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Original Article

Bionomics of Mosquito Larvae (Diptera: Culicidae) in Golestan National Park, a Biosphere Reserve, Northeastern Iran

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Abstract

Background: Golestan National Park is the first, oldest and most vast national park in Iran. It was registered as a biosphere reserve by UNESCO in 1977. The park is located in Golestan, North Khorasan and Semnan Provinces. There is no information about the mosquitoes (Diptera: Culicidae) in this park.

Methods: The larvae of mosquitoes were captured from various habitats using the standard 350 ml capacity dippers and also by pipettes, for small larval breeding sites, and buckets, for wells, during spring–autumn 2019. Larvae were preserved in lactophenol and mounted on microscope slides in Berlese medium and identified by morphological characters. The altitude and coordinates of the sampling localities, larval habitat characteristics and physicochemical features of habitat waters were recorded. Association and affinity indices were calculated.

Results: In total, 1349 larvae including 13 species across four genera were collected: *Anopheles claviger*, *An. maculipennis* s.l., *An. moghulensis*, *An. superpictus* s.l., *Culex hortensis*, *Cx. perexiguus*, *Cx. pipiens*, *Cx. theileri*, *Cx. torrentium*, *Cx. tritaeniorhynchus*, *Culiseta longiareolata*, *Cs. subochrea* and *Uranotaenia unguiculata*. *Anopheles moghulensis* and *Cx. torrentium* were new to the region. *Culiseta longiareolata* (62.6%) displayed the most abundance. *Anopheles maculipennis* s.l., *An. moghulensis* and *Cs. subochrea* were the least specimens (0.1%). Larval habitat characteristics, physicochemical features of habitat waters, association occasions, and percentages were presented. The nitrate of water samples displayed a significant difference among the species (P=0.003).

Conclusion: The study of bionomics of adult mosquitoes and detection of the vectors of different pathogens using serological or molecular-specific tests are recommended.

Keywords: Dominance; Larval habitat characteristics; Water physicochemical features; Affinity index; Association index

Introduction

Golestan National Park is the first, oldest, and most vast national park in Iran. This park has been registered as a biosphere reserve by UNESCO in 1977 (1). The plants of this park include the species from Irano-Turanian, Euro-Siberian, Mediterranean, and Saharo-Sindian floristic areas (2). This makes the park rich in flora and fauna and that is why Golestan National Park includes about 12% of the plant species, 19% of the vascular plant species, 33% of the avian species, and more than 50% of the mammal species of Iran (1, 2).

Although there are many documents about the diversity of mammals, amphibians, rep-

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tiles, birds, fishes, plants and fungi of Golestan National Park, a few data have been published about the arthropods of the park (1). As the authors know, a few published data on arthropods in this park are about Coleoptera (3), Collembola (4), Crustacea (5), Hemiptera (6, 7), Hymenoptera (8, 9), Lepidoptera (1, 10) and ticks (11), but there is no information about the mosquitoes (Diptera: Culicidae) of this biosphere reserve, despite of their medical and veterinary importance and probable influence on the wildlife.

Mosquitoes are the vectors of various protozoal, arboviral and filarial pathogens of humans and different animals. For this reason, they are the most significant family of arthropods in public health (12). The family includes two subfamilies, Anophelinae and Culicinae, 11 tribes, 41 genera, and 3726 species (13). Currently, Iranian mosquitoes include 73 species across eight genera (14–17). Seven arboviruses (causing agents of West Nile fever, Sindbis fever, bovine ephemeral fever, avian pox, Chikungunya fever, Rift Valley fever and dengue fever), two bacteria (causing pathogens of anthrax and tularaemia), four helminths (causal parasites of dirofilariasis, setariasis, dipetalonemiasis and lymphatic filariasis) and two protozoa (parasites of human and bird malaria) have been recorded in Iran. These pathogens are biologically or mechanically known or assumed to be vectored by mosquitoes (14, 18).

There are many published data on the larval breeding site characteristics of mosquitoes in Iran (19–35), however, the information on the physicochemical features of larval water samples and their exact correlation with the mosquito species presence and/or abundance is not very much (25, 32, 36–38). Also, there are a few articles that studied the affinity and association indices among Iranian mosquito species (24, 25, 28, 32, 39).

Basic knowledge about the fauna and ecology of the vectors is necessary to decrease the burdens of the diseases caused by agents that they transmit, applying the integrated vector management based on the One Health approach (14, 18). There is no such data about mosquitoes in Golestan National Park, despite their importance (14). To study the diversity of mosquitoes in Golestan National Park and their larval ecology, this research was carried out during 2019.

Materials and Methods

Study area

The surface area of Golestan National Park is about 91895 hectares and is located between 37º16' 34''–37º31'00''N and 55º43'00''–56º17'45''E. The elevation of the park ranges between 380 and 2410 meters. This park is located in three provinces of Golestan, North Khorasan and Semnan; however, the official administration and management of the park is in the Department of the Environment of Golestan Province (Fig. 1) (1).

Sampling and morphological identification

The larvae of mosquitoes were captured from various natural and artificial oviposition sites using standard 350 ml capacity dippers and also by pipettes, for small habitats, and buckets, for wells (40). The samplings were carried out through six rounds of collections during spring–autumn 2019. Only third- and fourth-instar larvae were identified. Larvae were preserved in lactophenol and mounted on microscope slides in Berlese medium and identified using morphological keys (41). Latitude, longitude and altitude of the sampling localities were obtained using the Garmin eTrex 20 GPS device and recorded in the relevant forms. The altitude and coordinates of the localities are listed in Table 1.

Measure of dominance

The measure of the dominant structure of a species is defined as the percentage of the species specimens in the whole sample. The following five categories are used as measures (percentage) of eudominat (ED) species (>30%), dominant (D; 10–30%), subdominant (SD; 5–10%), recedent (R; 1–5%) and subrecedent (SR; <1%) (42, 43).

Indices of affinity and association between species

The indices of association between pairs of species were calculated using two formulae: Fager's index of affinity and the index of association (44, 45). Fager's index of affinity is defined by $I_{AB}=2J/n_A+n_B$ where J=number of joint occurrences, n_A=total number of occurrences of species A and n_B=total number of occurrences of species B. The significance of the index was analyzed using a t-test (at a 5% probability level). The index of association is defined by I=2[J/A+B–0.5] where J=the number of individuals of both species in samples where they occur together, A=the total number of individuals of species A in all samples and B=the total number of individuals of species B in all samples. In this formula, the numbers of species individuals are taken into account. The formula has a range of +1 to –1.

