

Original Article**Applying Morphometric Variation of Seta 2 (Antepalmate Hair) among the Larvae of the Members of the Maculipennis Subgroup (Diptera: Culicidae) in Iran**S Doosti¹, H Vatandoost¹, MA Oshaghi¹, M Hosseini², *MM Sedaghat¹¹Dept. of Medical Entomology and Vector Control, School of Public Health and Institute of Public Health Research, Medical Sciences /University of Tehran, Iran²Dept. of Epidemiology and Biostatistics, School of Public Health and Institute of Public Health Research, Medical Sciences /University of Tehran, Tehran, Iran

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ABSTRACT

The members of *Anopheles maculipennis* subgroup (Diptera: Culicidae) are the most important vectors of malaria in the north, west, and central plateau of Iran. This study was carried out to differentiate the species composition of this subgroup based on morphometric variation seta 2-IV and V (antepalmate hair) among 149 larval specimens that were deposited at the Medical Arthropods Museum, the School of Public Health, Tehran University of Medical Sciences by using the light microscope. The mean numbers of larval seta 2-IV and V of the specimens belong to different locations of Iran, were calculated by SPSS (11.5) software package, followed by cluster analysis, and four different groups (clusters) were identified. The means were compared with the similar and available published data. After analyzing, four clusters recognized. The first cluster was fitted in ten localities in Esfahan, East Azarbaijan, West Azarbaijan, Khorassan, Kurdistan, and Mazandaran Provinces with its mean and standard deviation (SD) of 14.89 ± 1.13 (n= 79); the second group with one location in Gilan Province (11 ± 1.58 , n= 5); the third one with two locations in Fars and Western Azarbaijan Provinces (27.43 ± 0.31 , n=20), and the final group with four locations in Khuzestan, West Azarbaijan, and Qazvin Provinces (36.84 ± 1.91 , n= 45) were identified and corresponded to *Anopheles messeae*, *An. atroparvus*, *An. melanoon*, and *An. sacharovi* respectively. This work provides comparative information on the Maculipennis Subgroup based on morphometric examination at the larval stage in Iran.

Keywords: Maculipennis group, Malaria, Mosquito, Numerical taxonomy, Iran**INTRODUCTION**

The anopheline fauna in Iran is diverse and at least seven species have been incriminated as malaria vectors in the country. Among them the members of *Anopheles maculipennis* subgroup (Diptera: Culicidae) have involved with malaria transmission mostly in northern and central area of Iran (Sedaghat et al. 2003a).

According to the last classification, the genus *Anopheles* (Diptera: Culicidae) includes six subgenera and at least 484 species in the world

(Harbach 2004). The members of *Anopheles maculipennis* subgroup are the most important vectors of the western Palaearctic region (Sedaghat et al. 2003a). *An. maculipennis* was described and named for the first time by Migen in 1818 and for many years, all scientists expected that it was one species with single morphological type and biological characters (White 1978). In fact the Maculipennis Group was the first sibling species complex, to be discovered among mosquitoes (Falleroni 1926, van Thiel 1927). Since this discovery, extensive efforts

have been made to determine and explain species composition of the group.

Several methods including studies of egg morphology, ecology, hybridization, larval and pupal chaetotaxy, wing characteristics, cuticular hydrocarbons, DNA sequences, chromosome, and zymotaxonomy have been used for identification of this group (Linton et al. 2003).

These works contributed to the current recognition of twelve formal species in the Palaearctic region, including *An. artemievi*, *An. atroparvus*, *An. daciae*, *An. beklemishevi*, *An. labranchiae*, *An. lewisi*, *An. maculipennis*, *An. martinius*, *An. melanoon*, *An. messeae*, *An. persiensis* and *An. sacharovi* (White 1978, de Zulueta et al. 1983, Sedaghat et al. 2003 a, Harbach 2004, Nicolescu et al. 2004, Sedaghat and Harbach, 2005, Gordeyev et al. 2005).

The Maculipennis Group currently divided to three subgroups including Maculipennis, Quadrimaculatus and Freeborni Subgroups. The Maculipennis Subgroup (which previously named Maculipennis Complex) comprises all Palaearctic members of the group except *An. beklemishevi* (Harbach 2004).

