum, (+) longer than the length of pronotum.
49. Distal part of outer lobe of siphonal capsule.
(-) with prominent or acute projection, (+) with curved (forked or not forked) projection.
50. Lateral lobes (parameres) of phallobase (tegmen of male).
(-) black color, (+) brown color.
51. The outer margin of siphonal capsule.
(-) not dilated, (+) dilated.
52. Receptaculum seminis (Spermatheca) width.
(-) receptaculum seminis not wider than long, (+) receptaculum seminis wider than long.
53. Cornu of spermatheca shorter than ramus.
(-) absent, (+) present.
54. Cornu of spermatheca as long as ramus.
(-) absent, (+) present.
55. Cornu of spermatheca longer than ramus.
(-) absent, (+) present.

Table (3). Data matrix of morphometric characters of *C. undecimpunctata* L. and its 13 aberrations.

Character\OTU	sp.	ab. 1	ab. 2	ab. 3	ab. 4	ab. 5	ab. 6	ab. 7	ab. 8	ab. 9	ab. 10	ab. 11	ab. 12	ab. 13
1-	-	-	-	-	-	-	-	-	-	+	-	+	+	-
2-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
3-	-	-	-	+	-	-	-	+	+	+	-	-	+	-
4-	+	+	+	+	+	-	+	+	+	+	+	-	-	+
5-	-	-	+	-	+	-	-	-	-	-	-	+	-	-
6-	-	-	+	-	+	-	-	+	+	-	+	-	-	-
7-	+	-	+	-	+	-	-	+	-	+	+	+	-	+
8-	-	-	-	-	-	+	+	-	-	-	-	+	+	-
9-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
10-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
11-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
12-	-	-	+	+	+	+	+	+	+	-	+	+	+	-
13-	+	-	-	-	+	-	-	-	-	+	+	-	-	-
14-	+	+	-	+	+	+	-	+	-	+	+	-	-	+
15-	+	+	+	+	+	+	+	+	-	+	-	+	+	+
16-	+	+	-	+	+	+	+	-	+	+	-	+	-	-
17-	-	-	+	+	-	+	+	-	-	-	+	-	-	-
18-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
19-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
20-	-	-	-	-	-	+	-	-	-	-	+	-	-	-
21-	+	+	+	+	+	+	+	+	+	+	+	+	-	+
22-	+	+	-	+	+	+	+	-	+	+	-	+	+	+
23-	+	+	+	+	+	+	+	+	+	+	+	+	+	-
24-	+	+	+	+	+	+	+	+	+	+	+	+	-	+
25-	-	-	-	+	-	+	-	-	-	-	+	-	-	-
26-	-	-	-	-	-	+	+	-	-	-	+	-	-	-
27-	-	-	+	+	+	-	+	+	+	-	-	+	+	-
28-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
29-	-	+	-	+	-	+	-	+	+	+	+	-	?	?
30-	-	+	-	-	+	+	+	-	-	+	-	+	?	?
31-	+	+	+	+	-	+	+	+	-	-	+	+	?	+
32-	+	+	?	+	-	+	+	?	+	+	?	+	?	+
33-	-	-	-	+	-	-	-	-	-	+	-	+	?	?
34-	+	+	-	+	+	+	+	+	-	+	?	-	?	?
35-	-	+	-	+	-	+	+	+	-	+	?	-	-	?
36-	+	+	?	+	+	+	+	?	-	+	?	+	+	?
37-	+	-	?	+	+	+	-	?	+	+	?	+	?	-
38-	+	-	+	+	-	-	-	+	-	-	?	+	?	?
39-	-	-	-	-	-	-	-	-	+	-	-	-	+	-
40-	-	-	-	+	-	-	-	-	+	-	-	-	+	-
41-	+	+	+	-	+	-	-	+	+	+	?	+	+	?
42-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
43-	+	+	+	+	+	+	+	+	+	+	+	+	?	-
44-	+	-	+	-	-	-	-	+	+	-	+	-	?	-
45-	-	-	-	-	-	+	-	-	-	-	-	-	-	?
46-	-	-	-	-	-	-	-	-	+	-	-	-	?	-
47-	+	+	+	-	+	-	+	-	+	+	+	+	+	-
48-	-	-	-	+	+	+	+	+	-	-	+	-	-	-
49-	+	+	+	-	+	-	-	-	+	+	?	-	-	+
50-	+	+	+	-	+	+	+	+	+	-	?	-	-	+
51-	-	+	-	+	-	+	+	+	+	+	?	+	+	-
52-	-	+	-	+	-	-	+	-	+	-	+	+	+	?
53-	+	+	-	-	-	-	-	-	-	-	-	-	-	?
54-	-	-	+	+	-	-	+	-	-	-	+	+	+	?
55-	-	-	-	-	+	+	-	+	+	+	-	-	-	?