Larval habitat characteristics

Surface area (m^2), habitat situation (permanent or transient, still or running), habitat kind (natural or artificial), vegetation situation (with or without vegetation), bottom type (mud, sand, gravel, cement or plastic), sunlight situation (full or partial sunlight or shaded) and water turbidity (clear or turbid) were recorded.

Physicochemical analysis of water of larval habitats

For analysis of physicochemical characteristics, water samples were collected from different habitats in 1000 ml polyethylene bottles and transferred to the laboratory with cold boxes. The samples were analyzed for turbidity (NTU), acidity (pH), water temperature (°C), total dissolved solids (TDS) (mg/l), electrical conductivity (EC) (µS/cm), total hardness (mg/l), chemical oxygen demand (COD) (mg/l) and ions such as bicarbonate (HCO_3), calcium (Ca), carbonate (CO_3), chloride (Cl), fluorine (F), magnesium (Mg), nitrate (NO_3), nitrite (NO_2) and sulphate (SO_4). The analysis of physicochemical characteristics was carried out in the Vice-Chancellorship for Health, North Khorasan University of Medical Sciences. The physicochemical features of water samples were measured according to standard methods (46).

Statistical analysis

The normality assumption of physicochemical parameters of water samples was compared by the Shapiro-Wilk test of normality. The means physicochemical parameters of water samples among different species were compared by one-way analysis of variance (ANOVA) or Kruskal-Wallis test, as appropriate. Data were analyzed using SPSS software (Version 21 for Windows, SPSS Inc., Chicago, IL).

Results

In total, 1349 specimens including 13 species across four genera of mosquitoes were captured from 38 larval habitats and identified as: *Anopheles claviger*, *An. maculipennis* s.l., *An. moghulensis*, *An. superpictus* s.l., *Culex hortensis*, *Cx. perexiguus*, *Cx. pipiens*, *Cx. theileri*, *Cx. torrentium*, *Cx. tritaeniorhynchus*, *Culiseta longiareolata*, *Cs. subochrea* and *Uranotaenia unguiculata*. The occurrences of *An. moghulensis* and *Cx. torrentium* were new to the region. *Culiseta longiareolata* (62.6%) displayed the most abundance and was eudominant. *Anopheles maculipennis* s.l., *An. moghulensis*, *Cs. subochrea* as well as *Cx. tritaeniorhynchus* were captured with the least abundance (Subrecedent) (Table 2). *Culiseta longiareolata*, *An. claviger* and *Cx. hortensis* were collected more than other species from different larval habitats including 25, 16 and 13 occasions, while *An. moghulensis* and *Cs. subochrea* (each with one occasion) and *An. maculipennis* s.l., *Cx. perexiguus* and *Cx. tritaeniorhynchus* (each with two occasions) displayed the least collection occasions (larval breeding sites) (Table 3). The association percentages of larvae are shown in Table 4. *Culiseta longiareolata* larvae were captured alone (22.8%) more than any other species (Table 4). The indices of affinity and association of larvae are displayed in Table 5.

The significant affinities and associations of species pairs were displayed with bold numbers (also see discussion). The larval habitat characteristics and occurrence percentages of larvae are shown in Table 6. The surface area of most larval habitats was small (about 1–10 m^2, in one case about 25 m^2). All larvae were collected from clear (versus turbid) habitats. Ten species (out of 13) were mostly collected from natural habitats (75.8–100%). *Anopheles moghulensis* and *Cx. tritaeniorhynchus* were collected only from wells (Artificial habitats). Also, about 44% of *Cs. longiareolata* larvae were collected from artificial oviposition sites (Table 6). The physicochemical parameters of the water samples of larval habitats are shown in Table 7. The normality assumption was not met for any of the parameters (P<0.05), hence nonparametric analysis was used. There was no significant difference in physicochemical parameters (P>0.05) (Table 7). The physicochemical parameters of the habitat water samples of larvae for each species were displayed in Tables 8 and 9. Only nitrate displayed a significant difference among the species (P=0.003).

Table 1. Data collection of mosquito larvae for the localities in Golestan National Park, spring–autumn 2019

Locality/Station	Topography	Coordinates	Altitude (m)
Soulgurd	Plain	N 37º 26' 58", E 56º 08' 22"	1186
Degirmanli	Plain	N 37º 25' 29", E 56º 08' 48"	1289
Dareh-Zoghali	Plain	N 37º 24' 53", E 56º 09' 40"	1423
Bidak	Foothill forest	N 37º 16' 99", E 55º 53' 76"	1295
Alongside Dasht-e-Shad	Foothill forest	N 37º 16' 76", E 55º 50' 30"	1374
Dasht-e-Shad	Foothill forest	N 37º 18' 30", E 55º 49' 84"	1470
After tunnel	Foothill forest	N 37º 21' 01", E 56º 5' 22"	872
After Tangrah	Foothill forest	N 37º 23' 66", E 55º 44' 92"	412
Zav	Forest	N 37º 31' 33", E 55º 47' 07"	601
Beilidagh1	Forest	N 37º 30' 34", E 55º 50' 09"	852
Beilidagh2	Forest	N 37º 30' 02", E 55º 46' 21"	1339
Koeilar	Forest	N 37º 30' 43", E 55º 50' 45"	1329
Shor Cheshmeh	Plain	N 37º 30' 37", E 55º 54' 38"	1249
Ghare Ghovagh	Plain	N 37º 31' 11", E 55º 55' 23"	1147
Olang Cheshmeh	Forest	N 37º 24' 09", E 56º 00' 01"	2068
Ghajar Cheshmeh	Forest	N 37º 24' 08", E 56º 00' 00"	2072
Olang Cheshmeh alongside	Foothill	N 37º 31' 13", E 55º 58' 13"	1136
Ghoshe Cheshmeh	Forest	N 37º 26' 59", E 55º 45' 09"	1033
Mirza Baylu	Plain	N 37º 21' 36", E 56º 15' 20"	1299