Seven taxa of the members of the subgroup previously reported from Iran. These species include: *An. Atroparvus* (Dinparast-Jadid et al. 2001, 2007), *An. labranchiae* (Gholizadeh et al. 2004, Dinparast-Jadid et al. 2007), *An. maculipennis* s.s. (Dow 1953, Shahgudian 1960, Dinparast-Jadid et al. 1990, Glick 1992, Momeni et al. 1992, Dinparast-Jadid et al. 2001, Azari-Hamidian et al. 2003, Oshaghi et al. 2003, Sedaghat et al. 2003a, Dinparast-Jadid et al. 2007), *An. melanoon* (Dow 1953, Faghieh 1969, as *An. subalpinus*, Dinparast-Jadid et al. 1990, Momeni 1991, Glick 1992, Azari-Hamidian et al. 2003) *An. messeae* (de Zulueta et al. 1957, Minar 1974, Momeni 1991, Momeni et al. 1992, Dinparast-Jadid et al. 2001, Azari-Hamidian et al. 2003, Dinparast-Jadid et al. 2007), *An. Persiensis* (Sedaghat et al. 2003a, Sedaghat and Harbach 2005, Dinparast-Jadid et al. 2007) and *An. sacharovi* (Dow 1953, Etherington and Sellick 1946, Shahgudian 1960, Dinparast-Jadid et al.

1990, Glick 1992, Momeni et al. 1992, Dinparast-Jadid et al. 2001, Oshaghi et al. 2003, Sedaghat et al. 2003a,b, Sedaghat and Harbach 2005, Dinparast-Jadid et al. 2007). Although, Glick (1992) listed *An. martinius* among Iranian anopheline, the occurrence of this taxon has not been confirmed in the country (Sedaghat and Harbach 2005).

Among the members of *An. maculipennis* subgroup, five species of *An. Atroparvus*, *An. labranchiae*, *An. maculipennis*, *An. messeae* and *An. sacharovi* have been considered as malaria vectors in Eurasia (Jaenson et al. 1986, Ribeiro et al. 1988, Kasap 1990, Jetten and Takken 1994, Romi 1999, Romi et al. 2001, Alten et al. 2000, Faghieh 1969).

To date, *Anopheles maculipennis* s.s. has been discriminated as the major vector of malaria in the Caspian Sea littoral, and *An. sacharovi* is considered to be the principal vector in the central plateau (Faghieh 1969, Manouchehri et al. 1992).

Early studies on this group were based on morphological methods. Falleroni (1926) and van Thiel (1927), as first attempts to understand complexity of the Maculipennis Group, considered long- and short-winged forms of *An. maculipennis* sensu lato. The other morphological studies include: study on eggs (Missiroli et al. 1933); male genitalia (Martini 1933); wing scales (Ungureanu and Shute 1947); larval or pupal chaetotaxy (Bates 1939, Pichot and Deruaz 1981, Suzzoni-Blatger and Sevin 1981, Boccolini et al. 1986, Suzzoni-Blatger et al. 1990, Deruaz et al. 1991).

In the larval stage, situation of seta 3-C (outer clypeal hair) in comparison to lateral palatal brush in addition number and situation of seta 1-II was considered as a useful method (Abul-hab 1955). However, morphometric variation of seta 2-IV and V was regarded as a useful and reliable morphologic character to differentiate in the larval stage by some medical entomologists (Bates 1939, Romi et al. 2002).

There is little information on vector capacities and species distributions of these sibling

species in Iran. Besides, there were disagreements with the occurrence of some species in the country and some records need to be verified by classical morphology study, molecular method and using other advanced tools. Identification of species complex which show different biology, ecology, behaviour, host preference and vector potential aspect despite of their resemblance, is very important in malaria control programmes.

It is important to make statistical comparison of the amounts of branching of particular larval setae. This paper provides preliminary information on differentiation of species composition of *An. maculipennis* subgroup in the larval stage based on the morphometric variation of seta 2-IV and V.

MATERIALS AND METHODS

This study was carried out to separate the *An. maculipennis* subgroup larvae based on seta 2 (Ante palmate hair) in Iran. Two hundred and ninety one larval specimens which were deposited in the Medical Arthropods Museum, School of Public Health, Tehran University of Medical Sciences, Iran were examined. These specimens initially had been identified based on egg morphology. The number of seta 2 branches (ante palmate hair) of the fourth and fifth abdominal segments of each larva was counted using light microscope. For compared mean of seta 2-IV and V, those collection localities which had at least 5 specimens were considered in this study.

The mean numbers of larval setae 2-IV and V of the specimens belong to different locations of Iran, were calculated by SPSS (11.5) software package, followed by cluster analysis.