Table (4). Similarity matrix from morphometric data.

	sp.	ab.1	ab.2	ab.3	ab.4	ab.5	ab.6	ab.7	ab.8	ab.9	ab.10	ab.11	ab.12	ab.13
sp.	100.000													
ab.1	78.182	100.000												
ab.2	73.077	63.462	100.000											
ab.3	58.182	69.091	59.615	100.000										
ab.4	72.727	69.091	73.077	56.364	100.000									
ab.5	56.364	70.909	50.000	72.727	61.818	100.000								
ab.6	60.000	78.182	67.308	76.364	65.455	78.182	100.000							
ab.7	67.308	69.231	75.000	73.077	71.154	67.308	65.385	100.000						
ab.8	58.182	65.455	65.385	60.000	63.636	50.909	58.182	67.308	100.000					
ab.9	70.909	78.182	53.846	65.455	72.727	63.636	60.000	67.308	61.818	100.000				
ab.10	64.444	64.444	75.556	68.889	62.222	66.667	68.889	73.333	57.778	55.556	100.000			
ab.11	58.182	61.818	67.308	63.636	60.000	54.545	69.091	53.846	52.727	61.818	48.889	100.000		
ab.12	47.727	59.091	58.140	65.909	47.727	47.727	65.909	55.814	65.909	56.818	44.737	77.273	100.000	
ab.13	78.571	85.714	72.500	64.286	66.667	64.286	66.667	72.500	54.762	71.429	62.162	57.143	50.000	100.000

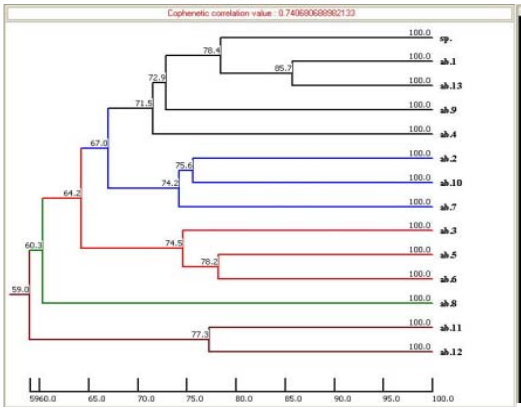


Fig. (4). Phenogram from morphometric data.

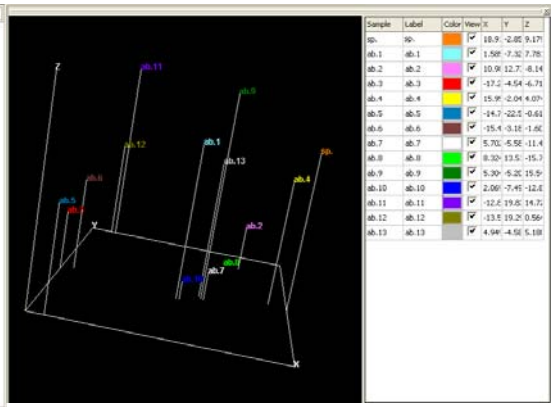


Fig. (5). PCA-plot from morphometric data.