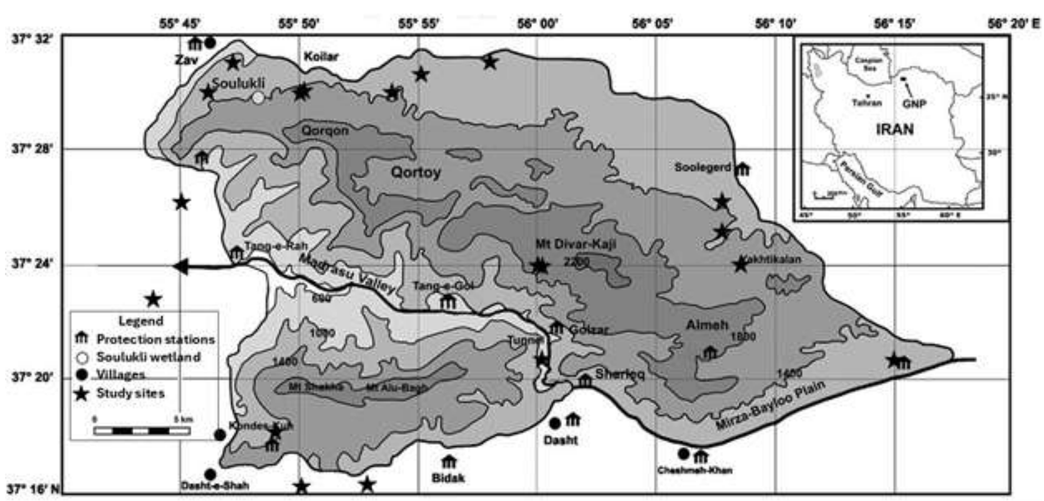

Fig. 1. Topographical map of Golestan National Park including collection sites, adopted from Akhani (1998)

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Table 2. Composition, abundance and dominance structure of mosquito larvae in Golestan National Park, spring–autumn 2019

Species	n	%	Dominance
Anopheles claviger	136	10.1	Dominant
***An. maculipennis* s.l.**	2	0.1	Subrecedent
An. moghulensis	1	0.1	Subrecedent
***An. superpictus* s.l.**	40	2.9	Recedent
Culex hortensis	183	13.6	Dominant
Cx. perexiguus	36	2.7	Recedent
Cx. pipiens	28	2.1	Recedent
Cx. theileri	21	1.5	Recedent
Cx. torrentium	24	1.8	Recedent
Cx. tritaeniorhynchus	8	0.6	Subrecedent
Culiseta longiareolata	845	62.6	Eudominant
Cs. subochrea	1	0.1	Subrecedent
Uranotaenia unguiculata	24	1.8	Recedent
Total	**1349**	**100**	

Table 3. Association occasions of mosquito larvae in Golestan National Park, spring–autumn 2019

Species	**Total occasions (out of 38)**	***Anopheles claviger***	***An. maculipennis* s.l.**	***An. moghulensis***	***An. superpictus* s.l.**	***Culex hortensis***	***Cx. perexiguus***	***Cx. pipiens***	***Cx. theileri***	***Cx. torrentium***	***Cx. tritaeniorhynchus***	***Culiseta longiareolata***	***Cs. subochrea***	***Uranotaenia unguiculata***
Anopheles claviger	16		2	1	6	6	2	3	3	-	1	6	1	3
***An. maculipennis* s.l.**	2	2		-	2	1	1	1	1	-	-	-	-	1
An. moghulensis	1	1	-		1	-	1	-	1	-	1	-	-	-
***An. superpictus* s.l.**	8	6	2	1		5	2	2	2	1	2	2	-	2
Culex hortensis	13	6	1	-	5		-	5	-	1	-	5	-	1
Cx. perexiguus	2	2	1	1	2	-		1	2	-	1	-	-	1
Cx. pipiens	6	3	1	-	2	5	1		1	-	-	4	-	2
Cx. theileri	4	3	1	1	2	-	2	1		1	1	1	-	2
Cx. torrentium	4	-	-	-	1	1	-	-	1		-	3	-	1
Cx. tritaeniorhynchus	2	1	-	1	2	-	1		1	-		-	-	-
Culiseta longiareolata	25	6	-	-	2	5	-	4	1	3	-		1	-
Cs. subochrea	1	1	-	-	-	-	-	-	-	-	-	1		-
Uranotaenia unguiculata	3	3	1	-	2	1	1	2	2	1	-	-	-	

Table 4. Association percentages of mosquito larvae in Golestan National Park, spring–autumn 2019

Species association	Abundance (%)
Anopheles claviger	**136 (100)**
Cx. hortensis, *Cx. pipiens*, *Ur. unguiculata*	42 (31.0)
Cs. longiareolata, *Cs. subochrea*	25 (18.4)
Cs. longiareolata	19 (14.0)
Alone	15 (11.0)
An. maculipennis s.l., *An. superpictus*, *Cx. hortensis*	12 (8.9)
An. superpictus, *Cx. hortensis*	7 (5.1)
Cx. hortensis	7 (5.1)
Cx. theileri	4 (3.0)
An. maculipennis s.l., *An. superpictus*, *Cx. perexiguus*, *Cx. pipiens*, *Cx. theileri*, *Ur. unguiculata*	2 (1.4)
An. superpictus, *An. moghulensis*, *Cx. perexiguus*, *Cx. theileri*, *Cx. tritaeniorhynchus*	2 (1.4)
Cx. pipiens, *Cs. longiareolata*	1 (0.7)
***Anopheles maculipennis* s.l.**	**2 (100)**
An. claviger, *An. superpictus*, *Cx. perexiguus*, *Cx. pipiens*, *Cx. theileri*, *Ur. unguiculata*	1 (50)
An. claviger, *An. superpictus*, *Cx. hortensis*	1 (50)

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Table 4. Continued ...