RESULTS

In this investigation 149 larval specimens from 17 different locations of Iran were studied to identify the species composition of the Maculipennis Subgroup based on seta 2. There

was not statistically significant between the number of the seta 2 branches in abdominal segments IV and V among the specimens. Means and Standard Deviations (SD) of different locations are shown in Table 1. Total mean of the seta 2 branches in segments 4 and 5 in different locations were statistically significant ($P < 0.0001$). The mean numbers of the larval seta 2-IV and V of the specimens, were calculated by SPSS (11.5) software package. After cluster analyzing, four different clusters were identified. These four groups include:

Group 1 with codes 4, 5, 6, 7, 9, 11, 12, 15, and 16 that they belong to Esmail Tarkhan (Esfahan Province), Takht Olia (West Azarbaijan), Dehriz (East Azarbaijan), Band Rezaie (East Azar-baijan), Ghagharalu (East Azarbaijan), Sarvelayat (Khorassan), Marsuk (Khorassan), Gheybisur (Kurdistan), Sarik (West Azarbaijan), group 2 with code 17 includes Fuman (Gilan), group 3 with codes 1 and 10 includes Dasht Khezri (Fars) and Dehjabal (East Azarbaijan), and group 4 with codes 2, 3, 8 and 13 includes Taher Abad (Qazvin), Nazar Abad (Qazvin), Jabal (East Azarbaijan) and Kaldusakh (Khuzestan) (Fig. 2, 3).

The mean numbers of the seta 2 branches for each of these clusters were compared with similar information from Bates (1939) and Romi et al. (2002). The first cluster was in ten locations in Esfahan, East Azarbaijan, West Azarbaijan, Khorassan, Kurdistan, and Mazandaran Provinces with mean and standard deviation (SD) 14.89 ± 1.13 , $n = 79$ was fitted to *An. messeae*, the second group with one location in Gilan Province (11 ± 1.58 , $n = 5$) fitted to *An. atroparvus*, the third one with two location in Fars and West Azarbaijan Provinces (27.43 ± 0.31 , $n = 20$) fitted to *An. melanoon* and the final group with four locations in Khuzestan, West Azarbaijan, Qazvin Provinces (36.84 ± 1.91 , $n = 45$) fitted to *An. sacharovi* (Table 1).

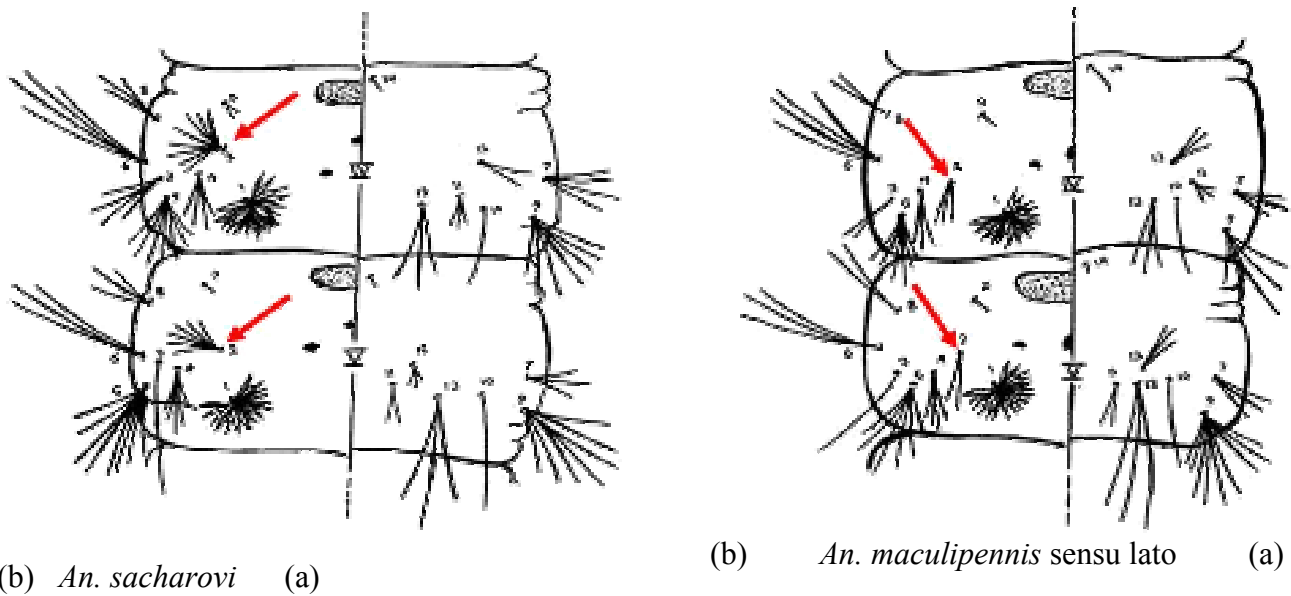


Fig. 1. Dorsal (a) and ventral (b) view the of the IV- V abdominal segments of larvae in *An. sacharovi* and *An. maculipennis* s.l. (setae 2 is shown by arrow)

Table 1. Location, mean and standard deviation (SD) of seta2 branches, and the number of larval specimens studied among Iranian *An. maculipennis* subgroup.

