Complete mitochondrial genome of *Scathophaga stercoraria* (Diptera: Scathophagidae) in wild plateau pika: genome descriptions and phylogenetic evolution

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Abstract

As a member of the Scathophagidae family, *Scathophaga stercoraria* (*S. stercoraria*) is widely distributed globally and is closely associated with animal feces. It is also a species of great interest to many scientific studies. However, its phylogenetic relationships are poorly understood. In this study, *S. stercoraria* was found in plateau pikas for the first time. The potential cause of its presence in the plateau pikas was discussed and it was speculated that the presence of *S. stercoraria* was related to the yak feces. In addition, 2 nuclear genes (18SrDNA and 28SrDNA), 1 mitochondrial gene (COI), and the complete mitochondrial genome of *S. stercoraria* were sequenced. Phylogenetic trees constructed based on 13 Protein coding genes (13PCGs), 18S and 28S rDNA showed that *S. stercoraria* is closely related to the *Calliphoridae* family; phylogenetic results based on COI suggest that within the family Scathophagidae, *S. stercoraria* is more closely related to the genus *Leptopa*, *Micropselapha*, *Parallelomma* and *Americina*. Divergence times estimated using the COI gene suggest that the divergence formation of the genus *Scathophaga* is closely related to changes in biogeographic scenarios and potentially driven by a combination of uplift of the Qinghai-Tibetan Plateau (QTP) and dramatic climate changes. These results provide valuable information for further studies on the phylogeny and differentiation of the *Scathophaga* genus in the future.

Introduction

Scathophaga stercoraria (yellow dung fly) is a species of fly in Scathophagidae, which is a small family of Muscoidea (Vockeroth, 1987). Most species of Scathophagidae are distributed in the Holarctic, mostly in northern latitudes (Mortelmans and Devillers, 2014). *S. stercoraria* is widespread and typically seen on the dung of large mammals, especially on cattle dung (Blanckenhorn, 1998). *S. stercoraria* attracted early attention as a possible biocontrol agent (Cotterell, 1920). Meanwhile, this fly is also a popular subject for many other studies, including sperm competition (Gress *et al.*, 2016), sexual selection (Sbilordo *et al.*, 2010), reproductive physiology (Reim *et al.*, 2006), growth and development (Hosken *et al.*, 2000; Walters *et al.*, 2022), genetic aspects (Demont *et al.*, 2008), thermobiology (Blanckenhorn *et al.*, 2021), immune-related research (West and Tracy, 2009). In addition, *S. stercoraria* is often used for ecotoxicological tests, such as ivermectin detection (Mahdjoub *et al.*, 2020; González-Tokman *et al.*, 2022).

The plateau pika (*Ochotona curzoniae*) (Lagomorpha, Ochotonidae) is a specialized native species of the Qinghai-Tibetan plateau (QTP) (Zhu *et al.*, 2022). It is important to the community of grasslands in the QTP (Zhang *et al.*, 2017). The digging activities of the plateau pika increase the abundance of plant species (Qin *et al.*, 2021) and the dug burrows can become habitats for other animals (lizards, and small birds) (Zhao *et al.*, 2020). Previous studies have shown that plateau pikas are susceptible to infestation by various parasites, including *Oestromyia leporina* (Fu *et al.*, 2016), *Echinococcus multilocularis* (Li *et al.*, 2018), *Taenia* spp. (Wu *et al.*, 2021), *Cryptosporidium* spp. (Zhang *et al.*, 2018), *Toxoplasma gondii* (Zhang *et al.*, 2013), *Enterocytozoon bieneusi* (Liu *et al.*, 2021). However, the presence of *S. stercoraria* in plateau pikas has never been reported before.

The QTP and its surrounding mountain systems are one of the hotspots of biodiversity (Wu *et al.*, 2022) and have played important roles in the evolution of organisms (Rahbek *et al.*, 2019). The uplift of the QTP and associated climate change has driven species diversity on the plateau (Mao *et al.*, 2021), while also causing the isolation and divergence of many species (Flantua *et al.*, 2019; Rahbek *et al.*, 2019). The mitochondrial genome is characterized by

low molecular weight and genetic conservation due to its largely haploid and uniparentally inherited feature. Thus, in recent years, the mitochondrial genome has been widely used in the study of phylogenetic relationships, molecular evolution and population genetics (Pyziel *et al.*, 2020).

In this study, the complete mitochondrial genome of *S. stercoraria* was sequenced and annotated, the phylogenetic tree was reconstructed using 40 mitochondrial genomes and complemented by phylogenetic trees generated on 18S rDNA, 28S rDNA and COI gene. The possible divergence time of *Scathophaga* in history was investigated by the COI gene. These studies have provided fundamental data to better understand the phylogenetic relationships and evolutionary history of *S. stercoraria* in the QTP area.

Materials and methods

Sample collection and DNA extraction, PCR, sequencing

Five larvae were collected subcutaneously from the hind limbs of plateau pika in Chenduo county (33°35′N; 97°12′E; altitude at 4377 m) of Qinghai Province, the People's Republic of China in June 2022. After being washed in phosphate saline buffer, all samples were stored in 70% ethanol. Genomic DNA were extracted from the samples using a commercial kit (TIANamp Genomic DNA Kit, TIANGEN Biotechnology, Beijing, China) according to the manufacturer's instructions. Partial sequences of COI (Otranto *et al.*, 2003), 18S rDNA (Nirmala *et al.*, 2001) and 28S rDNA (Otranto *et al.*, 2005) were determined using primers that have been previously reported. Primers were synthesized by Sangon Biotech (Shanghai, China), standard 25 μL PCR protocol was used to amplify the DNA fragments. The PCR products were purified using a TIANgel Midi Purification Kit (Cat. DP209-02, Tiangen, China), and finally sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing. The list of primers and PCR reaction conditions are shown in Supplementary Table 1.