Anopheles moghulensis	**1 (100)**
An. claviger, *An. superpictus*, *Cx. perexiguus*, *Cx. theileri*, *Cx. tritaeniorhynchus*	
***Anopheles superpictus* s.l.**	**40 (100)**
An. claviger, *An. maculipennis* s.l., *Cx. hortensis*	14 (35.0)
An. claviger, *An. moghulensis*, *Cx. perexiguus*, *Cx. theileri*, *Cx. tritaeniorhynchus*	7 (17.5)
An. claviger, *Cx. hortensis*	7 (17.5)
Cx. tritaeniorhynchus	7 (17.5)
An. claviger, *An. maculipennis* s.l., *Cx. perexiguus*, *Cx. pipiens*, *Cx. theileri*, *Ur. unguiculata*	3 (7.5)
Cx. hortensis, *Cx. pipiens*, *Cs. longiareolata*	1 (2.5)
Cx. hortensis, *Cx. torrentium*, *Cs. longiareolata*	1 (2.5)
Culex hortensis	**183 (100)**
An. superpictus, *Cx. pipiens*, *Cs. longiareolata*	82 (44.8)
An. claviger	41 (22.4)
An. claviger, *An. maculipennis* s.l., *An. superpictus*	21 (11.5)
Cx. pipiens, *Cs. longiareolata*	10 (5.5)
Cx. pipiens	10 (5.5)
An. claviger, *An. superpictus*	7 (3.8)
An. claviger, *Cx. pipiens*, *Ur. unguiculata*	4 (2.2)
Alone	4 (2.2)
Cs. longiareolata	3 (1.6)
An. superpictus, *Cx. torrentium*, *Cs. longiareolata*	1 (0.5)
Culex perexiguus	**36 (100)**
An. claviger, *An. maculipennis* s.l., *An. superpictus*, *Cx. pipiens*, *Cx. theileri*, *Ur. unguiculata*	34 (94.4)
An. claviger, *An. moghulensis*, *An. superpictus*, *Cx. theileri*, *Cx. tritaeniorhynchus*	2 (5.6)
Culex pipiens	**28 (100)**
Cx. hortensis	8 (28.6)
Cx. hortensis, *Cs. longiareolata*	7 (25.0)
An. claviger, *An. maculipennis* s.l., *An. superpictus*, *Cx. perexiguus*, *Cx. theileri*, *Ur. unguiculata*	4 (14.3)
An. claviger, *Cx. hortensis*, *Ur. unguiculata*	3 (10.7)
An. superpictus, *Cx. hortensis*, *Cs. longiareolata*	3 (10.7)
An. claviger, *Cs. longiareolata*	3 (10.7)
Culex theileri	**21 (100)**
An. claviger, *An. maculipennis* s.l., *An. superpictus*, *Cx. perexiguus*, *Cx. pipiens*, *Ur. unguiculata*	14 (66.6)
An. claviger, *An. moghulensis*, *An. superpictus*, *Cx. perexiguus*, *Cx. tritaeniorhynchus*	4 (19.0)
Cx. torrentium, *Ur. unguiculata*	1 (4.8)
An. claviger	1 (4.8)
Cs. longiareolata	1 (4.8)
Culex torrentium	**24 (100)**
Cx. theileri, *Ur. unguiculata*	13 (54.2)
Cs. longiareolata	9 (37.5)
An. superpictus, *Cx. hortensis*, *Cs. longiareolata*	2 (8.3)
Culex tritaeniorhynchus	**8 (100)**
An. superpictus	6 (75)
An. claviger, *An. moghulensis*, *An. superpictus*, *Cx. perexiguus*, *Cx. theileri*	2 (25)
Culiseta longiareolata	**845 (100)**
Alone	193 (22.8)
An. superpictus, *Cx. hortensis*, *Cx. pipiens*	177 (20.9)
Cx. theileri	165 (19.5)
Cx. hortensis, *Cx. pipiens*	143 (16.9)
An. superpictus, *Cx. hortensis*, *Cx. torrentium*	58 (6.9)
An. claviger, *Cs. subochrea*	52 (6.2)
An. claviger	33 (3.9)
Cx. torrentium	11 (1.3)
Cx. hortensis	9 (1.1)
An. claviger, *Cx. pipiens*	4 (0.5)
Culiseta subochrea	**1 (100)**
An. claviger, *Cs. longiareolata*	
Uranotaenia unguiculata	**24 (100)**
An. claviger, *An. maculipennis* s.l., *An. superpictus*, *Cx. perexiguus*, *Cx. pipiens*, *Cx. theileri*	12 (50)
An. claviger, *Cx. hortensis*, *Cx. pipiens*	11 (45.8)
Cx. theileri, *Cx. torrentium*	1 (4.2)

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Table 5. Indices of affinity and association of mosquito larvae in Golestan National Park, spring–autumn 2019. The lower part shows the index of association. The upper part shows Fager's index of affinity. For bold numbers (significant affinities and associations), see the discussion.

Fager's Index of Affinity / Index of Association	*An. claviger*	*An. maculipennis* s.l.	*An. moghulensis*	*An. superpictus* s.l.	*Culex hortensis*	*Cx. perexiguus*	*Cx. pipiens*	*Cx. theileri*	*Cx. torrentium*	*Cx. tritaeniorhynchus*	*Cs. longiareolata*	*Cs. subochrea*	*Ur. unguiculata*
Anopheles claviger		0.222	0.117	**0.500**	0.413	0.222	0.272	0.300	-	0.111	0.292	0.117	0.315
***An. maculipennis* s.l.**	–0.78		-	0.400	0.133	**0.500**	0.250	0.333	-	-	-	-	0.400
An. moghulensis	–0.96	-		0.222	-	**0.666**	-	0.400	-	**0.500**	-	-	-
***An. superpictus* s.l.**	–0.40	**0.22**	–0.62		0.476	0.400	0.285	0.333	0.333	0.400	0.121	-	0.363
Culex hortensis	–0.12	–0.78	-	**0.20**		-	**0.526**	-	0.117	-	0.263	-	0.125
Cx. perexiguus	–0.54	**0.84**	–0.84	**0.20**	-		0.250	**0.666**	-	**0.500**	-	-	0.400
Cx. pipiens	–0.34	–0.68	-	–0.68	**0.20**	**0.18**		0.200	-	-	0.258	-	0.444
Cx. theileri	–0.66	**0.30**	–0.56	–0.10	-	**0.88**	–0.28		0.250	0.333	0.068	-	**0.571**
Cx. torrentium	-	-	-	–0.92	–0.98	-	-	–0.38		-	0.206	-	0.285
Cx. tritaeniorhynchus	–0.96	-	–0.34	–0.10	-	–0.82	-	–0.60	-		-	-	-
Culiseta longiareolata	–0.74	-	-	–0.48	–0.08	-	–0.24	–0.62	–0.82	-		0.076	-
Cs. subochrea	–0.64	-	-	-	-	-	-	-	-	-	–0.88		-
Uranotaenia unguiculata	–0.18	0.00	-	–0.54	–0.86	**0.52**	**0.14**	**0.24**	–0.42	-	-	-	

