Heliyon 6 (2020) e04480

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Morphological and molecular identification of Culicidae mosquitoes (Diptera: Culicidae) in Lorestan province, Western Iran

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ARTICLE INFO

Keywords:
Ecology
Iran
Anopheles
Culex
Aedes
Fauna

ABSTRACT

Culicidae mosquitoes are main vectors of arboviruses that cause arboviral diseases in humans. Studies on fauna, ecology, biology, resting behaviors of Culicidae mosquitoes are important and greatly impacts the control of arboviral diseases that are transmitted by vectors. The aim of the present study was to determine fauna of mosquitoes (Diptera: Culicidae) based on morphological and molecular (genomic) identification and their habitats in Lorestan province, Western Iran. Meanwhile mosquito samples were examined for arbovirus infection.

Culicidae mosquitoes were caught in 2015 and 2016 from human homes, animal dwellings, storehouses and pit shelters in Lorestan province, Western Iran, using an oral aspirator (hand catch), total catch, human and animal bait and light trap methods. The samples were identified on the genus and species. Six species of Culex and eight species of Anopheles were caught. One complex species (*Cx. pipiens* complex) and a hybrid between *Cx. pipiens pipiens* biotype *pipiens* and *Cx. pipiens pipiens* biotype *molestus* were identified. Among all of the trapped mosquitoes (4211), 94.68% were from genus Culex mosquitoes (3987), which indicate that this genus is the dominant in Lorestan province, Western Iran. Anopheles comprised of 201 individuals out of the total catch. Arboviruses were not detected in these samples.

1. Introduction

Culicidae mosquitoes are main vectors of arboviruses that cause arboviral diseases in humans. Studies on fauna, ecology, biology, resting behaviors of Culicidae mosquitoes are important and have great impact to control arboviral diseases transmitted by vectors. Previous researche have studied the ecology, fauna and biology of Culicidae mosquitoes in adult and larval stages in Western provinces of Iran and different species of Anopheles, Culex and Aedes genera were reported (Moosa-Kazemi et al., 2015; Ghavami and Ladonni, 2005; Banafshi et al., 2013; Oshaghi et al., 2011; Khoshdel-Nezamiha et al., 2014; Abai et al., 2007). In some investigations resting and blood feeding behaviors of Culicidae mosquitoes and different larval habitats were reported (Yaghoobi-Ershadi et al., 2001; Kalandadze and Kaviladze, 1947; Shahhosseini et al., 2018a).

Infection of Culicidae mosquitoes to arboviruses, especially west Nile virus, in other studies have been investigated in Western Iran (Shahhosseini et al., 2016b, 2017a, 2017b, 2018b; Shahhosseini and Chinikar, 2016a; Chinikar et al., 2013a, 2013b; Meshkat et al., 2015; Shah-Hosseini et al., 2014). There are few studies related to fauna, ecology, biology, resting and blood feeding behaviors of Culicidae mosquitoes in

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https://doi.org/10.1016/j.heliyon.2020.e04480
Received 19 November 2018; Received in revised form 8 February 2019; Accepted 14 July 2020

Lorestan province, Western Iran. Mainly fauna and ecology of Anopheles genus have been investigated. *An. superpictus* have been reported the dominant and main vector of malaria in Lorestan province (Kayedi et al., 2001a; Kayedi and Rasi, 2001b; Kassiri and Amani, 2012; Amani et al., 2014).

The aim of the present study was to determine fauna of adult mosquitoes (Diptera: Culicidae) based on morphological and molecular (genomic) identification and their habitats in Lorestan province, Western Iran. Meanwhile, mosquito samples were examined for arbovirus infection.

2. Materials and methods

2.1. Mosquito collection

Lorestan province (33° 34′ 54.62″ N 48° 23′ 55.748″ E) is located in Western Iran, in the central Zagros area. Lorestan is a mountainous area with an elevation of 500–4050 m above sea level and mountains that cross from northwest to the southeast along the Zagros Dynasty. The province has a Mediterranean climate with an average annual rainfall of 450 mm. In general, there are three distinct climatic zones in the Lorestan province: warm in the south, moderate in the center and cold in the north and east of the province. The summer season is warm and dry without rainfall, and the rainy season starts from mid-November and continues until mid-June of the next year. In the moderate and cold regions of the province, snow is observed during the winter months.