Mitochondrial genome sequencing, assembly and annotation

DNA samples were sent to Sangon Biotech (Shanghai) Co., Ltd. for library construction and sequencing. Library construction using a whole genome shotgun (WGS) strategy was performed, followed by next-generation sequencing to obtain mitochondrial genome sequences. For the quality-checked fragments, sequencing was performed on the Illumina Hiseq platform using a double-end sequencing strategy. Low quality sequences were removed from raw sequences with FASTP v0.36 (Chen *et al.*, 2018) software to obtain a clean data dataset. SPAdes v3.15 (Bankevich *et al.*, 2012) software was used to splice and assemble the short fragment sequences (Clean reads) from high-throughput sequencing. After the assembly was completed, assembled sequences were compared with the known *S. stercoraria* genes in Gen Bank. Sequencing results were subsequently confirmed as the *S. stercoraria* mitochondria genome. The complete mitochondrial genome sequence of *S. stercoraria* was successfully obtained. The 37 genes of the *S. stercoraria* mitochondrial genome were annotated by the online software MITOS Web Server (http://mitos.bioinf.uni-leipzig.de/index.py) to determine the position of each gene and predict the secondary structure of tRNA (Bernt *et al.*, 2013). The annotation results were exported using SnapGene v7.0 software and manually corrected by referring to the reported mitochondrial genome annotation results of *S. stercoraria* in NCBI.

Phylogenetic analyses

To determine the phylogenetic relationships of *S. stercoraria*, 13 Protein coding genes (13PCGs), 18S and 28S rDNA, COI gene

in this study with those of other classified Diptera available in GenBank were used as ingroup, as 2 species (*Batocera horsfieldi*, *Trigoniopthalmus alternatus*) of insects belonging to different orders were chosen as outgroups. To analyse phylogenetic relationships, 4 datasets were collected which containing 108 species from 6 families (with evolutionary trees of 18S and 28S rDNA as a complement to the evolutionary tree of 13PCGs); due to little has been previously reported about the phylogeny of the genus *Scathophaga* with other species within the family Scathophagidae, 37 species were selected from 18 genera in the family Scathophagidae and the phylogenetic relationships were analysed (see Supplementary Tables 2–5 for details). Owing to limited data on different genes of the same species, the phylogeny of each dataset was performed independently. Sequence alignment was accomplished using the MAFFT v7.505 with auto option (Katoh and Standley, 2013). TrimAl v1.2 was used under the automated 1 option to trim the aligned sequences (Capella-Gutiérrez *et al.*, 2009). The phylogenetic tree was constructed using the maximum likelihood (ML) method with IQ-TREE v2.2.0 (Nguyen *et al.*, 2015). The selection of models is performed automatically by ModelFinder (Kalyaanamoorthy *et al.*, 2017). All other parameters were set to the default values. The online tool: tvBOT (Xie *et al.*, 2023) was used to view and modify phylogenetic trees.

Divergence times estimates

Due to the lack of a complete mitochondrial genome for other *Scathophaga* species, the COI gene was used to analyse the divergence time. Divergence times were estimated using BEAST v2.7.4 (Bouckaert *et al.*, 2014); the clock model was set to relaxed, uncorrelated log-normal and the gamma category count was set to 4; and the GTR substitution model was selected. For the tree prior the Calibrated Yule model (Heled and Drummond, 2015) was used. Due to the lack of *Scathophaga* fossils, a secondary calibration approach was used (Hedges and Kumar, 2004). Based on previous research on divergence time in *Scathophaga* (Junqueira *et al.*, 2016), the time calibration was set as 41 million years ago (Mya). The posterior probability estimates were drawn every 1000 steps out of the total 10 000 000 steps of each MCMC run. Other options were run on the default values. Tracer (v1.7.2) was used to determine whether the result converges. TreeAnnotator (v2.1.2) was used to annotate the tree by using maximum clade credibility tree and median heights settings with 10% burn-in.

Results

Characterization of the mitochondrial genome

The total length of the mitochondrial genome in *S. stercoraria* was determined to be 16 512 bp (GenBank ID: OR039275), consisting of circular DNA molecules. The mitochondrial genome exhibits features typical of insect mitochondrial genomes; double-stranded DNA molecules, including light-stranded L and heavy-stranded H, 2 rRNAs: 16s rRNA, 12s rRNA, 22 tRNAs, 13PCGs and a large control region (D-Loop region) (Fig. 1). The inferred gene boundaries and lengths are shown in Table 1. Our findings are consistent with previous reports (Li *et al.*, 2016).