Table 6. Larval habitat characteristics and occurrence percentages of mosquito larvae in Golestan National Park, spring–autumn 2019

Larval habitat characteristics	*Anopheles claviger*	*An. maculipennis* s.l.	*An. moghulensis*	*An. superpictus* s.l.	*Culex hortensis*	*Cx. perexiguus*	*Cx. pipiens*	*Cx. theileri*	*Cx. torrentium*	*Cx. tritaeniorhynchus*	*Culiseta longiareolata*	*Cs. subochrea*	*Uranotaenia unguiculata*
A) Habitat situation													
1. Permanent	67.5	50	-	63.6	31.3	-	10.7	5.0	91.7	-	2.9	-	50
2. Transient	32.5	50	100	36.4	68.7	100	89.3	95.0	8.3	100	97.1	100	50

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Table 6. Continued ...

3. Slow-running water	69.8	50		63.6	42.0		71.4	-	-		2.0		45.8
4. Stagnant water	30.2	50	100	36.4	58.0	100	28.6	100	100	100	98.0	100	54.2
B) Vegetation situation													
1. Without vegetation	31.0	50	100	87.9	72.7	100	89.3	100	100	100	99.8	100	54.2
2. With vegetation	69.0	50	-	12.1	27.3	-	10.7	-	-	-	0.2	-	45.8
C) Substrate type													
1. Mud	95.2	100	100	84.9	32.3	100	57.1	100	91.7	100	16.1	100	100
2. Sand	4.8	-	-	12.1	60.8	-	10.7	-	-	-	39.6	-	-
3. Gravel	-	-	-	-	5.7	-	28.6	-	-	-	-	-	-
4. Cement	-	-	-	3.0	0.6	-	-	-	8.3	-	13.0	-	-
5. Plastic	-	-	-	-	0.6	-	3.6	-	-	-	31.3	-	-
E) Sunlight situation													
1. Full sunlight	13.5	50	100	78.8	15.9	5.6	3.6	20.0	8.3	100	44.3	-	-
2. Partial sunlight	25.4	-	-	3.0	80.7	-	71.4	-	-	-	41.4	-	-
3. Shaded	61.1	50	-	18.2	3.4	94.4	25.0	80.0	91.7	-	14.3	100	100
F) Habitat type													
1. Natural habitat													
1a. Ground pool	20.6	-	-	3.0	46.6	-	21.4	-		-	52.1	100	-
1b. River bed pool	4.0	-	-	9.1	1.1	-	-	-	-	-	-	-	-
1c. River edge	-	-	-	-	-	-	-	-	-	-	-	-	-
1d. Spring	70.6	50	-	42.5	35.2	-	60.7	10.0	91.7	-	3.6	-	50
1e. Stream edge	3.2	50	-	21.2	15.9	94.4	14.3	70.0	-	-	-	-	50
2. Artificial habitat													
2a. Animal cement trough	-	-	-	3.0	0.6	-	-	-	8.3	-	13.0	-	-
2b. Water storage tank	-	-	-	-	0.6	-	3.6	-	-	-	31.3	-	-
2c. Well	1.6	-	100	21.2	-	5.6	-	20.0	-	100	-	-	-

Table 7. Physicochemical parameters of the water samples of larval habitats in Golestan National Park, spring–autumn 2019

Parameter	Mean	Standard deviation	Minimum	Maximum	Sample Number
Turbidity (NTU)	3.83	3.87	0.60	17.50	12
Acidity (pH)	7.42	0.23	7.03	8.00	14
Temperature (ºC)	19.7	4.9	9.0	24.9	14
Total dissolved solids (TDS) (mg/l)	672.1	685.7	180.3	4230.0	14
Electrical conductivity (EC) (µS/cm)	1213.5	1224.1	304.0	7620.0	14
Total hardness ($CaCO_3$) (mg/l)	514.0	464.3	200.0	3060.0	14
Chemical oxygen demand (COD) (mg/l)	19.6	19.2	0.0	70.0	12
Fluoride (F) (mg/l)	0.64	0.36	0.02	1.72	14
Chloride (Cl) (mg/l)	145.91	236.16	9.99	1399.56	14
Sulphate (SO_4) (mg/l)	313.8	804.6	1.0	5382.0	14

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Table 7. Continued ...

Carbonate (CO_3) (mg/l)	1.0	4.5	0.0	20.0	14
Bicarbonate (HCO_3) (mg/l)	302.0	112.0	97.6	658.8	14
Nitrite (NO_2) (mg/l)	0.007	0.017	0.000	0.083	14
Nitrate (NO_3) (mg/l)	42.3	104.8	0.0	341.0	14
Calcium (Ca) (mg/l)	129.9	123.1	9.6	704.0	14
Magnesium (Mg) (mg/l)	44.1	44.1	1.6	312.0	14

Table 8. Physicochemical parameters of the habitat water samples of mosquito larvae in Golestan National Park, spring–autumn 2019