During 2015, 2016, Culicidae mosquitoes were collected from nine counties of Lorestan province, in 24 rural areas (human places, animal shelters, pit shelter and storehouses) by hand catch method (oral/electronic aspirator), total catch method, human and animal bait method and New Jersey light trap (Figure 1). Mosquitoes were collected six times during field study for each collecting method and placed in 24 rural areas.

The description of catch methods used in this study is as follows:

Hand catch method: In each of the studied villages, human and animal places, storehouses, and pit shelters were selected and mosquitoes were collected using oral and electric aspirator, then they were stored in proper conditions until transferred to the laboratory (Ghavami and Ladonni, 2005; Banafshi et al., 2013).

Total catch method: In each of the studied villages, suitable human and animal places were selected and mosquitoes were collected (using standard method) and transferred to the laboratory (Khoshdel-Nezamiha et al., 2014).

Human and animal bait: Human and animal bait were performed in designated areas in the villages using a human volunteer and a cow before sunset (Shahhosseini et al., 2017a, 2018a). Light trap method: In this method, we used a New Jersey light trap. In the evening, the device was installed in a human or animal place and mosquitoes were collected

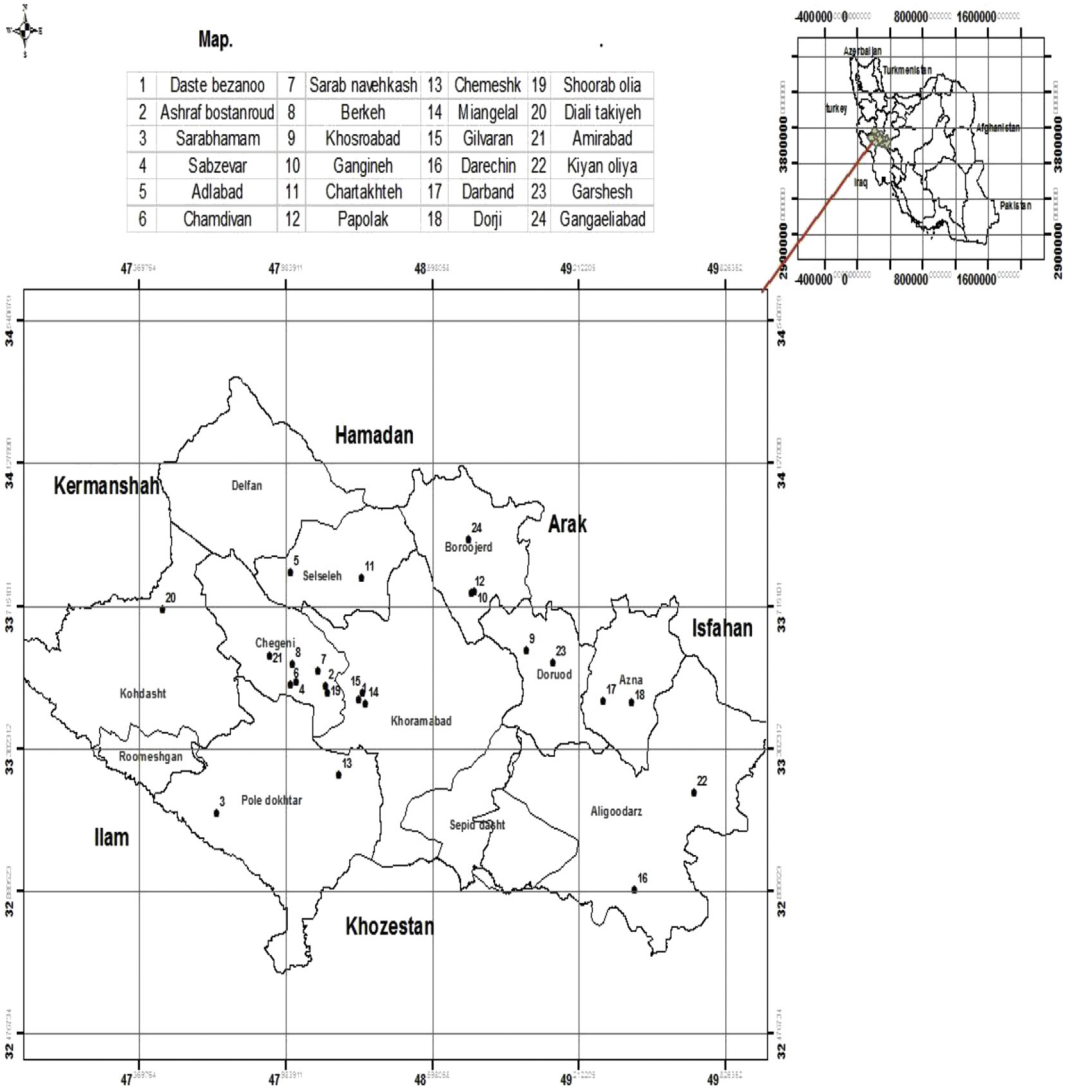

Figure 1. Map of Iran (right), province of Lorestan (left), and collecting sites (villages) of Culicidae mosquitoes in western Iran are indicated by number (above table).

Table 1. Mosquitoes that were caught by different methods according to collecting sites (villages) that were numbered in Figure 1.

No	County	Village	Culicidae mosquitoes
