Fleas from the Silk Road in Central Asia: identification of *Ctenocephalides canis* and *Ctenocephalides orientis* on owned dogs in Uzbekistan using molecular identification and geometric morphometrics

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**Abstract**

**Background:** The Silk Road connected the East and West for over 1500 years. Countries in Central Asia are valuable in addressing the hypothesis that parasites on domestic animals were introduced along the Silk Road. Adult fleas are obligate parasites, having worldwide distribution. In dogs, *Ctenocephalides canis*, *C. felis* and *C. orientis* are the most common species identified. The distribution of the Oriental cat flea, *C. orientis*, is restricted to southeast Asia. The purpose of this study was to determine the diversity of dog fleas from Uzbekistan, a country in Central Asia, with particular reference to *C. orientis*.

**Methods:** Fleas were collected from 77 dogs from 5 locations in Uzbekistan. The cox1 gene sequences from *Ctenocephalides* spp. were compared to global collection of *Ctenocephalides* cox1 haplotypes. Landmark-based geometric morphometrics have been applied to the head and curvature to compare *C. canis* and *C. canis* using canonical variate analysis and discriminant function analysis.

**Results:** Overall, 199 fleas were collected and identified as *C. canis* (*n* = 115, 58%), *C. orientis* (*n* = 53, 27%) and *Pulex irritans* (*n* = 22, 11%). None of the fleas were *C. felis*. All *Ctenocephalides* spp. fleas were subject to cox1 amplification and 95% (166/175) yielded DNA sequence. There were 25 cox1 haplotypes; 14 (22/25, 88%) were *C. canis* cox1 haplotypes and 3 (3/25, 12%) were *C. orientis* cox1 haplotypes. Molecular analysis confirmed the absence of *C. felis*. Four (4/22) and one (1/3) cox1 haplotypes were identical to cox1 haplotypes belonging to *C. canis* and *C. orientis* cox1 haplotypes identified elsewhere, respectively. Overall morphometric analysis confirmed significant differences between the head shape of *C. canis* and *C. orientis* and improved four–fivefold the species identification compared to traditional morphological key.

**Conclusion:** We report for the first time the presence of *C. orientis* in Uzbekistan. Differentiation of *C. orientis* from *C. canis* and *C. felis* remains difficult in regions where these species coexist. Studies in Central and Southeast Asia should...
Background

The order Siphonaptera (fleas) is a highly specialized order of holometabolous parasitic insects, comprising more than 200 genera with over 2500 recognized species. Adult fleas are obligate external parasites of birds and mammals, having a worldwide distribution, including cold areas (Antarctica). They can inhabit a large range of hosts and habitats [1–3]. In domestic carnivores, namely dogs and cats, fleas are common ectoparasites, responsible for up to 50% of all dermatological cases [4]. Responsible owners spend hundreds of dollars per year on ectoparasitic products against fleas [5]. Due to urbanization, specific services of some domestic dogs (hunting, security and shepherd), their contact with wild carnivores has significantly increased and represents an important spill-over of parasites, which could be a threat to human health due to the potential transmission of zoonotic pathogens [6, 7].

Three species of fleas are known to be the most common ones identified in domestic dogs and cats worldwide, apart from Antarctica, namely Ctenocephalides canis (Curtis, 1826), Ctenocephalides felis (Bouché, 1835) and Ctenocephalides orientis (Jordan, 1925) [8]. In Australia, Central and Southwestern Europe, South and Southeast Asia, the dominant species in dogs is C. felis [8–13]. Interestingly, in dogs of several Eastern Europe countries, a more common infestation by C. canis and even Pulex irritans Linnaeus, 1758, occurs [14]. The dog flea, C. canis, was dominant also in dogs from South Korea [15], while in India the most common species detected in dogs was C. orientis, a species previously associated with other animal species, such as ruminants [8, 16, 17]. The differentiation of C. canis from C. orientis requires fine morphological observation or molecular confirmation, and it is likely that past studies from Asia and Southeast Asia may have misidentified C. orientis as C. canis [8, 16]. Even though dog fleas are of great veterinary and human-health interest, there are very few to zero studies regarding the flea species diversity on companion animals and flea-borne pathogen occurrence in countries of Central Asia [18, 19].

