Original Article

Molecular Characterization and Phylogenetic Analysis of Flea Species in Human and Livestock Residence by Targeting ITS2 Region in East Azerbaijan Province, Iran

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Abstract

Background: Fleas are blood-sucking ectoparasites with complete metamorphosis. They belong to the order Siphonaptera and can infest both humans and animals, causing dermatitis and transmitting vector-borne diseases. Despite extensive study of their classification and biology, the phylogenetic relationship between fleas in Iran is not fully understood. This research aimed to identify the flea species collected from different parts of East Azerbaijan Province in northwest Iran, using morphological, molecular, and phylogenetic analysis.

Methods: From October 2019 to October 2020, we collected fleas using various methods such as hand catching for humans, brushing for dogs and cats, sticky traps for rodent burrows, light traps, and dishes with water for sheep and goats. After identifying the flea species using morphological identification keys, we extracted total genomic DNA and amplified it by targeting the ITS2 region. The PCR products were then directly sequenced to investigate the flea species.

Results: In total, 1929 flea specimens were collected, revealing three genera and four species. The breakdown of the specimens is as follows: *Pulex irritans* (n=1206; 62.5%), *Ctenocephalides canis* (n=345; 18%), *Ctenocephalides felis felis* (n=203; 10.5%), *Ctenocephalides felis orientis* (n=160; 8%), and *Xenopsylla nuttalli* (n=15; 1%). Phylogenetic analysis indicated low to moderate haplotype diversity (Hd: 0−0.524) across five distinct clades: *P. irritans*, *C. canis*, *C. felis felis*, *C. felis orientis*, and *X. nuttalli*.

Conclusion: This study represents the first in-depth analysis in East Azerbaijan Province, highlighting the significance of considering *P. irritans* as a major vector when assessing the risk of local disease transmission.

Keywords: Siphonaptera; Fleas; Phylogeny; ITS2; East Azerbaijan; Iran

Introduction

Fleas are external parasites that infect humans and other animals, causing dermatitis and the transmission of vector-borne diseases (1–3). There have been more than 2,500 identified species of fleas, belonging to 16 families and 238 genera worldwide (4). While they are found almost everywhere, certain species, such as those in the genus Xenopsylla, are restricted to tropical and warmer regions in some temperate countries (5–7). Fleas are small, wingless insects that undergo complete metamorphosis (Holometabolous). They are capable of jumping distances of 60–70 cm (5–7). These insects are the most significant ectoparasites of domestic animals, mammals, and birds (1, 2).

The most medically significant fleas are *Xenopsylla* species, as they can transmit Plague and Murine typhus. Fleas in the genus *Ctenocephalides* can serve as intermediate hosts for tapeworms. Fleas are also capable of trans-

mitting Tularemia, and the flea species Jigger can penetrate people's feet, potentially causing allergic dermatitis (2, 7, 8).

About 117 species or subspecies of fleas have been identified in Iran belonging to seven families and 35 genera (9, 10). Most of the reported species belong to the families Ceratophyllidae, Leptopsyllidae, Pulicidae, Ctenophthalmidae, and Coptopsyllidae.

Molecular markers can be used to identify flea species that are closely related. One well-known molecular marker for identifying flea specimens is the ITS2 region (11). The aim of this study was to use morphological, molecular, and phylogenetic analysis of the ITS2 region to identify flea specimens collected from various locations in East Azerbaijan Province, northwest Iran.

Materials and Methods

Study area

This study was carried out in East Azerbaijan Province, northwest Iran, bordering Armenia, the Republic of Azerbaijan, Ardabil Province, West Azerbaijan Province, and Zanjan Province. Fleas were collected biweekly from selected villages indoors and outdoors in 19 cities in the province (Table 1).

Sample collection

Fleas were collected using various catching methods including hand catching for humans, brushing for dogs and cats, sticky traps for rodent burrows, light traps, and dishes with water for sheep and goats from 2019 October to 2020 October (Fig. 1 and Table 1).