1	Khorramabad	1.Dase bezano	*Cx. theileri, Cx. pipiens, Cx. perexiguus, An. fluviatilis, An. superpictus, Ae. vexans, Cx. quinquefasciatus, Cx. pipiens molestus An. stephensi*
		14.Miangelal	*Cx. theileri, Cx. pipiens, Cx. perexiguus, Cx. tritaeniorthyncus, An. superpictus, An. sacharovi, An. dthali, An. maculipennis, An. apoci, An. stephensi, Culicoides*
		15.Gilvaran	*Cx. theileri, An. maculipennis*
2	Chegeni	2.Ashrafbostanroud	*Cx. theileri, Cx. pipiens, Cx. pipiens molestus, Cx. perexiguus, Cx. hortensis, An. maculipennis, Cs. Longiareolata, Ur. unguiculata*
		6.Chamdivan	*Cx. theileri, An. sacharovi, An. maculipennis*
		4.Sabzevar	*Cx. theileri, An. maculipennis*
		8.Berkeh	*Cx. theileri, Cx. pipiens, Cx. pipiens molestus, Cx. perexiguus, An. superpictus, An. sacharovi, An. dthali, An. stephensi*
		21.Amirabad	*Cx. pipiens, An. superpictus*
		19.Shorab olia	*Cx. theileri, An. sacharovi*
		7.Sarab navehkash	*Cx. theileri*
3	Boroujerd	24.Gangaeliabad	*Cx. theileri, An. superpictus*
		12.Papolak	*Cx. theileri, Cx. perexiguus, An. maculipennis*
		10.Gangineh	*Cx. theileri,*
4	Azna	17.Darband	*Cx. theileri, Cx. pipiens, Cx. perexiguus, An. superpictus, An. sacharovi, An. fluviatilis, Cs. longiareolata*
		18.Dorji	*Cx. theileri, Cx. perexiguus, An. fluviatilis, An. superpictus, An. dthali, Cs. annulata,*
5	Pole Dokhtar	3.Sarabhamam	*Cx. theileri, Cx. pipiens,*
		13.Chemeshk	*Cx. theileri, Cx. pipiens, Cx. perexiguus, An. sacharovi*
6	Selseleh	5.Adlabad	*Cx. theileri*
		11.Chahartakteh	*Cx. theileri, An. superpictus, Cx. pipiens molestus,*
7	Doroud	9.Khosroabad	*Cx. theileri, An. claviger*
		23.Garshesh	*Cx. theileri, Cx. pipiens, An. sacharovi*
8	Aligodarz	22.Kiyan oliya	*Cx. theileri, Cx. perexiguus, An. sacharovi, Cs. longiareolata*
		16.Darechin	*Cx. pipiens,*
9	Kohdasht	20. Diali takiyeh	*Cx. perexiguus*

and transferred to the laboratory the next morning (Chinikar et al., 2013a).