The only study on the flea fauna of dogs in Uzbekistan revealed the presence of C. canis and C. felis. However, details about their prevalence or other epidemiological characteristics are not given [19]. The Republic of Uzbekistan has a wide diversity of wild and domestic carnivores, including about 2.5 million domestic dogs kept as pets or for security reasons [20, 21] (Information from the State Committee for Veterinary and Livestock Development of the Republic of Uzbekistan as of January 1, 2022). Traditional animal husbandry is still practiced in this country, favoring a close contact of domestic dogs with wildlife [22].

The aims of the present study were to determine the fleas’ identity using a combination of traditional morphological identification, molecular identification and landmark-based geometric morphometric analysis.

Methods

The present study included a total of 77 dogs which were sampled over a period of 3 months during the winter season (December 2020 to February 2021) in Uzbekistan, Central Asia. The dogs were from the following five cities along the former Silk Road trade route between the East and the West: Jizzax, Buxoro, Farg’ona, Samarkand and Surxandaryo. The included dogs were housed outside, in rural areas, and served as security dogs for the protection of farm animals (cattle, sheep, poultry) from wild predators (wolves, jackals, foxes), being kept as free ranging. The owners were questioned about the ectoparasitic treatments, age and sex of each examined animal. The study was approved by the Animal Care Organization of the State Committee for Ecology and Environmental Protection.

The studied population of dogs was characterized according to age, gender (males and females) and geographical location. The age was recorded in months and based on this criterion, dogs were divided into two groups: juveniles (0 to 12 months) and adults (over 12 months). Fleas were opportunistically manually collected using fine entomological tweezers and by combing. The collection of ectoparasites was not related to a specific protocol and some of the specimens might have been missed.

Collected specimens were placed and stored in absolute ethanol in 2 ml labeled tubes. The preserved samples were sent to the Department of Parasitology and Parasitic Diseases of the University of Agriculture and Veterinary Medicine of Cluj-Napoca where genomic DNA was individually extracted with the preservation of the exoskeletons as previously described [11, 23] using a commercially available kit (Isolate II Genomic DNA, Bioline, UK). The remaining exoskeletons were further digested in 10% KOH for 2–3 h, washed in 10% acetic
acid for 30 min and then dehydrated using a series of ethanol washes (25%, 50%, 75%) for 30 min in each concentration. Before the final step, the fleas were kept in clove oil for 45–60 min and slide-mounted in natural Canada Balm (Roth, Germany). The slides were dried at 55 °C for about 4–6 weeks to facilitate the elimination of the air bubbles. The morphological identification was done to species level using specific descriptions and keys [8, 24]. Morphological identification of Ctenocephalides spp. was aided with subjective observation of the aspect of the angle of the cephalic profile and on the number and orientation of the setae as previously described [8].

Each specimen was photographed using a digital camera attached to a microscope, and images were used to build ‘tps’ files in tpsUtil V1.81 and tpsDig V2.32 [http://www.sbmorphometrics.org/soft-utility.html]. We identified five landmarks of the flea head with one curve that was resampled into ten landmarks (2 matched existing landmarks); a final dataset included 13 landmarks across the head of each flea for geometric morphometric analysis in MorphoJ V1.07a [https://morphometrics. uk/MorphoJ_page.html] [25]. Raw landmark coordinates were aligned and superimposed using Procrustes fit function to remove variation due to differences in scale, position and orientation from the coordinates. Head shape variation was analyzed using MorphoJ software [25]. Canonical variate analysis (CVA) was used to determine the most important feature as a possible discriminator between groups (sex or/and species). The statistical significance of pairwise differences in mean shapes was analyzed using permutation tests (10,000 rounds) with Mahalanobis distances and Procrustes distances. Additionally, a cross-validation test in discriminant function analysis (DFA) was used to assess the accuracy of classification based on Mahalanobis distances in a permutation test with 10,000 rounds using MorphoJ software [25].