DNA extraction and polymerase chain reaction (PCR)

All collected fleas were kept in 70% ethanol until examination. Morphological identification was performed using a standard identification key (12). DNA was extracted from the whole body of fleas according to the instructions of Yekta Tajhiz kit (Iran). The PCR amplification was carried out in 25 µL reaction volumes containing; (Master Mix: 12.5 µL, ddH2O: 5.5 µL, forward primer: 1 µL (10 pmol), reverse primer (10 pmol): 1 µL, DNA: 4 µL, BSR: 1 µL. The primers employed were forward: ITS2.5.8s: 5′-GGG TCG ATG AAG AAC GCA GC-3′ and reverse: ITS2.28s: 5′-TTT AGG GGG TAG TCT CAC CTG-3′) for *P. irritans*, and forward: ITS2:5.8s: 5′-GGG TCG ATG AAG AAC GCA GC-3′ and reverse: ITS2:28s: 5′-GCG CAC ATG CTA GAC TCC GTG GTT CAA G-3′ for other flea species (11, 13). The PCR amplification for the ITS2 region was set as follows: hot star at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. The thermal conditions used in PCR amplification to detect flea spp. were the same.

Sequencing and phylogenetic analysis

43 amplicons (PCR products) (including 32 isolates of *P. irritans* and 11 isolates of other species) were successfully sequenced using the both forward and reverse primers of the ITS2 marker (Codon company, Tehran, Iran). ITS2 sequences were edited in consensus positions compared to regional/global sequences using Chromas software. To confirm the phylogeny associations among the flea specimens inferred by 322−353-nucleotide ITS2 sequences, a phylogenetic tree was generated using MEGA 5 software (14), based on the maximum likelihood algorithm and Kimura 2-parameter model. The distance scale was estimated at 0.05. The analysis of molecular variance (AMOVA) was performed by DnaSP software to determine the genetic diversity indices (number of haplotypes (Hn) and haplotype diversity (Hd).

Results

Morphology

During the study period, a total of 1929

fleas were collected using various catching methods. The results of the morphological identification of flea species and their frequency in different regions of East Azerbaijan Province are summarized in Table 1. Five species in three genera of collected fleas were identified: *Pulex irritans* (62.5%), *Ctenocephalides canis* (18%), *Ctenocephalides felis felis* (10.5%), *Ctenocephalides felis orientis* (8%), and *Xenopsylla nuttalli* (1%) (Fig. 2). *Pulex irritans* was predominantly found in Azarshahr, Heris, Malekan, Osko, Shabestar, and Warzegan. However, *X. nuttalli* was only observed in Ahar. *Ctenocephalides canis*, *C. felis felis*, and *C. felis orientis* were distributed in different areas of the province.

PCR and phylogenetic findings

The amplified fragment size of ITS2 primers was 322 bp for *P. irritans*, 327 bp for *C. canis*, *C. felis felis*, and *C. felis orientis*, and 353 bp for *X. nuttalli*. It is noteworthy that *P. irritans* was identified based on its specific primer. Concerning other flea spp. the morphometric diagnostic keys and sequencing techniques were used to differentiate those isolates (*C. canis*, *C. felis felis*, *C. felis orientis*, and *X. nuttalli*) that have close PCR band sizes.

For authentication of the taxonomic status of flea spp. the phylogenetic tree was generated based on allelic differentiation. Twenty-four sequences were submitted to the GenBank database, and accession numbers were presented in Fig. 3. The topology of identified species indicated that flea spp. has been placed in five distinct clades including Clade I (*C. felis felis*; Accession numbers: OR769672−OR769678) Clade II (*C. felis orientis*; Accession nos: OR769684-OR769685), Clade III (*C. canis*; Accession numbers: OR769668−OR769671), Clade IV (*P. irritans*; Accession numbers: OR659493, OR659495, OR659496, OR659503, OR659504, OR659508, OR659511, OR659513, OR659489 and OR659500) and Clade V (*X. nuttalli*; Accession number: OR769686) (Fig. 3). In most cases, the bootstrap values of > 70% supported the topology on each clade or subclades.

Based on Table 2, a low to moderate genetic (haplotype) diversity of flea spp. was observed in *P. irritans* /*C. canis* (Hd; 0, Hn: 1)/ and *C. felis felis* (Hd; 0.524, Hn: 3), respectively. In this study, *X. nuttalli* clade (clade V) and *Nosopsyllus* spp. (clade VI) were placed in a different taxonomic position (Out-group) compared to the rest of the clades. Furthermore, the cladistic phylogenetic tree indicated that clades I-III (*C. felis felis*, *C. felis orientis*, and *C. canis*) have a sister relationship with the *P. irritans* (clade IV) (Fig. 3). Interestingly, clade *C. felis orientis* has a sister relationship with *C. canis* isolates.