According to the morphometric phenogram (figure 4), the 14 morphs of *C. undecimpunctata* L. were grouped into nine clusters including five independent clusters which were produced, in root point, by two main clusters at similarity level of 59.5%. The first is a major cluster, including 12 morphs, sp., ab.1, ab.2, ab.3, ab.4, ab.5, ab.6, ab.7, ab.8, ab.9, ab.10 & ab.13. The morph ab.8 has evolved early out (as an independent cluster) of the rest of the morphs at a similarity percentage of 64%. The rest of the morphs were divided into two main groups at a similarity level of 67.1%. The first one contained 3 morphs, ab.3, ab.5 & ab.6. The two morphs ab.5 & ab.6 were linked at a similarity percentage of 78.6% (cluster III). These two morphs were linked to morph ab.3 (independent cluster) at a similarity level of 74.5%. The second group was divided into two sub-groups at a similarity percentage of 71.8%. The first one contained 3 morphs, ab.7, ab.10 & ab.2. The two morphs ab.2 & ab.10 were attached at similarity level of 76% (cluster II). These two morphs were linked to morph ab.7 (independent cluster) at a similarity percentage of 74.5%. The second sub-group contained 5 morphs, ab.4, ab.9, sp., ab.13 & ab.1. The two morphs ab.4 & ab.9 (as two independent clusters) have evolved out of the rest of the morphs at similarity levels of 72.5% & 74% respectively. The morph sp. at a similarity percentage of 78.7% evolved from both morphs of ab.1 & ab.13 which were related to each other at a similarity level of 85% (cluster I).

The second cluster is contained two morphs; ab.11 & ab.12 which were attached at a similarity level of 76.9% (cluster IV).

In the principal component analysis (PCA) plot (figure 5), three groups can be observed; the first includes (9 morphs) sp., ab.1, ab.2, ab.4, ab.7, ab.8, ab.9, ab.10 & ab.13; the second includes (3 morphs) ab.3, ab.5 & ab.6 and the third includes (2 morphs) ab.11 & ab.12. The principal component Z differentiates sp., ab.1, ab.4, ab.9 & ab.13 from the first group and ab.11 from the third group. The principal component Y differentiates ab.2 and ab.8 from the first group.

RAPD-PCR technique:

Five primers were used for RAPD analysis. An average of 16 bands within a range of 12-24 bands was obtained for each primer in 14 morphs of *C. undecimpunctata* genotypes. Of a total of 80 clear and reproducible bands, 75 were polymorphic (table 5). The sizes of most amplified DNA fragments ranged from 130 to 1864 bp. An example of a RAPD pattern obtained from C15 is shown in (figures 6a-f). The highest molecular size was detected by using primer C07 with 8 morphs (sp., ab.1, ab.2, ab.3, ab.4, ab.5, ab.6 & ab.7) and the lowest molecular size was detected by using primer C16 with 6 morphs (sp., ab.1, ab.2, ab.4, ab.6 & ab.7).