Molecular identification for all Ctenocephalides spp. fleas followed previously established protocol [8]. An aliquot of the extracted DNA (30 μl) was sent to the Sydney School of Veterinary Science at the University of Sydney, Australia, for amplification of partial cytochrome c oxidase subunit 1 (cox1) and DNA sequencing. The partial cox1 (550–650 bp) fragment from individual flea DNA (n = 175) was amplified using a combination of LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) (Folmer et al. 1994) and/or Cff-F [S0367] (5′-AGA ATT AGG TCA ACC AGG A-3′) with Cff-R [S0368] (5′-GGA GTC AAG AAT GAT GT-3′) [11] primers. Amplification (30 μl) was performed with MyTaq Red Mix (BioLine), primers and 2 μl of DNA in nuclease-free water in a T100 cycler (Bio-Rad, Australia). The cycling condition included an initial denaturation at 95 °C for 1 min followed by 35 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 10 s, and the amplification was finished with a final elongation for 5 min at 72 °C. All reactions were run with a negative control that included sterile PCR water instead of DNA. The PCR products of the expected size (550–650 bp) were sequenced at Macrogen Inc. (Seoul, Korea). The raw bidirectional Sanger sequence chromatographs were assembled and compared to a reference dataset of 90 haplotypes (h1-h90) sensu [8] using CLC Main Workbench 21 (CLC bio, Qiagen, Australia). Newly obtained unique sequences were appended to the Ctenocephalides cox1 haplotype alignment from Lawrence et al. [8] and pairwise distances and phylogenetic tree using Kimura-2 (K2) distances constructed using Minimum Evolution (ME) method with bootstrap support (1000 replicates) in MEGA 11 [26].

The data were incorporated in a Microsoft Excel sheet, and proportion, standard error, prevalence and 95% confidence intervals (CI) were calculated for each of the obtained results. The descriptive data are available in the additional file in the “stats” sheet. The distribution map based on the presence of flea species was generated using ArcMap 10.6.1.

Results

We examined 77 dogs from Uzbekistan in Central Asia. The dogs originated from Jizzax (n = 24), Buxoro (n = 12), Farg‘ona (n = 19), Samarkand (n = 20) and Surkandaryo (n = 2). The age of the tested dogs ranged from 6 months to 2 years and 2 months, with an average age of 10.65 ± 5.26 months. Approximately equal numbers of dogs were females (n = 38) and males (n = 39). Dog owners reported that none had ever been treated with antiparasitic products.

On physical examination, 56% (43/77, 95% CI 45–67%) of dogs were found to be infested with fleas. Based on the dogs age, more infested dogs were < 1 year old (n = 33, 59% 95% CI 46–72%) compared to those > 1 year old (n = 8, 38%, 95% CI 17%-59%).

Overall, 197 fleas were collected and morphologically identified as C. canis (n = 94) (Fig. 1), C. orientis (n = 61), Ctenocephalides spp. (n = 19) and P. irritans (n = 22, 11%) (Fig. 2). None of the examined fleas were identified as C. felis. Nineteen specimens were classified as Ctenocephalides spp. without species identification due to the damage to their exoskeleton and/or ambiguous characteristics that precluded species identification.

