Molecular Divergence in Flea Ctenocephalides Canis From West and Northwest of Iran

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Research

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Abstract

Background

Fleas of the family Pulicidae are the most common ectoparasites infesting domestic livestock worldwide. The main aim of the present study was to demonstrate the degree of molecular divergence between *Ctenocephalides canis* fleas in the Western and Northwestern of Iran, based on nuclear and mitochondrial genes, including ITS1and ITS 2 and cytochrome c-oxidase 1 (cox1) mtDNA.

Methods

A total of 918 *C. canis* fleas was collected. The obtained morphometric data and DNA sequencing results did not show significant differences between *C. canis* specimens from the different regions or hosts. However, there was a significant degree of molecular divergence among the ten populations based on nuclear markers.

Results

The degree of molecular divergence between different isolates of *C. canis* based on ITS1and ITS 2 genes was 0.15% and 3.36%, respectively. But analysis of the sequencing results shows that there was no molecular divergence between the ten populations based on the Cox1 marker.

Conclusions

Study of internal transcribed spacer ITS1 and ITS2 of rDNA and the partial cox1 mtDNA gene showed that these fragments are useful tools for interspecific divergence rates, species-level differentiation and confirm the diagnosis of species *C. canis*.

Background

Fleas are very small insects, laterally compressed and wingless. These insects have a holometabolous development and belong to the Siphonaptera order. So far, 2574 species belonging to 16 families and 238 genera have been identified from these insects [16, 17, 5,14].

All species are parasitic in the adult stage. Some species are vectors of disease. Fleas transmit the disease in different ways including flea bites (*Yersiniapestis* and some viral pathogens), indirect method or fecal route (*Rickettsia typhi* and *Bartonella henselae*), and contaminated salivary glands by which *Rickettsia felis* is transmitted [3, 25,8].

Each flea has a different role in causing health complications and transmitting diseases that will have a different transmission cycle. Some species of fleas differ in susceptibility to pathogens and symbiosis, which can carry a high risk of transmitting the pathogen, so accurate identification of different species of

fleas is important. Based on recent studies in Iran and the latest available information, 117 species and subspecies have been identified [25, 29 [.

The identification of different species of fleas is based on morphological characteristics. However, there are still significant questions and concerns about the phylogenetic relationships of fleas and the identification of appropriate molecular markers in this important group of insects [28].

Variation in morphological characteristics is observed among different species, making it difficult to identify the species correctly. Researchers have been using molecular markers of various species of fleas such as ITS1 [10, 2, 33], ITS2 [27, 33] and parts of the mitochondrial genome such as cox1 [15, 24] to address their differences in molecular markers.

Ribosomal DNA (rDNA) has a very stable structure in all insects. There are five subunits in this structure including 28s, 18s, 5.8s, ITS1 and ITS2 that can be used for separation of species [27, 9, 2].

In the mitochondrial genome, the cox1 fragment is the largest protein-encoding gene in the mitochondria of metazoan organisms. This part of the genome has been extensively used as a derivative of species and phylogenetic studies and intra-species differences [22, 6].

The main objective of this study was to show molecular divergence between *C. canis* flea isolated from West and Northwest of Iran based on analysis of ITS1, ITS2, and cox1.

Materials And Methods

Study area

According to meteorological information, Iran has different climatic zones. Our study was done in The West and Northwest of Iran. In the Northwest, the winters are cold with heavy snowfall and subfreezing temperatures. Spring and fall are relatively mild, while summers are dry and hot. The western part is cold and mountainous, so the winters are cold, and the summers are hot. [23](Fig. 1). The annual rainfall in these areas is high, and these areas are rich in vegetation and have created favorable conditions for domestic livestock breeding. Animals commonly breed in these areas include cows, sheep, goats, and horses.

Flea sampling and identification

Adult fleas were collected from five provinces (two cities in each province) in the West and Northwest of Iran, including Kermanshah, Kordestan, Hamedan, Lorestan, and West Azerbaijan. Samples were collected using light traps, human baited traps, direct separation of fleas from the host body, and collection of fleas from home and animal farms. Totally out of 1937 were isolated, and 918 of them were *C. canis*.