Species (Total number of occurrences)	**Physicochemical parameters (Mean±SD, Minimum–Maximum, Number)**						
	Turbidity (NTU)	**Acidity (pH)**	**Temperature (ºC)**	**Total dissolved solids (TDS) (mg/l)**	**Electrical conductivity (EC) (µS/cm)**	**Total hardness ($CaCO_3$) (mg/l)**	**Chemical oxygen demand (COD) (mg/l)**
***Anopheles claviger* (16)**	1.93±0.97 (1.35–3.90, 6)	7.30±0.22 (7.03–7.70, 10)	19.2±5.2 (9.0–24.8, 10)	515.0±324.7 (180.3–1227.0, 10)	915.7±524.7 (304.0–2120.0, 10)	414.0±188.2 (200.0–750.0, 10)	11.1±8.7 (3.0–26.0, 6)
***An. maculipennis* s.l. (2)**	-	7.25 (1)	21.2 (1)	300.0 (1)	570.0 (1)	270.0 (1)	-
***An. moghulensis* (1)**	3.90 (1)	7.7 (1)	9.0 (1)	1227.0 (1)	2120.0 (1)	750.0 (1)	26.0 (1)
***An. superpictus* s.l. (8)**	4.00±0.14 (3.90–4.11, 2)	7.50±0.18 (7.25–7.70, 6)	19.7±5.4 (9.0–24.9, 6)	665.8±576.0 (230.0–1556.0, 6)	1208.6±1000.8 (472.0–2800.0, 6)	476.6±349.4 (210.0–1060.0, 6)	35.0±12.7 (26.0–44.0, 2)
***Culex hortensis* (13)**	3.09±2.35 (1.35–7.98, 7)	7.36±0.17 (7.08–7.69, 11)	19.6±3.8 (14.6–24.9, 11)	494.1±398.6 (180.3–1556.0, 11)	885.9±697.6 (304.0–2800.0, 11)	391.8±236.9 (200.0–1060.0, 11)	21.0±20.8 (0.0–49.0, 7)
***Cx. perexiguus* (2)**	3.90 (1)	7.70 (1)	9.0 (1)	1227.0 (1)	2120.0 (1)	750.0 (1)	26.0 (1)
***Cx. pipiens* (6)**	3.55±2.68 (1.55–7.98, 5)	7.33±0.20 (7.03–7.58, 6)	19.4±4.5 (15.0–24.9, 6)	386.6±151.3 (230.0–661.0, 6)	690.5±239.9 (465.0–1102.0, 6)	390.0±173.7 (220.0–720.0, 6)	29.6±19.2 (3.0–49.0, 5)
***Cx. theileri* (4)**	3.72±0.24 (3.55–3.90, 2)	7.85±0.21 (7.70–8.00, 2)	15.9±9.7 (9.0–22.8, 2)	792.0±615.1 (357.0–1227.0, 2)	1409.0±1005.5 (698.0–2120.0, 2)	500.0±353.5 (250.0–750.0, 2)	13.0±18.3 (0.0–26.0, 2)
***Cx. torrentium* (4)**	-	7.32 (1)	21.2 (1)	1556.0 (1)	2800 (1)	1060 (1)	-
***Cx. tritaeniorhynchus* (2)**	3.90 (1)	7.62±0.10 (7.55–7.70, 2)	15.1±8.6 (9.0–21.2, 2)	737.5±692.2 (248.0–1227.0, 2)	1296.5±1164.6 (473.0–2120.0, 2)	480.0±381.8 (210.0–750.0, 2)	26.0 (1)
***Culiseta longiareolata* (25)**	5.32±6.18 (0.60–17.50, 12)	7.46±0.26 (7.03–8.00, 14)	22.2±3.0 (15.0–24.9, 14)	940.4±1156.5 (205.7–4230.0, 14)	1728.7±2085.2 (423.0–7620.0, 14)	720.7±817.7 (220.0–3060.0, 14)	15.9±24.3 (0.0–70.0, 12)
***Cs. subochrea* (1)**	-	7.20 (1)	22.4 (1)	444 (1)	841 (1)	390.0 (1)	-
***Uranotaenia unguiculata* (3)**	-	7.32 (1)	21.3 (1)	431 (1)	813 (1)	380.0 (1)	-

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Table 9. Chemical parameters (ions) of the habitat water samples of mosquito larvae in Golestan National Park, spring–autumn 2019

Species (Total number of occurrences)	Chemical parameters (Mean±SD, Minimum–Maximum, Number) (mg/l)								
	Fluoride (F)	Chloride (Cl)	Sulphate (SO_4)	Carbonate (CO_3)	Bicarbonate (HCO_3)	Nitrite (NO_2)	Nitrate (NO_3)	Calcium (Ca)	Magnesium (Mg)
Anopheles claviger **(16)**	0.70±0.42 (0.28–1.72, 10)	92.96±120.42 (19.99–399.87, 10)	130.4±162.4 (1.0–477.0, 10)	0 (9)	326.9±136.0 (158.6–658.8, 10)	0.002±0.003 (0.000–0.009, 10)	35.8±107.2 (0.0–341.0, 10)	100.5±59.6 (9.6–200.0, 10)	32.0±19.6 (1.6–60.0, 10)
An. maculipennis **s.l. (2)**	0.40 (1)	29.99 (1)	48.0 (1)	0 (1)	256.2 (1)	0.005 (1)	1.6 (1)	60.0 (1)	28.8 (1)
An. moghulensis **(1)**	0.64 (1)	399.87 (1)	368.0 (1)	0 (1)	158.6 (1)	0 (1)	341 (1)	200.0 (1)	60.0 (1)
An. superpictus **s.l. (8)**	0.64±0.33 (0.38–1.24, 6)	143.28±176.88 (19.99–399.87, 6)	271.3±427.1 (3.0–1098.0, 6)	0 (6)	250.1±60.6 (158.6–341.6, 6)	0.005±0.005 (0.000–0.014, 6)	58.1±138.5 (0.8–341.0, 6)	114.6±83.0 (48.0–240.0, 6)	45.6±35.4 (14.4–110.4, 6)
Culex hortensis **(13)**	0.67±0.45 (0.28–1.72, 11)	75.42±101.78 (19.99–339.89, 11)	177.5±333.9 (2.0–1098.0, 11)	0 (11)	303.8±63.4 (195.2–390.4, 11)	0.002±0.003 (0.000–0.008, 11)	9.2±23.5 (0.0–80.2, 11)	94.5±52.3 (56.0–240.0, 11)	37.3±26.5 (14.4–110.4, 11)
Cx. perexiguus **(2)**	0.64 (1)	399.87 (1)	368.0 (1)	0 (1)	158.6 (1)	0 (1)	341.0 (1)	200.0 (1)	60.0 (1)
Cx. pipiens **(6)**	0.52±0.21 (.37–0.86, 6)	38.31±24.00 (19.99–79.97, 6)	47.3±52.8 (2.0–125.0, 6)	0 (6)	378.2±147.2 (231.8–658.8, 6)	0.0006±0.001 (0.000–0.004, 6)	15.2±31.8 (0.0–80.2, 6)	98.6±46.5 (64.0–192.0, 6)	34.4±16.7 (14.4–57.6, 6)
Cx. theileri **(4)**	0.52±0.16 (0.40–0.64, 2)	219.92±254.48 (39.98–399.87, 2)	227.5±198.6 (87.0–368.0, 2)	10.0±14.1 (0.0–20.0, 2)	225.7±94.8 (158.6–292.8, 2)	0.008±0.011 (0.000–0.016, 2)	171.0±240.3 (1.1–341.0, 2)	128.0±101.8 (56.0–200.0, 2)	43.2±23.7 (26.4–60.0, 2)
Cx. torrentium **(4)**	1.24 (1)	339.89 (1)	1098.0 (1)	0 (1)	280.6 (1)	0.004 (1)	1.1 (1)	240.0 (1)	110.4 (1)
Cx. tritaeniorhynchus **(2)**	0.54±0.14 (0.44–0.64, 2)	209.93±268.61 (19.99–399.87, 2)	188.0±254.5 (8.0–368.0, 2)	0 (2)	195.2±51.7 (158.6–231.8, 2)	0.007±0.009 (0.000–0.014, 2)	171.2±240.1 (1.4–341.0, 2)	124.0±107.4 (48.0–200.0, 2)	40.8±27.1 (21.6–60.0, 2)
Culiseta longiareolata **(25)**	0.61±0.42 (0.02–1.37, 14)	237.77±402.56 (9.99–1399.56, 14)	701.8±1517.8 (1.0–5382.0, 140	2.8±7.2 (0.0–20.0, 14)	316.3±128.2 (97.6–658.8, 14)	0.020±0.032 (0.000–0.083, 14)	10.0±22.1 (0.0–80.2, 14)	192.0±216.5 (52.0–704.0, 14)	57.7±78.1 (7.2–312.0, 14)
Cs. subochrea **(1)**	0.90 (1)	59.98 (1)	90.0 (1)	0 (1)	366.0 (1)	0.009 (1)	1.6 (1)	100.0 (1)	33.6 (1)
Uranotaenia unguiculata **(3)**	0.86 (1)	49.98 (1)	89.0 (1)	0 (1)	341.6 (1)	0.004 (1)	3.0 (1)	88.0 (1)	38.4 (1)