All fleas belonging to the genus Ctenocephalides were subject to cox1 amplification, and 95% (166/175) yielded DNA sequence. There were 25 cox1 haplotypes (UZBK1-25) among the Ctenocephalides DNA sequences; 14 (88%, 22/25) were C. canis cox1 haplotypes (UZBK1 (n = 56), UZBK2 (n = 20), UZBK3 (n = 7), UZBK4 (n = 2), UZBK5 (n = 2), UZBK6 (n = 1), UZBK7 (n = 1), UZBK8...
(n=7), UZBK9 (n=1), UZBK10 (n=1), UZBK11 (n=2), UZBK12 (n=1), UZBK13 (n=1), UZBK14 (n=1), UZBK15 (n=1), UZBK16 (n=1), UZBK17 (n=1), UZBK18 (n=2), UZBK19 (n=1), UZBK20 (n=2), UZBK21 (n=1), UZBK22 (n=1) and 3 (12%, 3/25) were C. orientis [UZBK23 (n=25), UZBK24 (n=18) and UZBK25 (n=10)]. Molecular analysis confirmed absence of C. felis. Phylogenetic analysis confirmed close relationship of the UZBK cox1 haplotypes with those previously identified in [8] (Fig. 3). Using Kimura-2 distance the maximum interspecies distance for the cox1 locus was 0.012 for C. canis, 0.064 for C. orientis and 0.092 for C. felis. Only four (4/22) cox1 haplotypes were identical to those cox1 haplotypes belonging to C. canis reported by Lawrence et al. [8]; UZBK2 = h10, UZBK8 = h31, UZBK19 = h30 and UZBK22 = h11. One out of three (1/3) C. orientis cox1 haplotypes were identical to previously identified haplotypes in Lawrence et al. [8]; UZBK23 = h48.

Comparing morphological with molecular identification of Ctenocephalides spp., we noted relatively good agreement for morphological identification of C. canis with 91–93% specimens correctly identified considering cox1 identified the species (Fig. 4a). Morphological identification appears to be more problematic for C. orientis based on morphology with overall 65% specimen identified correctly, and identification of females was marginally better reaching 69% correctness but for males reached only 43%. Misidentification was frequent for C. orientis, assuming morphological males of C. orientis were C. canis (57%, 4/7) based on cox1; morphological females of C. orientis were C. canis (31%, 16/51) (Fig. 4a, highlighted in red). Molecular identification unambiguously identified all 19 Ctenocephalides spp. that we were unable to assign to a species morphologically, 10 belonged to C. canis (1 male, 9 females), and 9 belonged to C. orientis (3 males, 6 females) based on cox1. The noted uncertainty revolving around morphological Ctenocephalides species identification was caused by key characteristics that traditionally differentiate the two species appearing variable. In particular, the numbers of setae-bearing notches on the hind tibia for C. canis and C. canis are eight and seven, respectively [8]. In the present dataset, there were specimens that had 6–9 setae bearing notches on hind tibia in combination with other characteristics made the species identification challenging. With the accessibility of the cox1 identification and retained exoskeletons, we considered using geometric morphometrics to review whether the head shape aligns with cox1 identity, and thus if it is species specific, and
potentially improves morphological identification (Additional file 1).

Head landmarks were collected for 165 specimens of *Ctenocephalides* spp. Based on *cox1*, there were 86 females of *C. canis*, 26 males of *C. canis*, 45 females of *C. orientis* and 8 males of *C. orientis* (Fig. 4b). There was a significant difference between the shape of the sexes and species (Table 1). Using canonical variate analysis (CVA), there were three canonical variates (CVs) that accounted for 100% variance in the datasets. First, two variates accounted for 97.3% of the variance (CV1: 79.3% and CV2: 18.8%) (Fig. 4b). Using a scatter plot and grouping based on *Ctenocephalides* species and sex showed clear clusters, and all groups were significantly different (*P* < 0.001) from each other using permutation tests (10,000 permutation rounds) for Mahalanobis and Procrustes distances among groups. We then restricted the dataset to only female and male specimens to undertake discriminant function analysis (DFA). Discriminant function between females or males of *C. canis* and *C. orientis* demonstrated a significant difference between pairs of groups of Procrustes and Mahalanobis distances (*P* < 0.0001 based on 1000 permutations). Reliability of the discrimination was then assessed by leave-one-out cross-validation showing that three (6%, 3/47) female *C. canis* have been morphologically classified as *C. orientis*, and one (1%, 1/84) female *C. orientis* has been classified morphologically as *C. canis*. For males, the leave-one-out cross-validation showed one (14%, 1/7) *C. canis* was classified morphologically as *C. orientis*, and two (7%, 2/27) *C. orientis* were classified as *C. canis* (Fig. 4c). Overall, the geometric morphometrics decreased the misclassification of both species and reduced the high misclassification of *C. orientis* females from 31 to 6% (5.2-fold improvement) and for males of *C. orientis* from 57 to 14% (4.1-fold improvement) misidentifications, if only head shape is considered, using geometric morphometrics (Fig. 4a, c).