The standard start codons of invertebrate PCGs include ATN, GTG and TTG (Wolstenholme, 1992*a*, 1992*b*). Except for CO1, which uses TCG in *S. stercoraria*, all PCGs start with standard start codons. Other Diptera mitochondrial genomes frequently contain these unconventional start codons. Twelve PCGs of *S. stercoraria* terminate with the common stop codons TAA or TAG, except for the *NAD4*, which ends with a single thymine
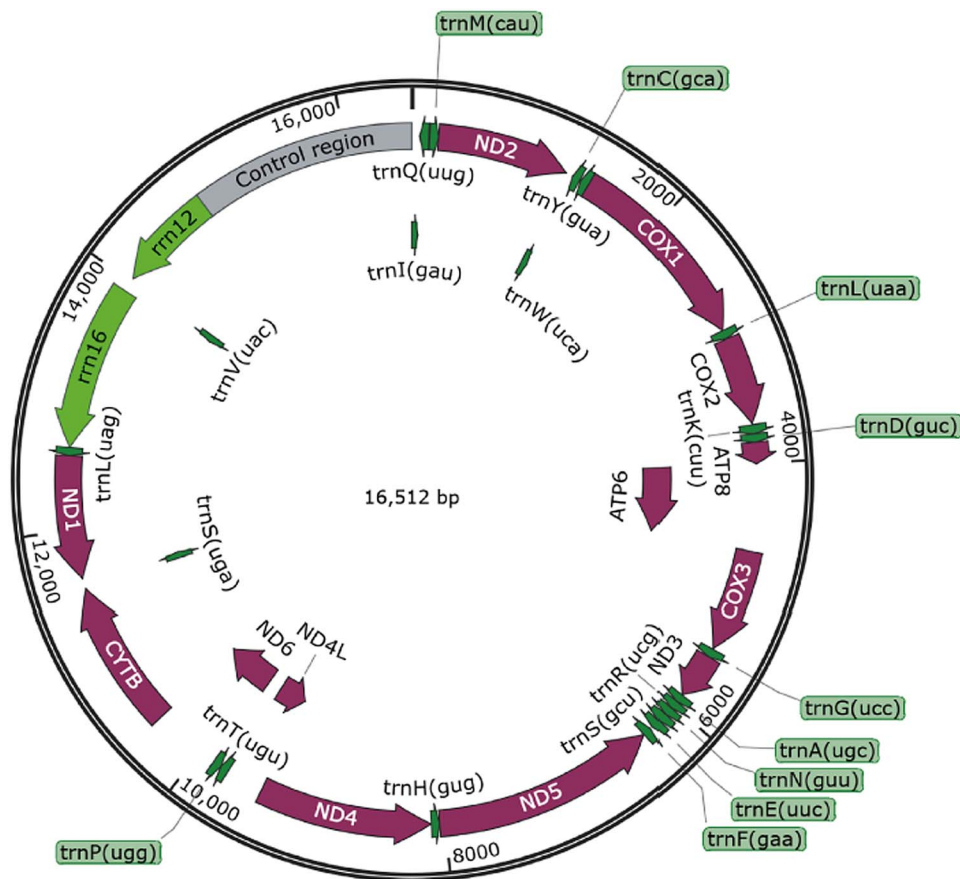
Figure 1. The diagram of complete mitochondrial genome of *S. stercoraria*. The mitochondrial genome consists of protein-encoding genes (plum), tRNAs (green), rRNAs (light green) and non-coding mitochondrial regions (Control region) (grey). The inferred gene boundaries of them are shown in Table 1.

stop codon (Table 1). Incomplete stop codons are hypothesized to be filled by polyadenylation during mRNA maturation (Ojala *et al.*, 1981). All tRNAs can be folded into typical cloverleaf structures, except trnSer1 for its DHU arm, forming a simple loop (Fig. 2), and this has been repeatedly reported in other metazoan mitochondrial genomes (Wolstenholme, 1992*a*, 1992*b*).

Phylogenetic relationships

The result of phylogenetic analysis constructed based on the sequences of 13PCGs showed that *S. stercoraria* has a close genetic affinity with the family *Calliphoridae* (Fig. 3), the same results were also obtained from the analysis of 18S and 28S rDNA (Fig. 4). Phylogenetic analysis based on COI gene showed that *S. stercoraria* itself, as a monophyletic group, has a close genetic affinity with *Leptopa filiformis*, *Micropselapha filiformis*, *Parallelomma medium*, *Parallelomma paridis* and *Americina adusta* (Fig. 5).

Divergence times analysis

The divergence time analysis based on the COI gene suggested that the most recent common ancestor of the twenty *Scathophaga* species existed at approximately 40.98 Mya, this is in accordance with previous reports (Junqueira *et al.*, 2016). The divergence time between *S. stercoraria* and the other twelve *Scathophaga* species was approximately 27.07 Mya, and the divergence time of the sixteen *Scathophaga* species all occurred within 1 Mya (Fig. 6).

Discussion

The presence of S. stercoraria in pika is related to yak feces

This is the first documentation of *S. stercoraria* collected in plateau pikas and the first report of the complete mitochondrial genome of *S. stercoraria* in the QTP. According to previous reports, *S. stercoraria* generally reproduces on dung and their larvae grow in the dung (Gress *et al.*, 2016). However, a new discovery has been made: the presence of *S. sterocoraria* in plateau pikas. As *S. stercoraria* has only been previously reported to be present in the dung of animals with no reports showing its presence in the animal itself, based on this, it was speculated that the presence of *S. stercoraria* in plateau pikas is possibly attributed to the contamination from the dung of yaks or other large mammals during their daily activities. Female *S. stercoraria* are accustomed to laying eggs on the surface of dung to avoid the living environment of eggs being too dry or too humid (Ward *et al.*, 1999). This creates conditions for plateau pikas to carry *S. stercoraria* eggs from the dung of large mammals. After the eggs are transferred from dung to the plateau pika, the environment where the eggs are located may not provide adequate conditions for their continued growth and development. Therefore, in order to survive, the eggs may have burrowed into the subcutaneous tissue of the plateau pika after developing into a larva.