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Discussion

This article presents the fauna of mosquitoes of Golestan National Park for the first time, including 13 species across four genera. Two species, *An. moghulensis* and *Cx. torrentium*, are found for the first time in the region. There are no previous records for them in Golestan, North Khorasan and Semnan Provinces where this park is located. *Anopheles moghulensis* is recorded only in Hormozgan Province and Sistan and Baluchistan Province in southern Iran (27, 47). Its record in northern Iran needs to be verified by collecting more specimens, especially adults. Also, it is not easy to distinguish the larvae and females of *Cx. torrentium* and *Cx. pipiens*. The most reliable characters for differentiating them are those of the male genitalia (41). *Culex torrentium* is recorded with certainty in Ardebil Province (48), Guilan Province (49, 50) and Mazandaran Province (30, 38, 39) in northern Iran. Other records in southern Iran, such as Bushehr (51) and Fars (52, 53) Provinces need to be verified.

Among the collected species, *An. maculipennis* s.l. and *An. superpictus* s.l. are the proven vectors of malaria parasites in Iran (14). *Anopheles maculipennis* and *Culex theileri* vector *Setaria labiatopapillosa*, causing agent of setariasis, and the dog heart worm *Dirofilaria immitis*, causing agent of dirofilariasis, respectively (48). *Culex pipiens* and *Cx. theileri* are known vectors of West Nile fever virus and probably Sindbis fever virus (54–56). Dirofilariasis and West Nile fever have been found in Golestan Province (14). Also, Bakhshi et al. (57) detected chikungunya virus in *An. maculipennis* s. l., *Cx. tritaeniorhynchus* and *Cs. longiareolata* in North Khorasan Province employing primers designed for the CHIKV Asian genotype, but they failed to isolate the virus and perform whole genome sequencing.

Culiseta longiareolata was the most prevalent species (Eudominant) and was collected mostly with other prevalent (Dominant) species: *An. claviger* and *Cx. hortensis* (Tables 2–4). This high association was also observed in Guilan Province of northern Iran (20). Moreover, high association of *Cs. longiareolata* and *Cx. hortensis* was observed in Chaharmahal and Bakhtiari Province (32). The high percentage of isolation of this species (22.8%) may be because of the predation behavior of *Cs. longiareolata* larvae against other mosquito larvae (58). This was observed in the Hormozgan Province of southern Iran (25: 36.6%), Isfahan Province of central Iran (28: 37.0%) and Kurdistan Province of western Iran (26: 96.9%), either. However, this isolation was not very high in Guilan Province (20: 10.6%). This may be because of the different climate of Guilan Province, temperate with higher precipitation, which causes more available larval habitats, or other physicochemical and biological features that influence larval population and their co-occurrences (32). It is noteworthy that the determination of the seasonal activity of the species was not among the goals of this study. However, the season of collection may influence the abundance and possibility of the collection of the species in different ways, especially via changes in temperature and precipitation (32).

Although some pairs of species displayed significant indices of affinity or association (Table 5), it is not possible to discuss them with certainty, because of the rarity of individuals of them. Only three species, *An. claviger*, *Cx. hortensis* and *Cs. longiareolata*, were captured in higher numbers of individuals (Eudominant and dominant). They did not show any significant affinity and association, but they had higher affinity and association indices with each other (Table 5). This is a more logical result than the significant indices of affinity and association for rare species. In a study in the Hormozgan Province of southern Iran, Hanafi-Bojd et al. (24) found that there were significant affinities between eight pairs of anopheline mosquitoes: *An. culicifacies* s.l./ *An. dthali*, *An. culicifacies* s.l./ *An. stephensi*, *An. culicifacies* s.l./ *An. turkhudi*, *An. culicifacies* s.l./ *An. moghulensis*, *An. dthali*/*An. stephensi*, *An. dthali*/