The only flea recorded in all five sampling locations was *C. canis*. A total of 88 *C. canis* male specimens and 26 female specimens were collected (sex ratio female: male = 0.31). The sex was not determined for one of the identified specimens because of the degradation status of the posterior part. The second most abundant flea was *C. orientis* identified on dogs from four locations: Jizzax, Buxoro, Farg‘ona and Samarkand. The sex ratio for
C. orientis between female (n = 45) and male (n = 8) fleas was 5.62. *Pulex irritans* were identified only on dogs from Samarkand with a sex ratio between females (n = 20) and two males (n = 2) of 10. The fleas for which the species was not identified were eight females and one male (Table 2). The geographical distribution of the flea species identified in domestic dogs is presented in (Fig. 5). The complete morphological identification and all the details about the sex, age and locations of the included animals and the results of the fleas' identification are available in the supplementary material.

**Discussion**

Present Uzbekistan lies on the ancient trade route that linked the Western world with the Middle East and Asia called The Silk Road. This road connected the worlds for...
over 1500 years, and recent evidence suggests that cat domestication might have taken place along the route [27]. Domestication of dogs and cats has been speculated to be the reason behind the success of the cat flea domination on our household pets [8]. Countries in Central Asia (Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, Uzbekistan) may be particularly valuable in addressing this hypothesis and tracing the genetic signatures of fleas on domestic animals along the ancient Silk Road. Presence of C. orientis in Uzbekistan suggests that this species may have been introduced from the east along the ancient Silk Road, because C. orientis is commonly known from South Asia, East Asia and Southeast Asia [8].

In this study that focused on owned dogs, we demonstrate that C. canis is the dominant flea infesting dogs in Uzbekistan, followed by C. orientis and P. irritans. To the best of our knowledge, up to now, there are no studies on flea species infesting dogs in the other countries of Central Asia (Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan). In Afghanistan, a geographically close country, two species of fleas parasitic in stray dogs were identified, C. canis and P. irritans [28], the first being dominant as in the present study. The identification and differentiation of C. orientis require mounting of fleas and precise observation of setae bearing notches on the hind tibia in combination with micro-setae above the antennal fossa together with further morphological features summarized in Lawrence et al. [8] and in the original descriptions [24, 29]. Secondary description and identification keys often are often based on European and North American veterinary literature and disregard C. orientis. Similarly, the previous recognition of the species C. orientis as a subspecies of C. felis renders the reliance on past literature for the distribution of Ctenocephalides species in Asia unreliable. This study where no C. felis was documented shows that traditional morphological identification of C. orientis can erroneously include 31–57% C. canis specimens. Even morphological identification of C. canis was incorrect in 6–9%. Traditionally, the differentiation relies on characters that in this case were variable. Besides the key presence or absence of characters and numerical features such as number of setae, subjectively the curvature of the cephalic profile has been used to discriminate C. orientis as C. canis. To remove the subjectivity, we employed geometric morphometrics to the shape or curvature of the head together with key head landmarks. Geometric morphometrics is a collection of approaches that provide a mathematical description of forms according to geometric definitions of their shape that transformed physical anthropology and other fields of biological sciences [30]. It is now argued that many so-called ‘cryptic species’ are not cryptic if analyzed using geometric morphometrics [31–35]. In our case with C. canis and C. felis, geometric morphometrics confirmed significant geometric differences in shape between these two species and their sexes. In addition, applying geometric morphometrics of only the flea head enabled us to improve by approximately four- to fivefold the species identification over the traditional morphological key.