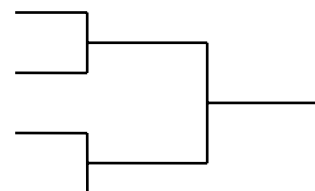
Province	No. larvae	Location	Species	Mean of seta2 branches ±SD
Fars	14	Dasht khezri	<i>An. melanoon</i>	27.21±4.28
Qazvin	13	Taherabad	<i>An. sacharovi</i>	36.92±3.79
Qazvin	9	Nazarabad	<i>An. sacharovi</i>	34.55±7.55
Esfahan	5	Esmailtarkhan	<i>An. messeae</i>	14.40±2.51
East Azarbaijan	13	Takhtolia	<i>An. messeae</i>	14.84±2.91
West Azarbaijan	7	Dehriz	<i>An. messeae</i>	16.57±4.57
West Azarbaijan	6	Band Rezaie	<i>An. messeae</i>	16.16±2.92
West Azarbaijan	6	A marsh near Jabal	<i>An. sacharovi</i>	36.66±8.47
West Azarbaijan	7	Ghagharalu	<i>An. messeae</i>	14.71±1.70
West Azarbaijan	6	Dehjabal	<i>An. melanoon</i>	27.66±7.22
Khorassan	17	Sarvelayat	<i>An. messeae</i>	15.47±4.001
Khorassan	6	Marsuk	<i>An. messeae</i>	15.66±2.80
Khuzestan	17	Kaldusakh	<i>An. sacharovi</i>	39.23±6.15
Kurdistan	5	Gheybisur	<i>An. messeae</i>	13.20±1.30
East Azarbaijan	5	Sarik	<i>An. messeae</i>	14.80±3.42
Mazandaran	8	Abdangeh	<i>An. messeae</i>	13.12±1.80
Gilan	5	Fuman	<i>An. atroparvus</i>	11±1.58



Fig. 2. Geographical distribution of *An. sacharovi* and *An. maculipennis* s.l. in Iran based on the mean number of seta 2 branches of available larval slides in the Medical Entomology Museum of School of Public Health, Tehran University of Medical Sciences, Iran. ■ *An. sacharovi*, ● *An. messeae*, ▲ *An. melanoon*, ◆ *An. atroparvus*. 1. East Azarbaijan, 2. West Azarbaijan, 3. Kurdistan, 4. Guilan, 5. Mazandaran, 6. Qazvin, 7. Esfahan, 8. Khorassan, 9. Khuzestan, 10. Fars.

An. messeae
An. atroparvus
An. melanoon
An. sacharovi

(A)



An. messeae
An. atroparvus
An. melanoon
An. sacharovi

(B)

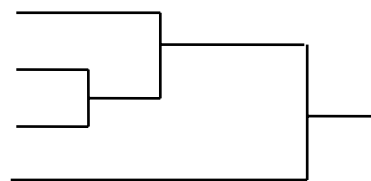


Fig. 3. (A) The simple dendrogram based on the mean number of seta 2 branches from this study (A) in comparison to the dendrogram (B) which taken from Marinochi et al. (1999) .

DISCUSSION

Although advanced tools provide diagnostic criteria, egg morphology has remained as a traditional and useful technique to identify the members of the complex for many years. However, seasonal variation in characters such as presence and absence of egg floats (Mer 1937), and intra-specific polymorphic egg surface patterns, indicate that recognition by egg morphology is not completely accurate (Linton et al. 2002b).

Recently, DNA sequencing of the mitochondrial cytochrome c oxidase gene (COI) (Linton et al. 2003, Sedaghat et al. 2003b), and the nuclear ITS2 rDNA have been employed to differentiate members of the complex (Marinucci et al. 1999, Proft et al. 1999, Romi et al. 2000, Linton et al. 2002a,b, 2003, Sedaghat et al. 2003a,b). DNA-based techniques are reliable and not limited to specific developmental stages or to specific sex (Collins and Paskewitz 1996). Nuclear ITS2 region has also been used to generate phylogeny of members of the complex (Marinucci et al. 1999, Kampen 2005, Dinparast-Jadid et al. 2007).