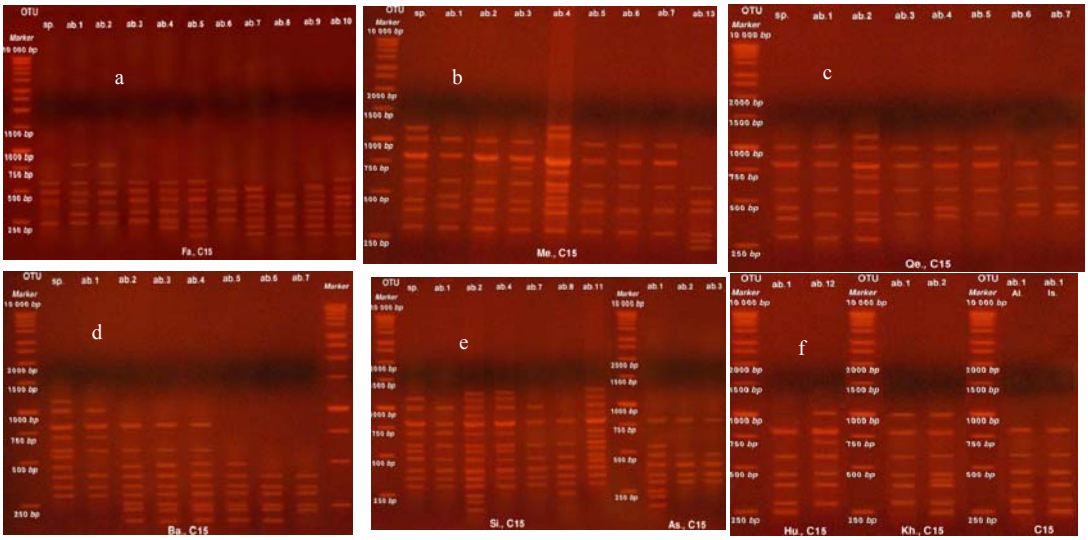
The fragments of DNA generated by using arbitrary primers were analyzed by the similarity coefficient. The values ranged from 40% to 93.75%. The aberrations (ab.9 & ab.10) were found to have the highest similarity coefficient of 93.75%. The aberrations (ab.6 & ab.11), (ab.10 & ab.11) and (ab.11 & ab.13) exhibited the lowest similarity coefficient of 40% (table 6). By using the UPGMA clustering method, a dendrogram was generated from similarity coefficient (figure 7) as well as the PCA plot was designed (figure 8).

Table (5). Primers used and the number of RAPD markers obtained from 14 morphs of *C. undecimpunctata* L.

Primer	Sequence	G+C (%)	No. of total RAPD markers	Polymorphic markers (%)
C07	5'-GTCCCGACGA-3'	70	17	16 (94.1)
C15	5'-GACGGATCAG-3'	60	24	23 (95.8)
C16	5'-CACACTCCAG-3'	60	14	13 (92.8)
C18	5'-TGAGTGGGTG-3'	60	12	11 (91.6)
K15	5'-CTCCTGCCAA-3'	60	13	12 (92.3)
Total			80	75 (93.7)

According to the phenogram which was obtained from RAPD data (figure 7), the 14 morphs of *C. undecimpunctata* L. were grouped into three main clusters and five independent clusters. Of these, ab. 1, ab.3 & ab.5 were classified with sp. in cluster I at similarity level 82.2%. The morphs ab.4 & ab.6 were classified with ab.7 in cluster II (79.4%). The morph ab.9 was classified with ab.10 in cluster III (93.8%). However, five morphs (ab.2, ab.8, ab.12, ab.13 & ab.11) did not belong to any of the aforementioned three main clusters. Therefore, they were identified as the independent clusters.

In the PCA plot (figure 8), four groups can be observed; the first includes (8 morphs) sp., ab.1, ab.2, ab.4, ab.5, ab.6 & ab.7; the second includes (4 morphs) ab.9, ab.10, ab.12 & ab.13; the third includes ab.8 and the fourth includes ab.11. Principal components X & Z are able to separate ab.2 from the first group, also principal components Y & Z are able to separate ab.13 from the second group and the principal component Z only differentiates ab.12 from the two morphs ab.9 & ab.10 in the second group.



Figs. (6a-f). RAPD banding pattern generated with random primer C15 from 14 morphs of *C. undecimpunctata* genotypes collected from 10 localities. Abbreviation: (OTU) Operational Taxonomic Unit, (Fa.) El-Fayoum, (Me.) El-Menya, (Qe.) Qena, (Ba.) Banha, (Si.) Siwa Oasis, (As.) Assiut, (Hu.) Hurghada, (Kh.) Kharga Oasis, (Al.) Alexandria, (Is.) Ismaileya.