Regional collections series and expert morphological characterization coupled with molecular confirmation based on cox1 provide the most reliable approach established for Ctenocephalides spp. [8]. The study by Lawrence et al. [8] was based on morphology and cox1 516 Ctenocephalides fleas across 56 countries; nevertheless, under-sampled areas of the world such as Central Asia, parts of the Middle East and Africa need further scrutiny [36–38]. This study from Uzbekistan added 166 Ctenocephalides flea cox1 sequences, thus increasing by one-third the sampling in Lawrence et al. [8]. In fact, Lawrence et al. [8] included only 58 C. canis cox1 sequences compared to 115 in this study from Uzbekistan. Our phylogenetic analysis of C. canis cox1 sequences demonstrates that this species is much less diverse (based on Kimura-2 distance) than C. felis. The reason for such difference may lie in the generalist nature of C. felis infecting at least 130 wildlife species of 20% of
all mammal species sampled for fleas while *C. canis* have been recorded only on 31 mammal species, mostly a narrow spectrum of canids, felids and murids [7].

In other studies, on dogs, *C. canis* was also the most prevalent flea species in Greece [39], Ireland [40], Argentina [41] and Korea [15]. The authors are aware that the number of examined dogs included in this study is not enough to have an overall picture of the actual distribution of *Ctenocephalides* fleas in Uzbekistan and that all the tested animals originated in the southeastern...
part of the country. The absence of studies in Central Asia together with the results of the present study calls for further epidemiological surveys of flea species in both domestic and wild carnivores from this region. Our results support the opinion that _C. canis_ prefers colder climates than _C. felis_ and it is a species restricted to temperate zones [8, 14, 15, 42]. As we tested only dogs kept outside, we could not compare the association of flea species with the dog housing conditions (inside/outside; rural/urban area), but a positive correlation between dogs kept outside and the infestation with the dog flea was previously reported [15, 42], and it is in correlation with the present results.

It is not clear which is the actual reason for the absence of infestation with _C. felis_, but similar results were obtained in South Korea [15] and confirmed by the characterization of _cox1_ from fleas on domestic dogs from South Korea that all belonged only to _C. canis_ [8]. There are several potential factors that could contribute to the lack of this species in examined dogs, such as a very low prevalence of this species in dogs, considering the small number of examined animals, or the absence of examined dogs kept inside as pets, the season when the fleas were collected (winter) or even the continental climate in Samarkand region. Interestingly, _C. felis_ was previously identified in domestic dogs from Uzbekistan [43].

In addition, in the same study, the authors did not report any infestation with _C. orientis_. Based on these and the lack of photos or complementary identification methods, we cannot exclude a misidentification of the fleas’ species in the only study previously done in Uzbekistan [43].

The second most frequent species, the oriental dog flea (_C. orientis_), is restricted to Asia [8], and our study represents the first report of _C. orientis_ in Central Asia. The species prefers tropical and subtropical zones and has a wide host spectrum, more commonly identified in ruminants than in dogs and cats [8, 12, 17]. Out of the five examined areas, the species was present only in four. Its absence in Surkxandaryo region is likely related to the low number of examined animals from that area or the higher altitude (Fig. 4). The exact geographical distribution of this species is not currently known, but there are some hypotheses that climate change and livestock movements could contribute to the spreading of this species [44] or even be a relic from the ancient times when the Silk Road connected Asia with the Mediterranean.

_Pulex irritans_, the human flea, were identified in dogs from a single region. Being a species that can infest a wide range of hosts, its absence in other geographical locations can be related to its host preferences, seasonality or climatic preferences [14]. Finding _P. irritans_ in dogs is not common but at the same time not unexpected, as it has been found in other regions of the world, e.g. [45].