Based on the above reasons, it was speculated that plateau pika carries the eggs of *S. stercoraria* from the dung of yaks. Study shows that the 2 species began to coexist at about 2.4 Mya and they compete for phytophagous food and overlapping spaces (Harris *et al.*, 2015). In addition, it was found that during winter, when food is scarce, plateau pikas survive by ingesting yak feces (Speakman *et al.*, 2021) and develop reciprocal relationships

Table 1. The list of mitochondrial genome annotation for Scathophage stercoraria

Genes	Positions	Strand	Length bp	Initiation and termination codons	Anticodons
*trn*I	1–64	+	64		GAT
*trn*Q	62–130	–	69		TTG
*trn*M	138–206	+	69		CAT
*nad*2	207–1223	+	1017	ATT/TAA	
*trn*W	1222–1288	+	67		TCA
*trn*C	1281–1343	–	63		GCA
*trn*Y	1351–1417	–	67		GTA
*cox*1	1419–2949	+	1531	TCG/TAA	
*trn*L	2950–3015	+	66		TAA
*cox*2	3020–3698	+	679	ATG/TAA	
*trn*K	3708–3777	+	70		CTT
*trn*D	3778–3842	+	65		GTC
*atp*8	3843–4007	+	165	ATC/TAA	
*atp*6	4001–4678	+	678	ATG/TAA	
*cox*3	4678–5466	+	789	ATG/TAA	
*trn*G	5473–5538	+	66		TCC
*nad*3	5539–5892	+	354	ATA/TAA	
*trn*A	5895–5958	+	64		TGC
*trn*R	5959–6022	+	64		TCG
*trn*N	6024–6088	+	65		GTT
*trn*S1	6089–6156	+	68		GCT
*trn*E	6157–6223	+	67		TTC
*trn*F	6242–6308	–	67		GAA
*nad*5	6292–8043	–	1752	ATT/TAA	
*trn*H	8044–8107	–	64		GTG
*nad*4	8107–9446	–	1340	ATG/T	
*nad*4L	9440–9733	–	294	ATG/TAA	
*trn*T	9739–9805	+	67		TGT
*trn*P	9806–9872	–	67		TGG
*nad*6	9875–10 399	+	525	ATT/TAA	
cytb	10 399–11 535	+	1137	ATA/TAG	
*trn*S2	11 534–11 600	+	67		TGA
*nad*1	11 617–12 567	–	951	ATT/TAA	
*trn*L	12 569–12 631	–	63		TAG
*rrn*16S	12 611–13 916	–	1306		
*trn*V	13 958–14 029	–	72		TAC
*rrn*12S	14 028–14 811	–	784		
Control region	14 812–16 512	+	1701		

through horizontal transmission of the gut microbiota (Fu *et al.*, 2021). Meanwhile, *S. stercoraria* is thought to occur more often in cattle dung (Blanckenhorn *et al.*, 2010). Therefore, it was speculated that the plateau pika carries the eggs of *S. stercoraria* from the yak dung, and the larvae parasitize in plateau pikas in order to survive. As this is the first discovery of *S. stercoraria* 'parasitism' in animals, further study is needed to determine whether this parasitic behaviour of *S. stercoraria* is an accidental event or an adaptive change made to adapt to the harsh living environment of the QTP.

Mitochondrial genomic characterization and phylogenetic analysis

The complete mitochondrial genome of *S. stercoraria* was sequenced and annotated, and the sequence was similar to the reported mitochondrial whole genome of *S. stercoraria* (Li *et al.*, 2016). They share similarities in the rRNA, tRNA and protein-encoding genes regarding length, gene order and composition. The difference between the two lies in the control region (D-Loop region). The control region in this study was approximately 400 bp longer than that from previous
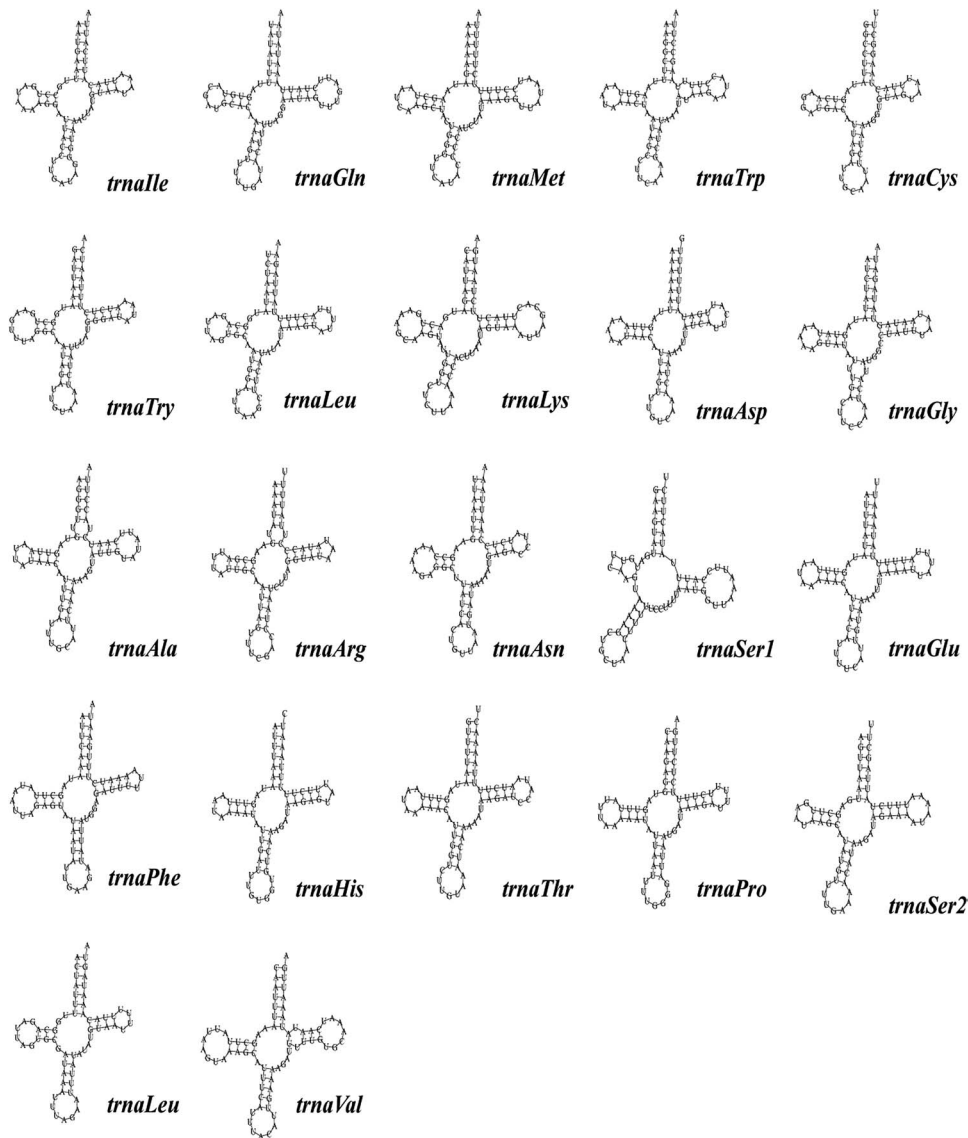
Figure 2. The structure of 22 tRNAs derived from the *S. stercoraria* mitochondrial genome. Structures of 22 tRNAs with base pairs are shown, with the names of the tRNAs and anticodons displayed in the bottom right of each structure.