Morphological identification of mosquitoes: Samples were shipped to the Medical Entomology Laboratory, School of Public Health, Tehran University of Medical Sciences in a cool box for morphological identification of mosquitoes using identification keys of adult Culicidae mosquito species of Iran (Shahgudian, 1960; Azari-Hamidian and Harbach, 2009), and subsequently sent to Bernhard Nocht Institute for Tropical Medicine (Hamburg, Germany) for arbovirus screening and molecular taxonomy. This research was approved by the Ethical Committee of Lorestan University of Medical Sciences (Code: 200/5686).

2.2. Nucleic acid extraction, DNA-barcoding PCR and sequencing

From each morphologically identified adult mosquito species, individual mosquitoes were selected for confirmation using DNA-barcoding. Each individual mosquito was homogenized and the RNA and DNA extracted using the procedure previously described (Shahhosseini et al., 2017b). RNA extractions were treated to test for Arthropod-borne viruses (Arboviruses) (Shahhosseini et al., 2017b).

For PCR, amplification was conducted with primers targeting an mtDNA gene, cytochrome-oxydase subunit 1 (597-bp fragment). The primer pair used were: CI-J-1632 (5′-TGATCAAATTTATAAT-3′) and CI-N-2191 (5′-GGTAAAATTAAAATATAAACTTC-3′) (Korba et al., 2016) [34]. Reactions were made in 25 μl volume containing 10 μl DNA template, 2× HotStar Taq plus Master Mix 4.20 μl, 25 mM MgCl2 1.6 μl, 10 μM of each primer 0.6 μl (QIAGEN). The temperature profile consisted of an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 40 °C for 45 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min [Phylogeography of *Aedes (Stegomyia) aegypti* (L.) and *Aedes (Stegomyia) albopictus* (Skuse) (Diptera: Culicidae) based on mitochondrial DNA variations. Genet Res. 2005;]. An aliquot of 5 μl of each PCR product was subjected to electrophoresis on a 2% agarose gel stained with Midori-green and photographed with Gel Doc system. When bands with expected size were visualized, the remaining PCR products were used for Sanger sequencing (LGC genomic, Berlin) and the sequences were compared to existing sequences from publicly available databases.

2.3. Culex taxonomy real-time PCR

All mosquitoes morphologically identified as members of the *Cx. pipiens* complex were further typed using previously designed primers for *Culex pipiens* 1725-F (5′-GCGGCCAAATATTGAGACTT-3′) and 1726-R (5′- CGTCCTCAAACATCCAGACA-3′) and probes *Cx. pipiens* all (5′-Cy55- GGAACATGTTGAGCTTCGGK -BBQ-1 -3′), *Cx. pipiens pipiens* biotype *pipiens* (5′-JOEGCTTCGGTGAAGGTTTGTGT-BHQ1 -3′) and *Cx. pipiens pipiens* biotype *molestus* (5′- Rox-TGAACCCTCCAGTAA-GGTATCAACTAC- BHQ2 -3′). *Cx. torrentium* was molecularly detected using the primers *Cx. torrentium* F (5′-GACACAGGACGACAGAAA-3′), and R (5′- GCCTACGCAACTACTAAA-3′) and the probe *Cx. torrentium* (5′-FAM- CGATGATGCCTGTGCTACCA-BHQ1-3′). Multiplex real-time PCR was performed in a 20 mL reaction volume using HotStarTaq Master Mix Kit (Qiagen). Real-time PCR was performed with an initial denaturation step 95 °C (15 min), followed by 50 cycles denaturation at 95 °C (15 s), annealing at 60 °C (20 s), extension at 72 °C (30 s), and a final elongation at 40 °C (30 s) (Shahhosseini et al., 2018b).

2.4. Larva collection

Culicidae larvae (ages 3 and 4) were collected six times during field studies in 2015 and 2016 from larval habitats. Samples were shipped to the Medical Entomology Laboratory, School of Public Health, Tehran University of Medical Sciences for morphological identification using discrimination keys of Culicidae mosquito species of Iran (Shahgudian, 1960; Azari-Hamidian and Harbach, 2009).

3. Results

A total of 4211 adult Culicidae mosquitoes and biting midges were caught and collected (Table 1), of which 3987 (94.68%) were Culex, 201

Table 2. The Number and percentage of trapped Culex and Anopheles mosquitoes from Lorestan province in 2015–2016.