Table (6). Similarity matrix of DNA band patterns by using five primers among 14 morphs genotypes according to Nei and Li's coefficient of similarity.

	sp.	ab.1	ab.2	ab.3	ab.4	ab.5	ab.6	ab.7	ab.8	ab.9	ab.10	ab.11	ab.12	ab.13
sp.	100.000													
ab.1	82.500	100.000												
ab.2	73.750	78.750	100.000											
ab.3	81.250	91.250	72.500	100.000										
ab.4	78.750	81.250	75.000	77.500	100.000									
ab.5	82.500	85.000	81.250	86.250	73.750	100.000								
ab.6	80.000	82.500	73.750	81.250	83.750	80.000	100.000							
ab.7	71.250	81.250	72.500	80.000	75.000	78.750	83.750	100.000						
ab.8	63.750	76.250	60.000	75.000	70.000	68.750	73.750	77.500	100.000					
ab.9	51.250	53.750	40.000	60.000	50.000	56.250	58.750	50.000	67.500	100.000				
ab.10	55.000	57.500	43.750	61.250	53.750	60.000	62.500	48.750	66.250	93.750	100.000			
ab.11	55.000	55.000	43.750	56.250	48.750	52.500	47.500	46.250	63.750	56.250	55.000	100.000		
ab.12	63.750	58.750	50.000	62.500	55.000	63.750	66.250	55.000	60.000	75.000	76.250	58.750	100.000	
ab.13	56.250	58.750	45.000	65.000	52.500	61.250	56.250	50.000	62.500	67.500	68.750	63.750	70.000	100.000

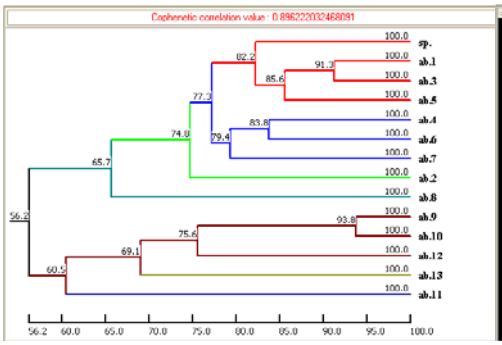


Fig. (7). Phenogram from RAPD data by using 5 primers

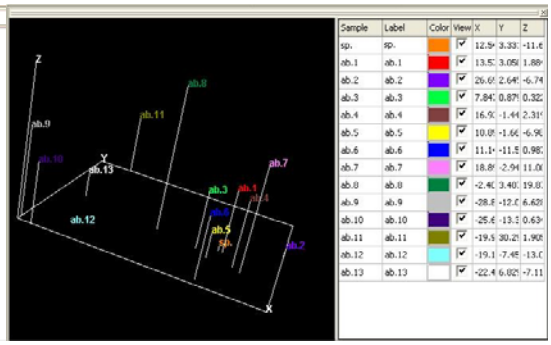


Fig. (8). PCA plot from RAPD data by using 5 primers.

The phenogram which was obtained from the combination of data matrix from both morphometric and RAPD data (figure 9), showed that the 14 morphs of *C. undecimpunctata* L. were grouped into three main clusters and five independent clusters. Of these, ab.2 & ab.4 were classified with sp. in cluster I at similarity level 73.9%. The morphs ab.1, ab.3 & ab.6 were classified with ab.5 in cluster II (79.4%). The morph ab.9 was classified with ab.10 in cluster III (80.0%). However, five morphs (ab.7, ab.8, ab.13, ab.12 & ab.11) did not belong to any of the abovementioned three clusters. Therefore, they were identified as the independent clusters.

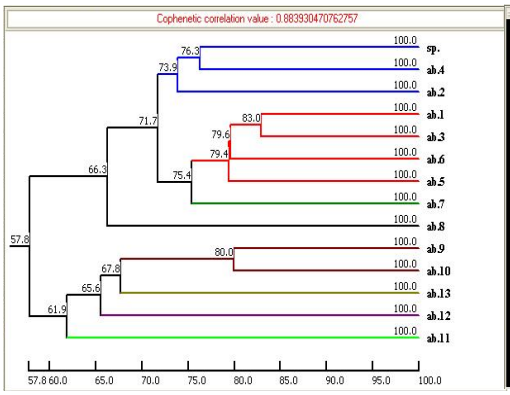


Fig. (9). Phenogram from morphometric data and RAPD data by using 5 primers.