studies. The differences are potentially attributed to the fact that *S. stercoraria* collected in our study versus the *S. stercoraria* from previous studies were collected in different places, resulting in differences in their adaptation to the local environment. The phylogenetic tree shows that *S. stercoraria* is more closely related to *Lucilia*, *Calliphora* and *Chrysomya* of the family *Calliphoridae*, which is consistent with previous findings (Ding *et al.*, 2015). The phylogenetic tree obtained based on the COI gene within the family Scathophagidae shows that *S. stercoraria* is closely related to the genus *Leptopa*, *Micropselapha*, *Parallelomma* and *Americina*. This adds to the previously reported phylogenetic relationships within the Scathophagidae family; in addition, it was found that the affinities of *S. stercoraria* and *Americina adusta* in this study were inconsistent with previously reported studies (Bernasconi *et al.*, 2000*a*, 2000*b*), and it was speculated that this is potentially due to the location of sample collection (*S. stercoraria* in Bernasconi's study was from Switzerland) and the selection of different outgroups.

Divergence time and evolution of the *Scathophaga*

The evolution, differentiation, or diversity of organisms may be influenced by biotic factors such as competition, intraspecific interactions, and abiotic factors such as tectonic events and climate, or by the combination of both (Antonelli and Sanmartín, 2011). Biotic factors tend to influence organisms over a short period (less than 1 Mya), while abiotic factors drive evolutionary differentiation over a longer period (millions of years or even longer) (Benton, 2009). Because the events associated with the uplift of the QTP span tens of millions of years, the effects of biotic factors on organisms during this process are likely to be lower than those of abiotic factors (Favre *et al.*, 2015). Our divergence time analysis based on the COI gene estimated that there are 2 important divergence times for the genus *Scathophaga*, 27 and 20 Mya, and except for *S. suilla*, *S. soror*, *S. apicalis*, *S. stercoraria*, the rest of *Scathophaga* underwent divergence at approximately 1 Mya. This indicates that the geological and climatic events during these periods (late Oligocene to early Miocene, Pliocene, Pleistocene) (Harrison *et al.*, 1992; Ni *et al.*, 2016) may have played an important role in the differentiation of *Scathophaga*.