After collection, samples were stored in 70% ethanol and transferred to the laboratory of the Faculty of Veterinary Medicine, Urmia University, Iran. Fleas were identified under a stereomicroscope according to identification keys [1].

DNA isolation, PCR amplification, and DNA sequencing

Total DNA was extracted from the individual flea using a DNA extraction kit (MBST, Tehran, Iran) and following the manufacturer instructions. The extracted DNA samples were stored in sterile microtubes at -20 °C until use. The primers used by Vobis et al. (2004) [27] were applied to identify flea's *C. canis*. These primers were specific to identify ITS1 and ITS2 (Table 1). Primers used by Lawrence et al. (2014) [15] were used to amplify the fragment of the mitochondrial gene cox1 of *C. canis*(table 1).

PCR analysis performed in 50 µL total volume including10 ng of DNA, 10x PCR buffer, 1.25 U Taq Polymerase, 1 µL of each primer (20 µM), 1 µL dNTP, (100 µM), 0.75 mM MgCl2 (50mM) all materials obtained from Sinaclon, Iran. Program to perform PRC reaction for fragments ITS1 and ITS2 including 5 min incubation at 94°C to denature double-stranded DNA, followed by 30 cycles of 1min at 94°C (denaturation), 1 min at 54°C (annealing) and 1min at 72°C (extension) and an additional extension step at 72°C for 5 min these steps. The PCR program was also set up to replicate the cox1 fragment 95 ◦C for 5 min, followed by 35 cycles of denaturation at 95 ◦C for 1min; annealing at 55 ◦C for 45 s and extension at 72 ◦C for 45 s; with a final extension step at 72 ◦C for 10. The PCR products were electrophoresed on 1.5% agarose gel and then stained with Safe stain and visualized under the light. The PCR products were then purified for sequencing. The phylogenetic tree was plotted using MEGA 6 software based on the maximum likelihood method with bootstrap 1,000(1,000 replicates).

Results

Out of 1937, flea were collected from humans, sheep, dogs, goats, home, and animal farms (table 2) from which 918 *C. canis* were isolated (Fig. 2).

The amplicons obtained from the PCR for ITS1, ITS2 (rDNA) and cox1 (mtDNA) were approximately800bp, 500bp, and 700bp, respectively (Fig. 3,4).

The sequencing length for the ITS1 and ITS2 genomes in the study sites were 800bp and 500bp, respectively. When the ITS1 sequence in *C. canis* was compared in ten different regions with the CLUSTAL O software, the sequences were 99.85% similar, and the degree of molecular divergence was 0.15% (Fig. 5). But after comparing the ITS2 gene sequences in the ten studied regions, the molecular divergence between *C. canis* based on ITS2 gene was 3.36%. (Fig. 6).

Comparison of cox1 sequencing from the nuclear genome (mtDNA) in five provinces (accession number: MN173762-771) showed that the sequences were 100% similar and all species belong to *C. canis* species (Fig. 7).

Discussion

Using molecular methods in the identification of ectoparasites has enabled the identification and evolution of species with high morphological similarities [18]. The present study provided morphometric, phylogenetic, and molecular divergence comparative data between fleas *C. canis* isolated from Western and Northwestern Iran. The morphological characteristics of family Pulicidae fleas in this study were in agreement with previous studies [29, 21, 25, 12, 4, 7].

The host specificity might affect the level of intraspecific genetic divergence because generalist parasite species will show a higher level of intraspecific genetic variation, enabling them to infest a broader host range [26]. Hornok et al. (2018)[11]. studying mitochondrial sequences demonstrated divergence in some synanthropic flea species such as *C.felis* and *Pulex irritans*. In our study, morphological data indicated that there was no difference between *C. canis* specimens from Western and Northwestern of Iran. The result of the morphological study was in line with the results of the molecular study. Nucleotide sequences of *C. canis* from different regions showed 100% identity with. *C. canis* in Turkey: KY865411.1 (NCBI), they also had 95.59 % identity with *C. orientis* in India (KX 467332.1), 95.32 % with *C. orientis* in Iran (MF380397.1), 93.48 % with *C. felis* in Iran (MF380394.1). This study is in agreement with the results of Seyyedzadeh et al. (2018)[20] which observed that there were no differences between the isolates of *C. canis* samples of different regions of West Azerbaijan, Iran. The results also in agreement with those of Zurita et al. (2019)[33] showed no significant difference between the morphological data of *P. irritans* in Spain and Argentina. There was a considerable degree of intraspecific similarity between both populations based on mitochondrial genes. Our results agree the studies of Hornok et al. (2018),[11] who observed no morphological differences between human and wild carnivorous *P. irritans* specimens in Hungary and Croatia. In contrast, the studies of Krasnov et al. (2015)[13] showed that fleas species isolated from different hosts in different geographic regions have morphological differences that can indicate a high level of genetic diversity. We concluded that the cox1 region is a useful marker to approach intraspecific similarity in *C. canis* and confirming its diagnosis.