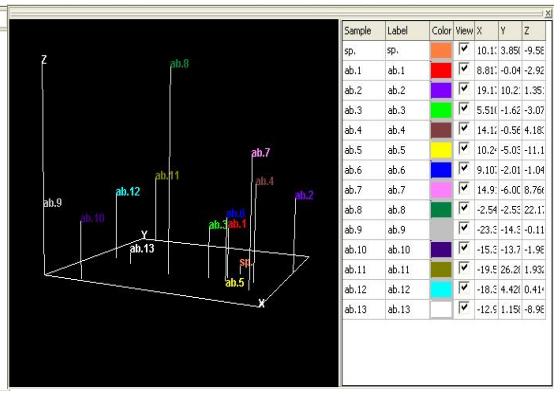


Fig. (10). PCA plot from morphometric data and RAPD data by using 5 primers.

In the PCA plot (figure 10), four groups can be observed; the first includes (8 morphs) sp., ab.1, ab.2, ab.4, ab.5, ab.6 & ab.7; the second includes (5 morphs) ab.9, ab.10, ab.12 & ab.13; the third includes ab.8 and the fourth includes ab.11. Principal component Y is able to separate ab.2 from the first group and also is able to separate ab.9 & ab.10 from the second group. Principal component Z differentiates ab.13 from the second group.

DISCUSSION AND CONCLUSION

Color pattern polymorphism occurs in many coccinellid lady beetles, posing a challenge to those who wish to identify or characterize the species. Modern taxonomists find that the male genitalia often provide a definitive means for associating members of a polymorphic species, but this method is less useful if the key structures are simplistic or evolutionary changes have been conservative (Gonzalez & Vandenberg 2006). The aberrations of *C. undecimpunctata* treated in the present paper exemplify many of the difficulties encountered in studies of lady beetle taxonomy. Thus, this study intends to combine numerical phenetic taxonomy using morphometric analysis with RAPD molecular study to represent the degree of variations and the genetic structures of the taxa that will lead eventually to understand the phenetic relationships among these aberrations.

In order to determine the morphometric analysis and phenotypic features of the aberrations of *C. undecimpunctata* L. to support the phenetic method, both sexes of the morphs under study are investigated [except for ab.10 (♀♀) & ab.13 (♂♂) only]. This study has given a clear picture and large numbers of the essential and diagnostic morphological characters of these morphs.

The morphometric phenogram (figure 4) clearly differentiates between the species and the morphs into two main clusters according to the proportion of epipleuron width to elytral length. The first is a major cluster (contains eight clusters), including 12 morphs, sp., ab.1, ab.2, ab.3, ab.4, ab.5, ab.6, ab.7, ab.8, ab.9, ab.10 & ab.13. The morph ab.8 (as independent cluster) has evolved early out of the rest of the morphs based on the diameters of lateral and marginal spots. The second cluster contains two morphs, ab.11 & ab.12 (cluster IV) which are attached to each other depending on length of head and pronotum, colors of scutellum and pronotum and the presence or absence of scutellar & apical spots on the elytra. The obtained phenogram clearly differentiate the 14 morphs of *C. undecimpunctata* L. into nine main clusters including five independent clusters.

The principal component analysis (PCA) plot (figure 5), separates the aberrations into three groups the first includes (9 morphs) sp., ab.1, ab.2, ab.4, ab.7, ab.8, ab.9, ab.10 & ab.13 according to length of flagellum, length & width of pronotum, shape of distal part of outer lobe of siphonal capsule and lengths of the cornu & ramus of spermatheca; the second group includes (3 morphs) ab.3, ab.5 & ab.6 depending on the fusion of elytral spots and the distances between lateral-discal spots, discal-marginal spots & lateral-marginal spots; and the third group includes (2 morphs) ab.11 & ab.12 based on body length, head width, epipleuron width and color of lateral lobes of male tegmen.