An. superpictus s.l., *An. dthali*/ *An. moghulensis* and *An. superpictus* s.l./ *An. moghulensis*. In a study in the Isfahan Province of central Iran, there was no significant affinity or association between species pairs when they occurred together except for the pair of *An. dthali*/ *An. turkhudi* that each was collected one time as one larva (28). In Mazandaran Province of northern Iran, Nikookar et al. (39) observed an association between *An. hyrcanus*/ *An. pseudopictus*, but the exact difference between these two species in the larval stage and their taxonomy needs to be investigated extensively (14, 41). In another investigation in Hormozgan Province, the pair of *Cx. sinaiticus*/ *Cx. tritaeniorhynchus* showed significant affinity using Fager and McGowan's test and the pair of *Ae. caballus*/ *Ae. vexans* (with low abundance) displayed significant association according to the index of association (25). Omrani and Azari-Hamidian (32) found significant affinities between the following pairs of species in Chaharmahal and Bakhtiari Province: *Cx. theileri/ An. maculipennis* s.l., *Cx. theileri/ Cx. perexiguus*, *An. superpictus* s.l./ *Cx. perexiguus*, *An. superpictus* s.l./ *Cx. theileri*, *Cx. perexiguus/ An. maculipennis* s.l. and *Cs. longiareolata/ Cx. hortensis*. These affinities and associations may show similar ecological requirements of these pair species. To the best of our knowledge, there is no more data about the affinity and association indices of mosquitoes in Iran.

As expected, most of the species specimens were collected from natural habitats (75.8–100%), because of the rarity of artificial habitats, but rare larvae of *An. moghulensis* and *Cx. tritaeniorhynchus* were collected only from wells (Artificial habitat) (Table 6). The rice field mosquito *Cx. tritaeniorhynchus* was found mostly (80.59%) in natural larval breeding sites in the Guilan Province of northern Iran, but more than 66% of the collected specimens from artificial habitats were found in rice fields (21). Also, more than 95% of the specimens of this species were found in natural oviposition sites in the Hormozgan Province of southern Iran (25). On the other hand, Zaim (19) reported that 76 % of the specimens of the species were collected from man-made (artificial) habitats, including more than 68% from rice fields, in various areas of the country. Also, Shoraka et al. (33) found 62.7% of larvae of this species in artificial habitats, including about 10% in rice fields, in Golestan Province. Interestingly, about 44% of *Cs. longiareolata* larvae were captured from artificial habitats (Table 6). Also, 41.4, 50, 51.3, 52 and 88.8% of this species larvae were collected from artificial larval breeding sites in Charmahal and Bakhtiari Province (32), Kashan County of Isfahan Province (34), Mazandaran Province (30), Hamedan Province (23) and Golestan Province (31), respectively. However, the species larvae were collected from natural habitats in as much as 78% of the whole country (19), 81.5% of Hormozgan Province (35), 89 to 100% of Kurdistan Province (26, 29), and 100% of Guilan Province (20). These differences may be because of sampling and different climates which cause differences in precipitations and available habitats. In this point of view, for example, Guilan and Kurdistan Provinces with more precipitations provide much more natural oviposition sites for mosquitoes. Also, most of the available habitats in Hormozgan Province, with an arid climate, are permanent or seasonal temporary rivers or streams (natural). Another abundant species of this investigation, *Cx. hortensis*, was collected 98.8% from natural habitats (Table 6). The species was also collected from natural habitats as much as 71.3–100% in Kurdistan Province (26, 29), 85% in Guilan Province (21), 93.5% in the whole country (19) and 100% in Isfahan Province (28). Another prevalent species of this study, *An. claviger*, was collected from natural habitats as much as 98.4% (Table 6). This species was also captured from natural habitats in Mazandaran Province as much as 77.4% (30), 97.9% in Guilan Province (22) and 100% in Kurdistan Province (26). In general, it seems, these species prefer natural habitats.

In the present investigation, among the physicochemical parameters of the habitat water samples of larvae, only nitrate displayed a significant difference among the species (Tables 8 and 9). Ghanbari et al. (36) found a significant correlation between the larvae of the following anophelines with some physicochemical features in southeastern Iran: *An. culicifacies* s.l. (Phosphate, EC, and calcium), *An. stephensi* (Nitrate), *An. superpictus* s.l. (Total hardness, calcium), *An. turkhudi* (pH, total hardness, nitrate, calcium) and *An. multicolor* (pH, sulphate). In two studies in Iran, there were no significant correlations between physicochemical and/or microbial features and the presence of larvae in Qom Province (37) and Hormozgan Province (25). In a study in northern Iran, *Cx. pipiens* larval abundance showed a significant positive correlation with EC, alkalinity, total hardness and chloride (38). In an investigation in Chaharmahal and Bakhtiari Province, pH, dissolved oxygen and temperature significantly differed between the larval breeding sites of different mosquito species (32). As far as we know, there is no more information in this regard in Iran. According to a systematic review and meta-analysis, pH, turbidity, EC, dissolved oxygen, nitrogen and phosphorus displayed a significant positive pooled correlation with mosquito presence and abundance, while alkalinity showed a nonsignificant null pooled correlation (59).

One of the limitations of the present investigation was the rarity of specimens of some species. They were collected in a few numbers; thus, it was not possible to analyze their larval habitat characteristics or physicochemical features of habitat water with certainty. The reason probably was the rarity of available larval habitats, sampling limitation or the relatively low population of some species. In this regard, several limitations of the study of mosquito larvae have been listed by Banafshi et al. (26) and Omrani and Azari-Hamidian (32). Also, the possibility of the sampling of some species larvae is low, for example, some aedine species, especially tree-hole species. Hence, adult collections using manual aspirators while landing on animal or human baits, light traps with or without CO_2, BG lure traps and ovitraps may increase the possibility of finding new species in the region. Another important limitation of sampling was the inaccessibility of many localities. Most areas of the park are mountainous forests without any roads for vehicles. Thus, in this study, most sampling sites were limited to those areas which were close to the present available roads.

Conclusion

Among the identified species, there are many potential vectors of pathogens that can infect humans and animals. The investigation of the ecology of mosquitoes, especially using the collections of adults, and the detection of the exact vectors using serological or molecular-specific tests are recommended for future studies.

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Ethical considerations

The ethical code IR.GUMS.REC.1398.283 has been registered for this study.

Conflict of interest statement

The authors declare there is no conflict of interest.

Dedication

This article is dedicated to the late Mr Dordy-Mohammad (Freydoon) Aghatabai, one of the colleagues of the present project, who passed away during conducting this research.

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