Table 1. Flea species and their abundance were collected from various regions of East Azerbaijan Province, Iran, from October 2019 to October 2020

City	*Pulex irritans*	*Ctenocephalides canis*	*Ctenocephalides felis felis*	*Ctenocephalides felis orientis*	*Xenopsylla nuttalli*
Azarshahr	13	0	0	0	0
Ahar	0	38	0	33	15
Ajabshir	43	15	0	0	0
Bostanabad	221	18	156	0	0
Bonab	102	0	9	17	0
Hashtroud	16	5	4	21	0
Heris	58	0	0	0	0
Jolfa	118	8	2	0	0
Kaleybar	0	25	4	24	0
Maragheh	38	0	0	0	0
Malekan	13	0	0	0	0
Marand	3	23	0	0	0
Miyaneh	10	63	0	42	0

Table 1. Continued ...

Osko	58	0	0	0	0
Qareh-Aghaj	11	0	3	7	0
Sarab	180	74	0	0	0
Shabestar	40	0	25	0	0
Tabriz	229	76	0	16	0
Warzegan	53	0	0	0	0
Total: 1929(%)	1206 (62.5)	345 (18)	203 (10.5)	160 (8)	15 (1)

Fig. 1. Map of Iran showing flea sampling sites in East Azerbaijan Province from October 2019 to October 2020. The study locations are highlighted with red circles

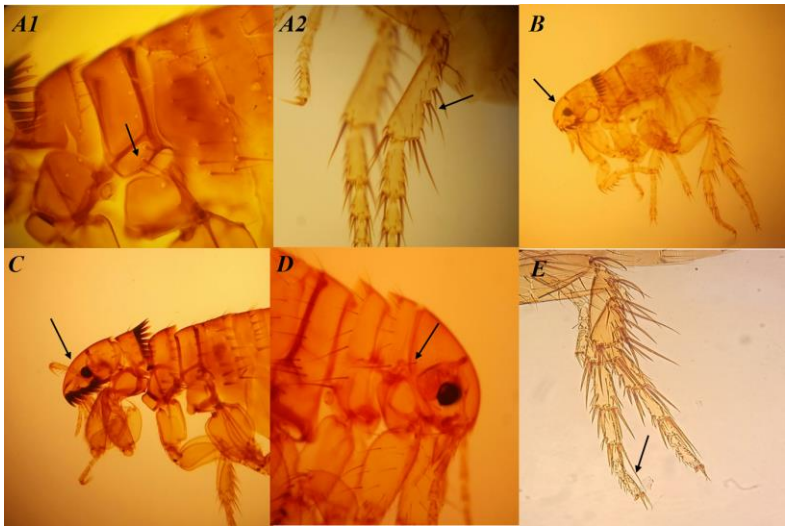

Fig. 2. Flea species isolated from East Azerbaijan Province (2019−2020). A1 and A2: *Ctenocephalides canis* (A1: The metepisternum has 3 spines A2: there are 2 small and strong spines in the distance between the middle and the side of the apex of the posterior tibia), B: *Ctenocephalides felis felis* (Head is long and its front part is not vertical, the forehead border is long and narrow) C: *Ctenocephalides felis orientis:* The head is short and its anterior part is vertical also the forehead is small D: *Pulex irritans:* There is a hair on the back of the head and behind the antenna gap, and there is another hair under the eye and E: *Xenopsylla nuttalli*: 2−3 long spine at the apex of the second tarsal segment reach the end of the fourth segment

Table 2. Diversity indices of flea species using ITS2 sequences across different regions of East Azerbaijan Province, Iran, 2019−2020

Flea species	Number of isolates	Number of Haplotypes	Haplotype (gene) diversity	Variable (polymorphic) sites	Total number of InDel sites analyzed
Pulex irritans	32	1	0.000	0	0
Ctenocephalides felis felis	7	3	0.524	1	2
Ctenocephalides canis	4	1	0.000	0	1