### Conclusions

This study aimed to investigate the flea infestation in privately owned dogs kept outdoors in five locations in Uzbekistan. The most common flea species infesting dogs is _C. canis_ followed by _C. orientis_ and _P. irritans_. No dogs were infested with _C. felis_. We report for the first time the presence of _C. orientis_ in a country in Central Asia, Uzbekistan, using a robust and reproducible methodology that employed both _cox1_ DNA sequencing and geometric morphometrics. A landmark-based geometric

### Table 1

Summary of the Procrustes ANOVA using shape for _Ctenocephalides canis_ and _C. orientis_

| Effect                  | SS   | MS   | Df  | F    | P-value (param.) |
|-------------------------|------|------|-----|------|------------------|
| Flea sex                | 0.12271 | 0.00558 | 22  | 38.49 | <0.0001          |
| Flea species            | 0.09495 | 0.00432 | 22  | 29.78 | <0.0001          |
| Flea sex * species      | 0.00319 | 0.00015 | 22  | 2.88  | <0.0001          |
| Residual                | 0.17843 | 0.00005 | 3542 |      |                  |

_F_ statistics and parametric _P_-values for each of the effects in the ANOVA

SS sums of squares, _MS_ mean squares, df degrees of freedom

*Significantly different

### Table 2

Number of animals examined from each geographical location of Uzbekistan and the flea species identified in each of the locations

| Region       | Dogs examined | Dogs infested | Fleas collected, _N_ | _C. canis_ | _C. orientis_ | _P. irritans_ | Unidentifiable | Total |
|--------------|---------------|---------------|----------------------|------------|---------------|---------------|----------------|-------|
|              | M  | F  | T  | N= | %  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T  | M  | F  | T |
morphometric analysis of flea heads is a useful additional method for species discrimination. This study highlights the ambiguity of identification of *Ctenocephalides* spp. when *C. orientis* is present based on traditional morphological features. Studies across Asia are needed to expose the actual distribution of *C. orientis* to improve our understanding of the role of the Silk Road connecting Asia with the Mediterranean played in parasite dispersal.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05477-3.

Additional file 1: Table S1. Table showing the prevalence of infested dogs. Table S2. The number of examined and positive dogs regarding their sex. Table S3. The number of examined and positive dogs regarding their age. Table S4. The number of dogs infested with *C. canis* from each of the locations. Table S5. The number of dogs infested with *C. orientis* from each of the locations. Table S6. The number of dogs infested with *P. irritans* from each of the locations. Table S7. The number of dogs infested with unidentified fleas from each of the locations. Table S8. Number of infested dogs in each location. Table S9. Species of fleas for each of the age groups. Table S10. The sex of the identified fleas.

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**Author contributions**
GD mounted and morphologically identified the fleas, extracted the DNA, analyzed the data and wrote the draft of the manuscript; AS did the fieldwork and collected the fleas; ADM financially supported the DNA extractions and critically revised the paper; XCS and RW assisted with coding morphology and geometric morphometrics; JS performed the molecular characterization and geometric morphometrics analysis, interpreted the results and revised the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**
Details about the tested dogs and identified fleas are available in the additional file. The nucleotide sequence data generated in this study were deposited in GenBank (NCBI) under the accession numbers ON247050-ON247215. Sequence comparative data and the associated additional data including geometric morphometrics (images, MorphoJ, tps) are available at LabArchives (https://dx.doi.org/10.25833/c9qi-ha67). The mounted fleas are preserved in the collection of the Department of Parasitology and Parasitic Diseases from USAMV Cluj-Napoca Romania and are available from the first author on reasonable request.

**Fig. 5** Map showing the geographical distribution of the identified flea species related to the altitude. Pie charts illustrate the proportions of identified fleas for each geographical location and the number of fleas identified for each species.
Declarations

Ethics approval and consent to participate
The study was approved by the Animal Care Organization of the State Committee for Ecology and Environmental Protection.

Consent for publication
Not applicable.

Competing interests
The authors declare they have no competing interests.

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