In a conclusion, the numerical phenetic taxonomy allowed giving efficient summarization of phenetic similarities and affinities among the aberrations; it is difficult for the human mind to manipulate efficiently a large volume of multivariate data for any sizable taxonomic group. It is obvious from the phenogram and PCA plot which obtained from the phenetic taxonomy, the high degree of variations and affinities of the different aberrations (Similarity coeffeciant ranged from 44.7 to 85.7%) (table 4) resulted from numerical phenetic method and principal component analysis according to the morphometric measures (these results suggest a good morphological differention), therefore the present work aimed to apply the molecular analysis (RAPD-PCR) technique to study the genetic variations of these aberrations and to compare it with the numerical phenetic taxonomy.

Five RAPD primers (C07, C15, C16, C18 and K15) are used to investigate molecular markers among the 14 aberrations of *Coccinella undecimpunctata* L. genotypes based on random amplified polymorphic DNA (RAPD) analysis to determine the phenetic relationship among them and to identify the DNA polymorphisms. In fact, different thermal cyclers, brand of DNA polymerase, annealing temperature, and concentration of $MgCl_2$, primer and template DNA can affect the reproducibility of RAPD assay (Ellsworth *et al.*, 1993). Thus, standardized methodology should be devised for RAPD assay to obtain identical RAPD pattern.

RAPD-PCR of 14 morphs of *C. undecimpunctata* L. produces DNA fragments ranged from 130 to 1864 bp and five monomorphic fragments are resulted by using five random primers. These fragments are measured by the genetic distance, where the values ranged from 0.6 to 0.09. From (table 6), the highest value 0.6 is detected between morphs (ab.2 & ab.9) and the lowest value 0.09 is detected between morphs (ab.1 & ab.3).

In the comparison between both phenograms which were produced from RAPD data by using 5 random primers (figure 7) and from morphometric data (figure 4) respectively, the 14 morphs are grouped into 8 clusters from the RAPD-phenogram against 9 clusters are obtained from the other phenogram. Therefore both phenograms are discrepancy to each other in the design, arrangement and the number of aberrations in each cluster that is obviously in the root (point of origin) of the phenograms. Discrepancies among the topologies of phenograms or PCA plots obtained from different markers have also been observed in other taxa (Li, 2000 and Casiva *et al.*, 2002).

The arrangement of some aberrations in RAPD-phenogram is roughly consistent with that from morphometric-phenogram; for example both aberrations ab.11 & ab.12 which are related to each other in the second cluster, from the root, as two independent clusters in the former phenogram or as forming cluster IV in the latter phenogram. The aberration ab.8 is considered as independent cluster in the first cluster, from the root, of both phenograms.

The PCA from RAPD collective data (figure 8) allowed us to differentiate four groups in comparison to three groups which were differentiated from the PCA morphometric data (figure 5).

High levels of genetic variability, such as those observed in the present study, may be related to mating system and geographic distribution (i.e. produced generations of random mating with different populations in the field, more and more of the genome of the original marked strain will be scattered). This result is agreed with Roehrdanz and Flanders (1993), who identified the DNA polymorphisms in some coccinellid predators.

The concept of the numerical phenetic taxonomy is based on the use of maximum number of characters which may not be necessarily derived only from external or internal morphology but may include any attributes of the OTU (biochemical, cytological, molecular marker and ecological … etc.) according to Kapoor (1994).