The uplift of the QTP has caused environmental and climatic changes that have driven the evolution of associated biotas (Favre *et al.*, 2015). During the Oligocene and Miocene periods, the uplift of the QTP advanced to the north and south, which caused the extension of the QTP (35 to 20 Mya) (Mulch and Chamberlain,
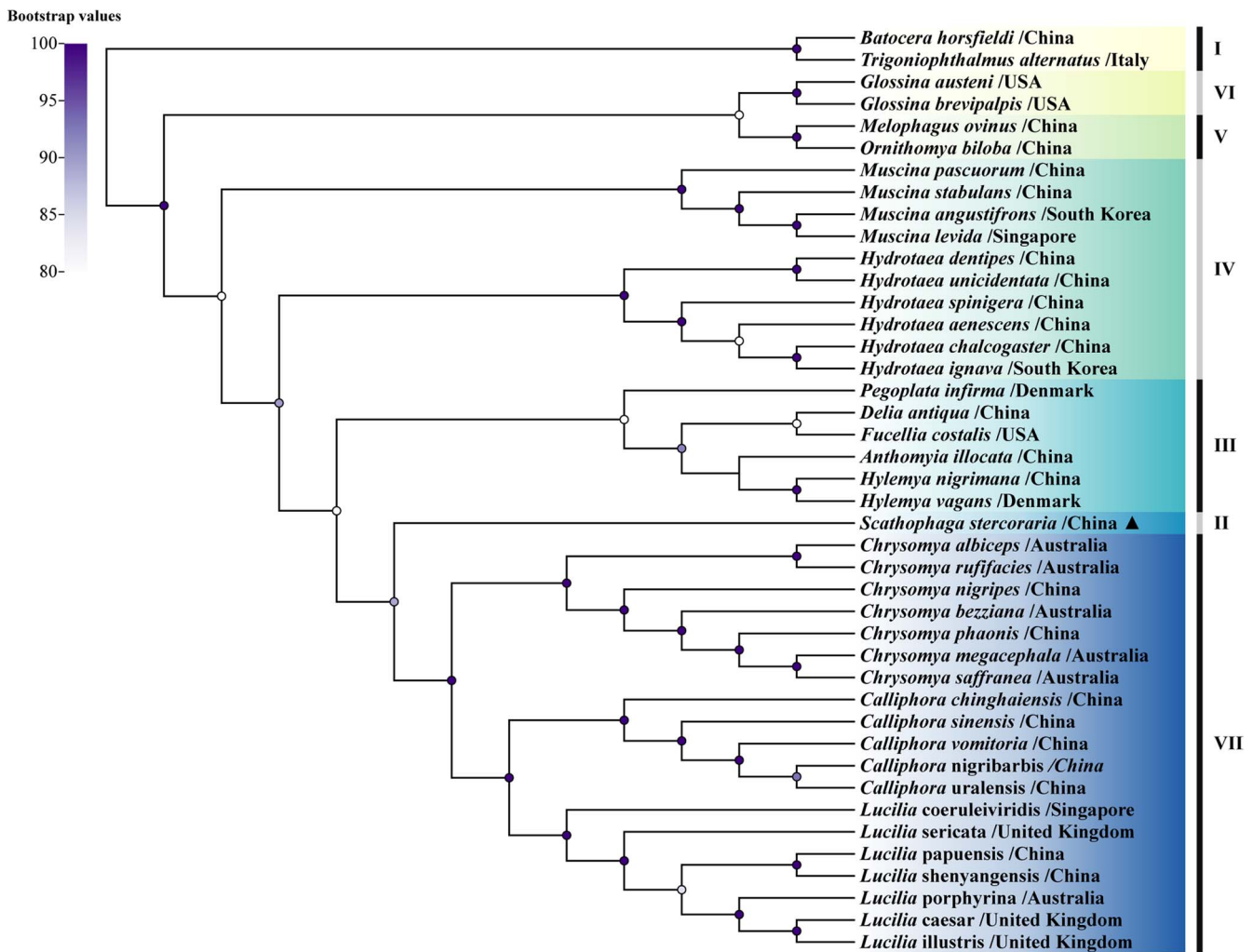
Figure 3. Maximum likelihood analyses of *S. stercoraria* based on 13PCGs. The different coloured bars and the Roman numerals to the right represent outgroups and different genus names (i.e. I: outgroup, II: Scathophagidae, III: Anthomyiidae, IV: Muscidae, V: Hippoboscidae, VI: Glossinidae, VII: Calliphoridae). ▲ was used to mark the *S. stercoraria* of this study. Nodes with Bootstrap values > 80% are displayed.