No	Mosquito species	Total
1	*Cx. theileri*	3674 (92.15)
2	*Cx. pipiens. pipiens biotype pipiens*	228 (5.72)
3	*Cx. pipiens.pipiens biotype molestus*	3 (0.07)
	Cx. hybrid molestus/pipiens	12 (0.30)
4	*Cx. quinquefasciatus*	1 (0.03)
5	*Cx. perexiguus*	64 (1.61)
6	*Cx. hortensis*	3 (0.07)
7	*Cx. tritaeniorthyncus*	2 (0.05)
	Total Culex species	3987
8	*An. sacharovi*	57 (28.36)
9	*An. maculipennis*	6 (2.98)
10	*An. superpictus*	111 (55.22)
11	*An. stephensi*	14 (6.97)
12	*An. claviger*	1 (0.50)
13	*An. dthali*	6 (2.98)
14	*An. fluviatilis*	5 (2.49)
15	*An. apoci*	1 (0.50)
	Total Anopheles species	201
Total Culex + Anopheles species		4188

(4.77%) were Anopheles, 2 (0.047%) were Aedes, 16 (0.38%) were Culiseta, 3 (0.07%) were Culicoides (Diptera: Ceratopogoindae) and 2 (0.047%) were Uranotaenia.

Six species of Culex were caught, including *Culex (Cx) theileri* (3674), *Cx. pipiens* complex (243), *Cx. perexiguus* (64), *Cx. hortensis* (3), *Cx. quinquefasciatus* (1) and *Cx. tritaeniorthyncus* (2) (Table 2). One complex species (*Cx. pipiens* complex) and a hybrid between *Cx. pipiens pipiens* from *pipiens and Cx. pipiens pipiens* from *molestus* were identified.

Two biotypes and one hybrid form were identified by genomic identification of *Cx. pipiens* complex. Of the 243 female *Cx. pipiens* complex; 93.83% were recognized as *Cx. pipiens pipiens* biotype *pipiens*, 1.23% as *Cx. pipiens. pipiens* biotype *molestus* and 4.94% were identified as a hybrid (*molestus/pipiens*) form.

Eight species of Anopheles were identified, including *Anopheles (An) superpictus* (111), *An. sacharovi* (57), *An. maculipennis* (6), *An. stephensi* (14), *An. dthali* (6), *An. fluviatilis* (5), *An. claviger* (1), and *An. apoci* (1) (Table 2).

For the first time ever in Lorestan province, one species of Aedes *(Ae), Ae. vexans* (2), one species of Culiseta, *Cs. annulata* (1), and one species of Uranotaenia *(Ur. unguiculata)* (2) were caught. Arboviruses were not detected in samples.

A total of 764 larvae of Culicidae mosquitoes were caught and collected, of which 703 (92.02%) were Culex, 40 (5.24%) were Anopheles and 21 (2.74%) were Culiseta. Four species of Culex were caught, including *Culex (Cx) theileri* (449), *Cx. pipiens* complex (240), *Cx. perexiguus* (12) and *Cx. sitiens* (2). Three species of Anopheles were identified, including *Anopheles (An) superpictus* (1), *An. maculipennis* (38), and *An. stephensi* (1), One species of Culiseta, *Cs. Longioairolata* (21) were caught.

Larva were collected from different larval habitats (Table 3). As it has been shown in Table 3, 598 larvae species were collected from rice fields, 129 from streams and 37 from river banks.

4. Discussion

Among all the trapped mosquitoes (4211), 94.68% were Culex (3987), which indicates that the dominant genus in this area of Iran is the Culex. Anopheles accounted for 4.77% of the total catch (201).

Only 2 (0.047%) of the total 4211 trapped Culicidae mosquitoes were Aedes, identified as *Ae. vexans*. During the 2 years of the study, none of the species of *Ae. aegypti* and *Ae. albopictus*, which are known vectors of arboviral diseases such as Yellow fever, Dengue fever, Zika virus, and Chikungunya virus, were caught in Lorestan province. However, considering that the methods of catching Aedes mosquitoes are completely different from Anopheles and Culex mosquitoes, and in this study, these methods of catching were not used, the results of this study cannot be generalized to Aedes mosquitoes. Therefore, in order to obtain reliable results for the species of Aedes in the region, separate studies should be carried out with emphasis on specific catch collection methods of Aedes mosquitoes in the province.