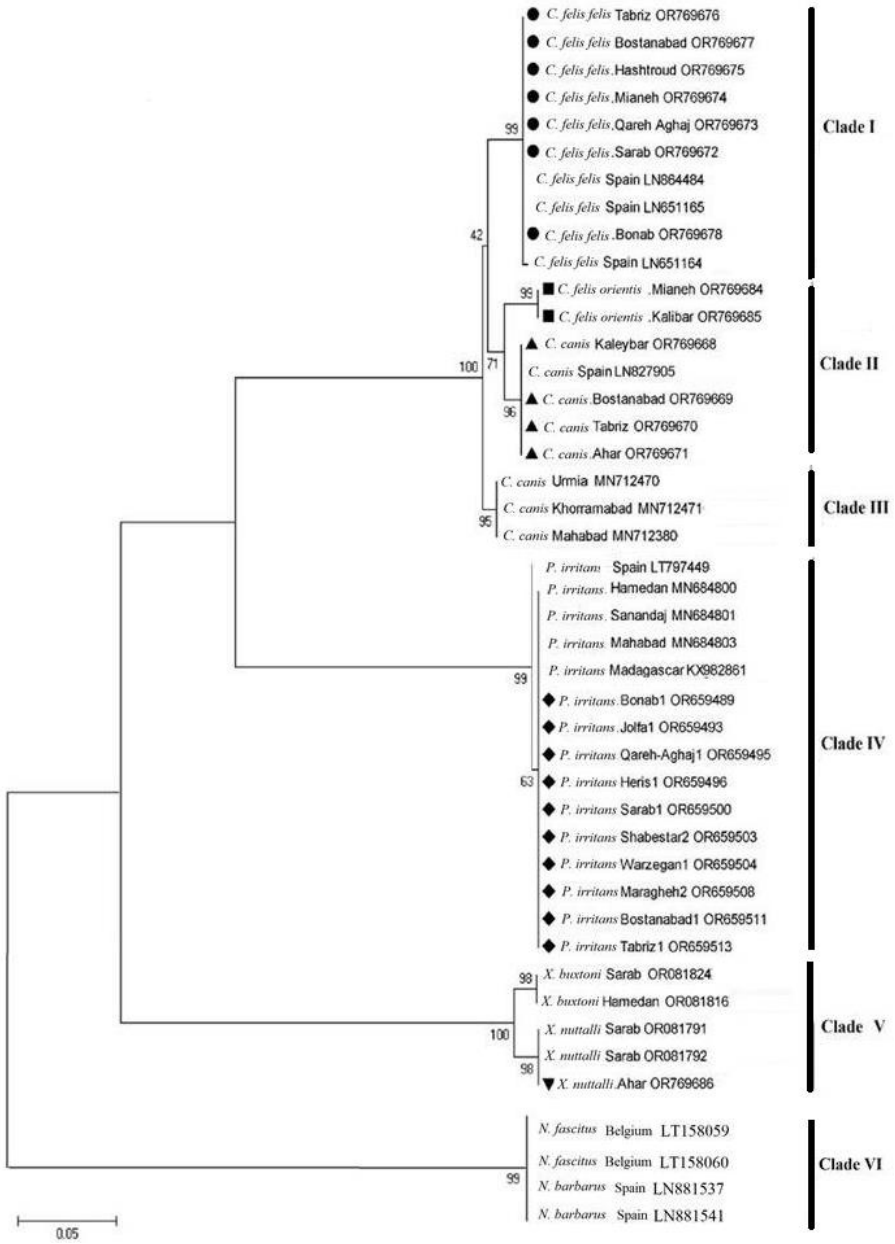

Fig. 3. Phylogenetic tree inferred from 320 bp of ITS2 sequences of four flea species collected in West-Azerbaijan Province, I.R.Iran, (2019−2020), alongside sequence data retrieved from Genbank. The tree was constructed using the maximum likelihood algorithm and Kimura2-parameter model in MEGA 5. Bootstrap values supporting the tree topology are indicated at each node. Sequences obtained from this study are highlighted with geometric shapes

Discussion

In the present study, for the first time using morphological and phylogenetic analysis of ITS2 sequences in different parts of East Azerbaijan Province, northwest Iran, the flea spp. (*P. irritans, C. canis, C. felis felis, C. felis orientis* and *X. nuttalli*) was identified.