Figure 4. Maximum likelihood analyses of *S. stercoraria* based on 18S rDNA (a) and 28S rDNA (b) fragments. The different coloured bars and the Roman numerals to the right represent outgroups and different genus names (i.e. I: outgroup, II: Scathophagidae, III: Anthomyiidae, IV: Muscidae, V: Hippoboscidae, VI: Glossinidae, VII: Calliphoridae). ▲ was used to mark the *S. stercoraria* of this study. Nodes with Bootstrap values > 80% are displayed.
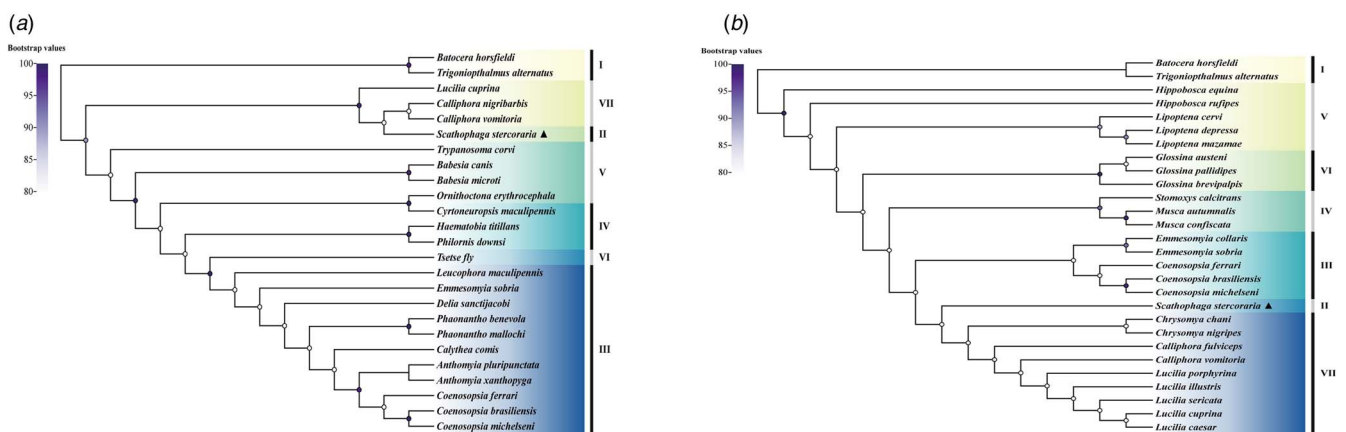
2006; Qiang *et al.*, 2011; Miao *et al.*, 2012). The carbon dioxide content in the atmosphere during the Oligocene was lower than that during the Eocene, which resulted in a warmer global climate during the Oligocene (Villa and Persico, 2006; O'Brien *et al.*, 2020). During the Miocene period, orogenic movements in high mountain ranges, such as the Himalayas led to the gradual expansion of the uplift of the QTP (Tapponnier *et al.*, 2001; Wang *et al.*, 2008). In addition, paleobotanical data indicates that the southeastern edge of the QTP was dominated by a warm and humid climate during the Miocene period, primarily influenced by the monsoon winds from East and South Asia (Sun and Wang, 2005; Jacques *et al.*, 2011). The Earth's climate underwent a fundamental change during the Pleistocene, known as the Middle Pleistocene Transition (MPT); during this time, the climate changed more and more
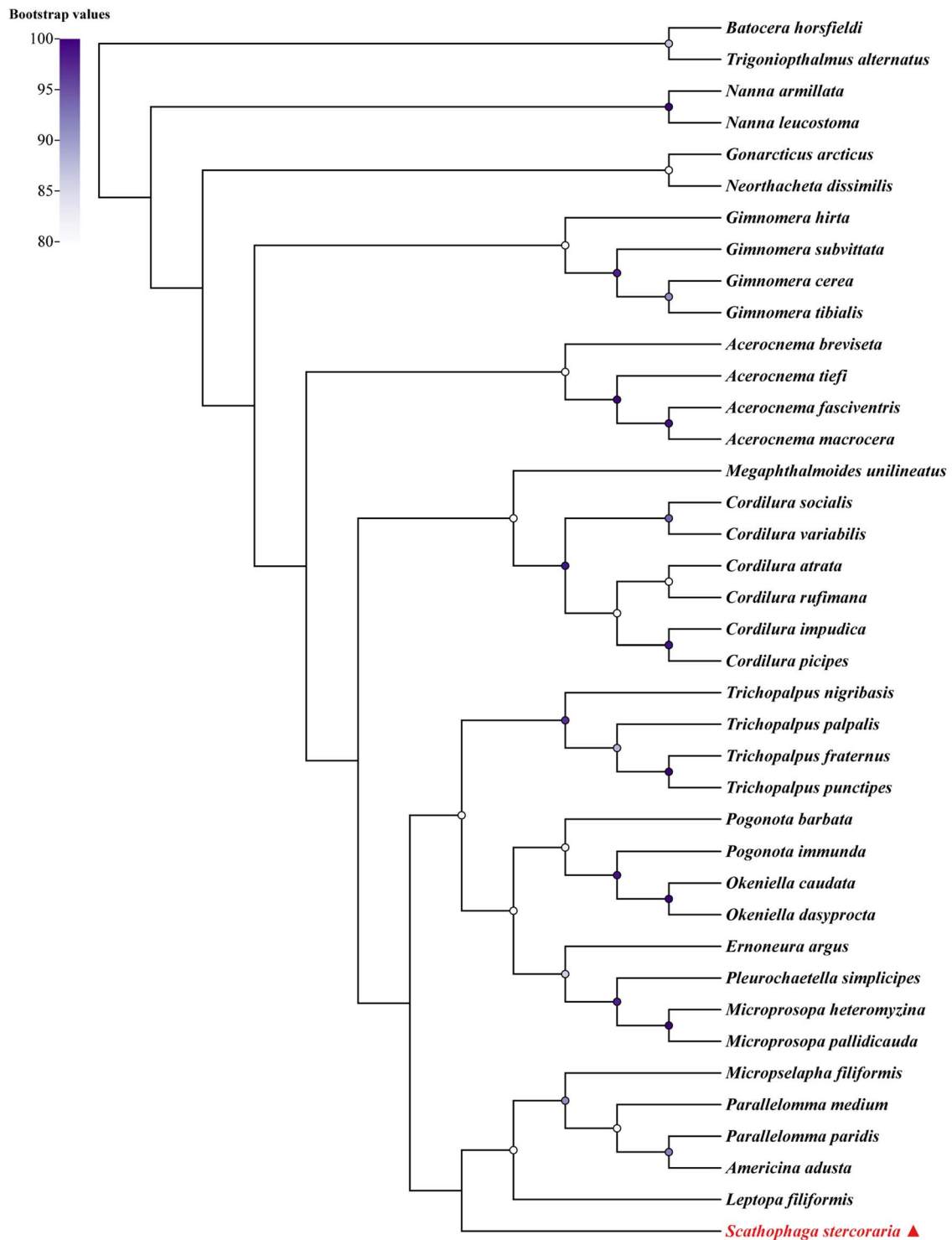
Figure 5. Maximum likelihood analyses of *S. stercoraria* based on COI fragments. *S. stercoraria* of this study is marked with ▲ and red. Nodes with Bootstrap values > 80% are displayed.

drastically, and the Northern Hemisphere became increasingly glaciated (Pena and Goldstein, 2014; Sun *et al.*, 2019).