A phylogenetic tree based on the similarity between our sequences with registered sequences in

GenBank showed 2 subclades for *C. canis*: one subclade including *C. canis* of recent study and *C. canis* from Turkey and the other with *C. canis* of Iran, Urmia and, Iran, Makoo. *C. felis*, *C. orientis* and *P. irritans* were in separate clades (Fig. 8).

Studies have shown that ITS1 and ITS2 are one of the best molecular markers for analyzing phylogenetic relationships at the species level in fleas [27, 19]. In our study, a comparison of nucleotide sequences of the ITS1 gene in five different provinces showed 99.85% similarity. Comparison of nucleotide sequences also showed single-nucleotide transversion at position 294, which caused the substitution of adenine for thymine in the isolate from Kuhdasht, Urmia, and Kermanshah. This result is in agreement with the results of Ghawami et al. (2018),[10] which compared the nucleotide sequence of the ITS1 gene in *P. irritans* in two different geographic regions and showed only one nucleotide difference with 99.85% similarity. Our results are similar to Vobis et al. (2004) study, which observed that the nucleotide sequence

of the ITS1 gene was relatively constant in different populations of *C. felis*. Therefore, it can be concluded that ITS1 is a useful marker for the diagnosis of *Ctenocephalides* genus.

A phylogenetic tree based on ITS1 registered sequences in GenBank showed 2 subclades for *C. canis*: one subclade including *C. canis* isolated in Kamiyaran, Sanandaj, Mahabad, Ghilangharb, Hamedan, Bahar and Khorramabad and the other *C.canis* which was isolated in Kuhdasht, Urmia, and Kermanshah. *P. irritans* and *C. felis* were in separate clades (Fig. 9).

The ITS2 nucleotide sequence in different sampled regions showed 96.64% similarity. There are five isolates found.

The results of the current study agree with the survey by Zurita et al. (2015),[30] which showed intraspecific variation in four clones of *C. canis* based on the ITS2 fragment is between 90.1% and 100%. Vobis et al. (2004) also used the ITS2 sequence to illustrate intra-species differences and identify different species of fleas.

A phylogenetic tree based on ITS2 in the present study showed 4 subclades for *C. canis*: one subclade including *C. canis* isolate in Kuhdasht, Mahabad, Urmia, Khorramabad and Kermanshah, next subclade including isolate in Kamiyaran, Sanandaj, and Bahar. The isolates of Ghilangharb and Hamedan were in separate subclades. *P. irritans* were in a separate clade, and *C. felis* were in separate subclades (Fig. 10).

Conclusion

In conclusion, we have found that the morphological characteristics in *C. canis* are consistent with the molecular findings. The analysis of the cox 1 partial gene is a useful tool to assess intraspecific similarity and confirm the diagnosis of species *C. canis*. ITS1 and ITS2 sequences could be used as useful markers for species-level differentiation and interspecific divergence rates. As a result, the Cox1 gene is more useful than the ITS1 and ITS2 genes in detecting genus and species and showing intra-species similarity

Declarations

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Ethics approval and consent to participate

The ethical issues are not applicable to the article, due to no human and animals involvement.