Several reports have been reported ectoparasites infestations among dogs in Iran (15−18). Research by Darvishi et al. (19) was done in 2014, and during that investigation on ectoparasites of five *Mus musculus* in Semnan Province, Iran, 15 fleas were collected. After precise study, all examined specimens were recognized as *Leptopsylla aethiopicus aethiopicus*. Also, an investigation was conducted by Maleki et al. in different regions of Iran (10) in 2017, which showed that the fauna was dominated by seven families, namely the Ceratophyllidae, Leptopsyllidae, Pulicidae, Ctenocephalidae, Coptopsyllidae, Ischnopsyllidae and Vermipsyllidae and rodents are the common hosts of five flea families. A study like this research was conducted by Azarm et al. (20) in Iran. That morphological study indicated, that from the 1053 fleas, which were collected from cats and dogs, 74 specimens belonged to human fleas, *P. irritans*. In addition, molecular analysis showed a high sequence similarly (99.5%) with *P. irritans* from Spain country and Zanjan of Iran available in GenBank.

In a study conducted by Tavassoli et al. (17) among dogs in other parts of Iran, the highest frequency was related to *C. felis felis*. However, in our study, the frequency of C. canis is greater than that of *C. felis felis*. *Pulex irritans* has a nearly cosmopolitan distribution (7) and is the only species of the genus Pulex distributed in different parts of Iran. It was the most frequent flea in our study (21). The most significant finding of this study was the high homogeneity (low haplotype diversity) of the flea specimens collected from different parts of the province into each of the flea families which is associated with their host interactions and potential effects on public health in the region. This study revealed a consistency between morphological and phylogenetic classifications of the collected fleas. Although we sampled only some parts of the East Azerbaijan Province, these sites define the occurrence of flea species diversity in this province.

In this study a taxonomic variation of flea spp. (*C. felis felis, C. felis orientis, C. canis, P. irritans* and *X. nuttalli*) including five distinct clades (I-V) characterized in the province.

In a similar study, a molecular divergence of flea *C. canis* was observed from west and northwest Iran (West Azerbaijan), however, no significant nucleotide differences of ITS1 and ITS2 loci were observed (Unpublished data). The phylogenetic investigation among 31 fleas studied by Vobis et al. by nucleotide sequence comparison of ITS1, ITS2, and mt−16SrDNA revealed that all are useful for discriminating different flea species which is somehow consistent with the results of this research (18). A study conducted by Seidi et al. (22) has shown that molecular divergence of *C. canis* based on ITS1 and ITS2 sequences from human and domestic animals were 0.15% and 3.33%, respectively. Notably, the analysis of the COX1 marker revealed no molecular divergence among the partial sequences representing the studied isolates from *C. canis*. A similar study conducted by Tajadin et al. (23) has shown that *X. nuttalli* is one of the ectoparasites of *Rhombomys opimus* in endemic foci of zoonotic cutaneous leishmaniasis in Iran. Also, Mostafavi et al. (24) have reported the *X. nuttalli* and other flea spp. in small mammals in northwestern Iran.

Previously, there have been few reports on *C. felis orientis* since 1930 in Iran (21). Furthermore, Seyyed-zadeh et al. (25) characterized the presence of *C. felis orientis* in West Azerbaijan inferred by COI fragment. In this study, *C. felis orientis* clade has a sister re-

lationship with *C. canis* isolates, however, *C. felis orientis* is a subspecies of *C. felis* (25). Thus, it has been suggested that, so this new finding elevates it to a distinct species, supported by molecular evidence (26) as well as using anatomical traits (27).

The most important limitation of the present research was that the ITS2 nucleus sequences obtained from collected fleas were partially small. Therefore to infer the extensive genetic diversity of fleas concatenated mitochondrial genomes should be conducted on large flea populations in different regions of Iran.

Conclusion

The study represents the initial comprehensive examination of flea species in East Azerbaijan Province. Through morphological analysis, three genera and four species were identified. Given the abundance of flea species, special consideration should be given to *Pulex irritans*, a major vector in the region, when evaluating the risk of local disease transmission.

Phylogenetic analysis indicates that at least five distinct clades of flea spp. (*C. felis felis*, *C. felis orientis*, *C. canis*, *P. irritans* and *X. nuttalli*) with different frequencies unequivocally circulating in the region.

Acknowledgements

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Ethical Considerations

This study was approved by the ethical committee of Tabriz University of Medical Sciences and followed the Helsinki Declaration (approval number: IR.TBZMED.REC. 1399.355).

Conflict of interest statement

The authors declare that there is no conflict of interest.

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