The present study is the first attempt to combine two taxonomic studies (numerical phenetic method and RAPD molecular analysis) to show the advantages of the combination against one-dimensional taxonomy (phenetic method or RAPD technique). Where, the phenogram (figure 9) which resulted from the combination between the morphometric data and RAPD data is clearly differentiated, in root point, into 2 major clusters, the first contains 9 aberrations including 2 main clusters and 2 independent clusters and the second contains 5 aberrations including one main cluster

and 3 independent clusters. The arrangement of these aberrations is completely consistent in root point and in the second cluster which contains (ab.9, ab.10, ab.11, ab.12 & ab.13) with RAPD-phenogram (figure 7), while it does not agree with morphometric-phenogram alone (figure 4).

The arrangement of some aberrations in lower area of phenogram based on morphometric and RAPD data is roughly consistent with that from morphometric-phenogram and RAPD-phenogram such as both aberrations ab.11 & ab.12 which related to each other in the second cluster, from the root. The aberration ab.8 is considered as independent cluster in the first cluster as in the two latter phenograms. Other aberrations are not agreed with morphometric-phenogram or with RAPD-phenogram.

The PCA (figure 10) based on morphometric and RAPD data in this work allowed us to differentiate four groups, that is completely consistent with that from PCA based on RAPD data (figure 8), in comparison to three groups which can be observed from the PCA based on morphometric data (figure 5).

In a conclusion, from these results, the present work show that both aberrations (ab.11 & ab.12) entered to the new niche (Siwa Oasis and Hurghada) according to the migratory habits as an initial step of parapatric speciation and after that, may be the reproductive isolation is completing within few years. That is confirmed by the results of the morphometric measures which applied in both aberrations especially on the genitalia which differed in shape from the original population (sp.) as well as by the results of different topologies of phenograms which obtained from different markers.

We recognize that this study also has raised additional taxonomic questions that will require further study and possibly the application of other molecular techniques and other alternative approaches.

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ARABIC SUMMARY

دراسات مقارنة مظهرية عن الأشكال المتعددة لخنفساء *كوكسينيلا أنديسمنكتاتا* لينيس باستخدام القياسات المورفولوجية و تقنية التضخيم العشوائى متعدد الأأشكال من دنا و ذلك لأول مرة فى مصر

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اشتملت الدراسة الحالية على تطبيق التحليل العددى الظاهرى و تقنية دلائل التضخيم العشوائي متعدد الأشكال من المادة الوراثية (دنا) على خنفساء *كوكسينيلا أنديسمنكتاتا* لينيس و الأشكال المتعددة التابعة لها و التى قدرت بـ ١٣ شكل و التى تم جمعها من ١٠ أماكن مختلفة من مصر.
لقد تم استخدام القياسات المورفولوجية لتحديد الصفات التشخيصية الهامة من الأشكال المتعددة لهذه الخنفساء للحصول على الشكل الشجرى و الذى يعبر عن مدى درجات التشابه أو التباين بين الأشكال المختلفة. و قد أوضح الشكل الشجرى درجة عالية من التباين (و قد تراوح معامل التشابه ما بين ٤٤.٧% - ٨٥.٧%). كذلك اشتملت الدراسة على عمل أنماط البصمة الوراثية لكل الأشكال المتعددة من خنفساء أبو العيد عن طريق استخدام ٥ بادئات وراثية عشوائية لتحديد العلاقات الظاهرية و للتحقق من الدلائل الجزيئية للتراكيب الوراثية لهذه الأشكال. و قد تراوحت نسبة التباين الوراثى بين الأشكال من ٤٠%-٩٣.٧٥%.
كما تم عمل مقارنة بين كلا من الشكلين الشجريين الناتجين من تقنية التضخيم العشوائي متعدد الأشكال و من التحليل الظاهرى على التوالى و قد ظهر أن تقنية التضخيم العشوائي تقسم الأشكال المتعددة الى ٨ مجموعات فى مقابل ٩ مجموعات ناتجة من التحليل الظاهرى. أما بالنسبة لنتيجة الرسم البيانى (PCA plot) الناتجة من تقنية التضخيم العشوائي فقد قسمت الأشكال الى ٤ مجموعات فى مقابل ٣ مجموعات ناتجة من التحليل الظاهرى.