The timeline of geological and climate change mentioned above is close to the timeline of *Scathophaga* differentiation obtained in this study (Fig. 4). It was speculated that the uplift of the QTP, along with global climate change, drove the divergence of the *Scathophaga* genus. The complex mountainous regions of western China (including the QTP, the Himalayas, the Hengduan Mountains, and the Three Gorges Mountains) are responsible for the isolation and divergence of many plants and animals (Yuan *et al.*, 2008; Zhang *et al.*, 2010). During the Oligocene and

Miocene periods, the uplift of the QTP and the resulting climate change drove the formation of species and the diversity of their populations in western China (He *et al.*, 2001; Che *et al.*, 2010); and the species of *Scathophaga* gradually differentiated. In addition, the warm and humid climate of the Miocene period created a suitable environment for the development of biodiversity (Barry *et al.*, 2002; Badgley *et al.*, 2008; LaRiviere *et al.*, 2012), which accelerated the differentiation of the *Scathophaga* genus. Finally, the drastic climate changes during the Pleistocene period resulted in ecological variability, which in turn affected all aspects of flora and fauna (Hofreiter and Stewart, 2009); this may also be the reason why
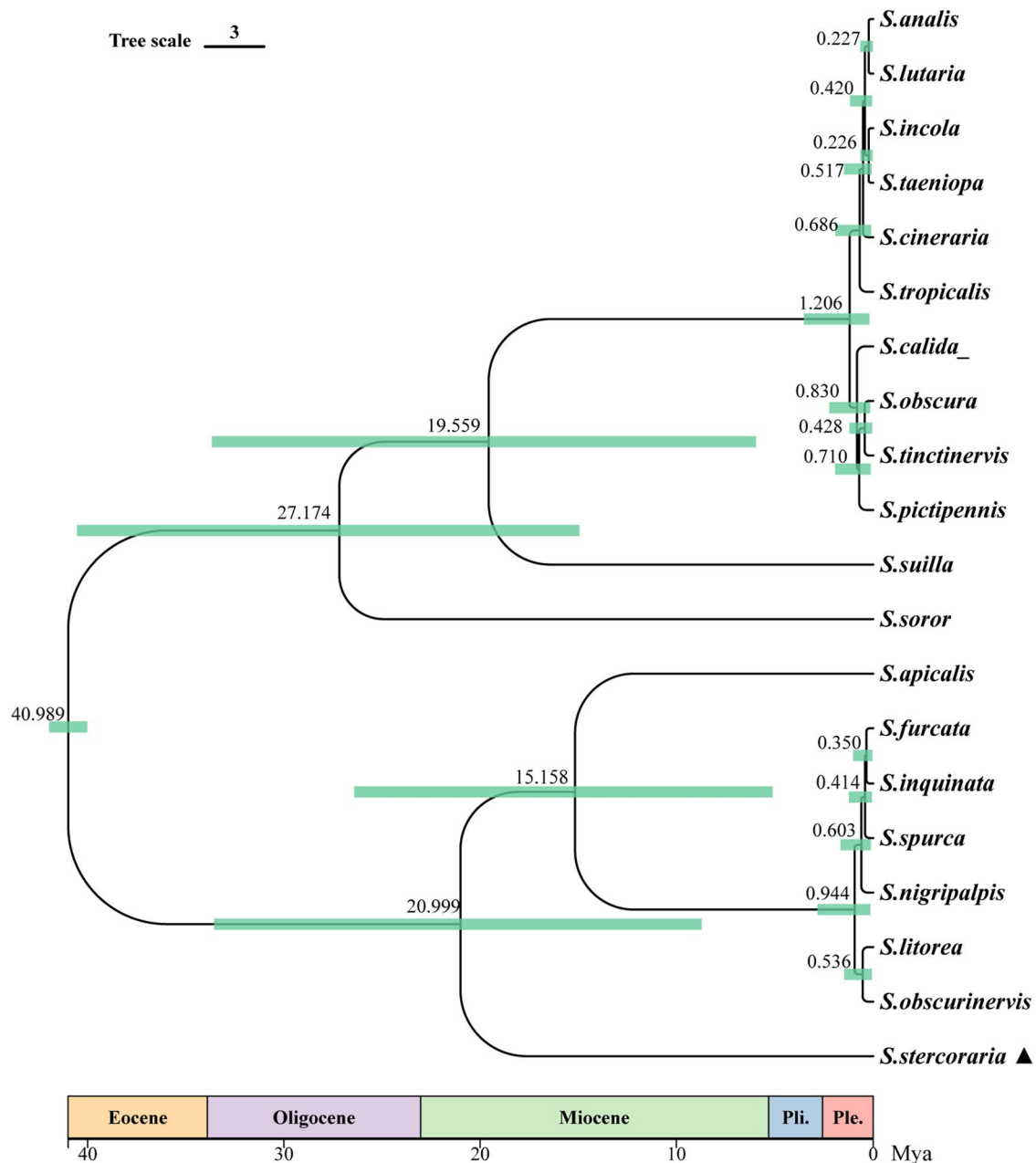
Figure 6. Divergence time of *S. stercoraria* was estimated based on COI fragments. ▲ was used to mark the *S. stercoraria* of this study. The green bar represents an interval of 95% highest probability density. Different colours indicate different periods (Eocene, Oligocene, Miocene, Pliocene and Pleistocene).

most *Scathophaga* species differentiated and formed during the Pleistocene. Therefore, it was inferred that climate change during the Pleistocene and the uplift of the QTP are the 2 most important factors influencing *Scathophaga* differentiation.

Conclusion

In conclusion, in this study, the phylogenetic analysis of *S. stercoraria* was conducted for the first time using mitochondrial genes, 18S rDNA and 28S rDNA. Additionally, the divergence time of *Scathophaga* was estimated for the first time using the COI gene. It was suggested that *S. stercoraria* in plateau pikas may be derived from yak feces, and *S. stercoraria* was differentiated and formed around the early Miocene (21 Mya) due to the uplift of the QTP and climate change. This study provides fundamental information for the subsequent study of the kinship and differentiation of *S. stercoraria*. However, due to the lack of reports on *S. stercoraria* in the QTP, more samples need to be collected to further study the growth

and development of *S. stercoraria* in the QTP and to elucidate the phylogeny and differentiation of *Scathophaga* in more detail.

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