The Culex species captured in this study are consistent with some species from other studies of this genus that were caught in the west, southwest, and center of Iran (Dehghan et al., 2014; Reusken et al., 2010). The most abundant was *Cx. theileri* (92.15% of the total Culex) and the second was *Cx. pipiens* complex (6.09%). Zahirnia and Zendehfili (2014) reported the presence of *Cx. theileri, Cx. pipiens* and *Cx. antennatus* in Hamadan province, which borders the Lorestan province in the north. The predominant *Cx. theileri*, with 49% of the total catch, was the most abundant. This result is consistent with our study. However, *Cx. theileri* abundance in Lorestan was almost twice the same in Hamadan province, and the *Cx. antennatus* species were not caught from Lorestan. Lorestan has a warmer climate than Hamadan, and perhaps may explain the reason behind larger populations of *Cx. theileri* in Lorestan, moreover there are widespread rice fields in Lorestan, so that we cannot find them in Hamadan.

New methods of molecular (genomic) identification of species, subspecies, biotypes, races and forms that has been performed in recent years are more precise and reliable than older morphological identification methods of species complex. *Culex pipiens* complex has been collected in different climatic zones, especially temperate regions of the world. They have biotypes, forms and hybrids that demonstrate a different ecology, blood feeding behavior and physiology. These differences may affect vectorial capacity of transmission of pathogens (Dehghan et al., 2014; Reusken et al., 2010; Osório et al., 2014; Becker et al., 2012). In our study, molecular identification was performed on *Culex pipiens* complex to identify biotypes, forms and hybrids of this species.

Culex pipiens complex habitats can be found mostly in temperate-humid zones, thus we expect to find larger numbers of these

Table 3. Number of Culicidae larvae species that were collected from larval habitats in Lorestan province (2015–2016).

No	Culicidae larvae species	Rice fields	Streams	River banks	Total
1	*Cx. theileri*	360	72	17	449
2	*Cx. Pipiens complex*	179	47	14	240
3	*Cx. perexiguus*	3	8	1	12
4	*Cx. sitiens*	0	2	0	2
5	*An. maculipennis*	34	0	4	38
6	*An. superpictus*	0	0	1	1
7	*An. stephensi*	1	0	0	1
8	*Cs. longiareolata*	21	0	0	21
9	Total	598	129	37	764

mosquitoes in Western Iran with its Mediterranean climate (hot and dry in summer) compared to other, temperate zones. In Northern Iran (Southern provinces of Caspian sea) where the weather is warm and humid in the summer, other studies have reported *Culex pipiens* complex as the predominant Culex species in the area (Nikookar et al., 2010).

The results of three proportions of three forms of *Culex pipiens* complex in the present study are consistent with the results of Zittra et al. (2016) who worked in Eastern Austria. They found that 87.33% of collected mosquitoes were *Culex pipiens pipiens* form *pipiens*, 3.25% form *molestus* and 5.62% hybrids of both forms (Zittra et al., 2016). In contrast, the results of two studies conducted in Tunisia and Algeria showed higher proportions of *molestus* and *molestus/pipiens* hybrid forms compared to our study and Eastern Austria study (Beji et al., 2017; Korba et al., 2016).

Declarations

Author contribution statement

Mohammad Hassan Kayedi, Ehsan Mostafavi, Sadegh Chinikar, Gary Wong, Nariman Shahhosseini, Seyed Hassan Moosa Kazemi: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Fariba Sepahvand, Hamid Mokhayeri, Ali Chegheni Sharafi: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Funding statement

This work was supported by Deputy of Research and Technology of Lorestan University of Medical Sciences (project Code: 200/5686).

Competing interest statement

The authors declare no conflicts of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

We would like to thanks to Dr. Jonas Schmidt-Chanasit and Renke Luhken that made this research possible. The authors appreciate the villagers who participated in this study and other collaborators who collaborated in the research.

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