In spite of the role of the members of Maculipennis Subgroup in transmission of malaria and reporting different species of the Subgroup in Iran, so far they have not been differentiated in the larval stage. The information provided in this study is the first one on *An. maculipennis* subgroup in the larval stage in the country which compared with those available in the world. Bates (1939) and Romi et al. (2002) used the number of branches of setae 2-IV and V to separate the species composition of *An. maculipennis* complex.

In this study, *An. sacharovi* was found in Qazvin, Khuzestan, and East Azarbaijan. Before, this species was reported from this provinces based on the morphological character of egg and adult and/or molecular method (Dow 1953, Dinparast-Jadid et al. 1990, Momeni et al. 1992, Dinparast-Jadid et al. 2001, Sedaghat et al. 2003a). Recently, Dinparast-Jadid et al.

reported this species based on the molecular information from Gilan and Ardebil Provinces (Dinparast-Jadid et al. 2007).

Anopheles atroparvus has been reported from Gilan and Mazandaran based on egg pattern and/or molecular method (Dinparast-Jadid et al. 2001, 2007).

An. melanoon was found in East Azarbaijan and Fars Provinces in this study. Although, this species previously was reported in north and north-eastern areas of the country based on the egg morphology (Dow 1953, Dinparast-Jadid et al. 1990, Azari-Hamidian et al. 2003), there is no information about the distribution of this taxon in Fars Province; different authors only mentioned the occurrence of *An. sacharovi* and *An. maculipennis sensu lato* in Fars.

An. messeae was found in Esfahan, Kurdistan, East Azarbaijan, West Azarbaijan, Khorassan and Mazandaran in this study. Before, *An. messeae* has been reported from Gilan or Mazandaran Provinces based on the molecular method, adult morphology (wing scale index) and egg morphology (Minar 1974, Momeni et al. 1992, Dinparast-Jadid et al. 2001, Azari-Hamidian et al. 2003, Dinparast-Jadid et al. 2007). This species was reported based on the egg morphology from Esfahan by de Zulueta et al. (1957). However, the occurrence of *An. messeae* has not been confirmed in Esfahan by other authors.

This study showed that the means of the seta 2 branches of *An. messeae* from different areas was more similar than *An. maculipennis* from different areas. The results of this study can be used as preliminary information in the future works on sibling species.

Based on the comparison of two dendograms, A from this study and B, taken from Romi et al. (2002); it showed that there was conformity to separate *An. atroparvus* and *An. sacharovi* in two divide monophyletic groups, but there was no conformity for *An. melanoon* and *An. messeae* in two dendograms (Fig. 3).

In dendogram B, which was based on ITS2 sequence data, *An. sacharovi* placed in a basal position within the rest of taxa. Previous studies

derived from rDNA sequence data isolated *An. sacharovi* from the other siblings (Kampen 2005, Dinparast-Jadid et al. 2007). This position also supported the phylogenetic relationships inferred from the previous analyses of polytene chromosome banding patterns (White 1978, Stegnii 1981). Moreover a parsimony analysis of mtDNA data generated by Sedaghat supported this position of *An. sacharovi* (Sedaghat 2003).

In this study, *An. atroparvus* + *An. messeae* formed a separate clade. This position is in agreement with the phylogenetic relationships based on ITS2 sequences defined by Kampen (2005). However, Dinparast-Jadid et al. could not show such relation between *An. atroparvus* + *An. messeae* (Dinparast-Jadid et al. 2007).

The method which employed in this paper can be applied in integrated systematics studies. It is not possible to provide a meaningful morphological diagnosis of *An. maculipennis s.l.* at this time because the other members of the subgroup have not been studied in detail. To date, the life stages of only *An. maculipennis*, *An. sacharovi* and *An. daciae* from this subgroup have been described in detail (Linton et al. 2003, Sedaghat et al. 2003b, Nicolescu et al. 2004, respectively). However, despite these studies there are not enough publications that give a detailed morphological description of each taxon, while a thorough morphological knowledge of all *Anopheles* species is essential. The taxonomic recognition of these species should be based on a combination of morphological, biological and molecular methods on the adults, egg, larva and pupa of the members of the group. A detailed comparative studies of associated all life stages of unambiguously identified samples using of progeny broods, is an important resource for systematic studies.

Although malaria transmission in north and central areas of Iran was completely controlled, travelling from endemic southern areas of the country and neighbouring countries and also presence *An. maculipennis* subgroup as poten-

tial vectors, always are threats of recurrence of the disease in these areas.

In conclusion, considering the medical importance of the *Maculipennis* Subgroup, and differences in vector capacities and species distributions, it is important to elucidate reliable methods to unambiguously identify the species group.

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