Consent for publication

All authors are agree for published article in Parasite & Vector.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table. 1 Nucleotide sequence and specificity of primers used

References	Annealing temperature (°C)	fragment length (bp)	Primer nucleotide sequence	Gene name
Vobis et al., 2004	54 °C	800bp	Sen: GTA CAC ACC GCC CGT GCG TAC T Rev: GCT GCG TTC TTC ATC GAC CC	ITS1
Vobis et al., 2004	54 °C	500bp	Sen: GGG TCG ATG AAG AAC GCA GC Rev: GCG CAC ATG CTA GAC TCC GTG GTT CAA G	ITS2
Lawrence et al., 2014	55 °C	700bp	Sen: GGT CAA CAA ATC ATA AAG ATA TTG G Rev: GAA GGG TCA AAG AAT GAT GT	cox1

Table. 2 GenBank accession numbers of ITS1, ITS2 and cox1 gene sequences of individuals *C.canis* obtained in the present study.

Location/cities/province	Species	cox1 Host	Number of fleas	Base pairs (bp)	Accession Number
Kermanshah/Ghilangharb	*Ctenocephalides canis*	sheep, goats,farm, home, dog	30	700bp	MN173764.1
Kermanshah/Kermanshah	*Ctenocephalides canis*		25	700bp	MN173767.1
Kordestan/Sanandaj	*Ctenocephalides canis*	sheep, goats,farm, home, dog	20	700bp	MN173770.1
Kordestan/kamiyaran	*Ctenocephalides canis*		20	700bp	MN173766.1
West Azerbaijan/Urmia	*Ctenocephalides canis*	sheep, goats farm, home	15	700bp	MN173771.1
West Azerbaijan/Mahabad	*Ctenocephalides canis*		18	700bp	MN173769.1
Hamedan/Hamedan	*Ctenocephalides canis*	dog, goat,sheep	58	700bp	MN173765.1
Hamedan/Bahar	*Ctenocephalides canis*		17	700bp	MN173763.1
Lorestan/Khorramabad	*Ctenocephalides canis*	farm,home dog, sheep	16	700bp	MN173768.1
Lorestan/Kuhdasht	*Ctenocephalides canis*		32	700bp	MN173762.1
	ITS1				
Kermanshah/Ghilangharb	*Ctenocephalides canis*	sheep, goats, farm, home, dog	51	800bp	MN684799.1
Kermanshah/Kermanshah	*Ctenocephalides canis*		30	800bp	MN684785.1
Kordestan/Sanandaj	*Ctenocephalides canis*	sheep, goats, farm,home, dog	39	800bp	MN684804.1
Kordestan/kamiyaran	*Ctenocephalides canis*		21	800bp	MN684805.1
West Azerbaijan/Urmia	*Ctenocephalides canis*	sheep, goats	41	800bp	MN684789.1
West Azerbaijan/Mahabad	*Ctenocephalides canis*	farm,home	23	800bp	MN684802.1
Hamedan/Hamedan	*Ctenocephalides canis*	dog, goat,sheep	61	800bp	MN684798.1
Hamedan/Bahar			32	800bp	MN684788.1

	Ctenocephalides canis				
Lorestan/Khorramabad Lorestan/Kuhdasht	*Ctenocephalides canis* *Ctenocephalides canis*	farm,home dog, sheep	50 55	800bp 800bp	MN684783.1 MN684806.1
	ITS2				
Kermanshah/Ghilangharb Kermanshah/Kermanshah	*Ctenocephalides canis* *Ctenocephalides canis*	sheep, goats, farm, home, dog	50 57	500bp 500bp	MN684808.1 MN684795.1
Kordestan/Sanandaj Kordestan/kamiyaran	*Ctenocephalides canis* *Ctenocephalides canis*	sheep, goats, farm, home, dog	42 15	500bp 500bp	MN712340.1 MN712469.1
West Azerbaijan/Urmia West Azerbaijan/Mahabad	*Ctenocephalides canis* *Ctenocephalides canis*	sheep, goats, farm, home	56 18	500bp 500bp	MN712470.1 MN712380.1
Hamedan/Hamedan Hamedan/Bahar	*Ctenocephalides canis* *Ctenocephalides canis*	dog, goat, sheep	22 34	500bp 500bp	MN684807.1 MN684791.1
Lorestan/Khorramabad Lorestan/Kuhdasht	*Ctenocephalides canis* *Ctenocephalides canis*	farm,home, dog, sheep	13 27	500bp 500bp	MN712471.1 MN712467.1