Tropical Biomedicine 41(4): 512-517 (2024)
https://doi.org/10.47665/tb.41.4.012

RESEARCH ARTICLE

Cryptic genetic diversity and molecular detection of *Trypanosoma theileri* complex in the deer fly *Chrysops dispar* Fabricius from Thailand

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ARTICLE HISTORY

Received: 2 October 2024
Revised: 25 October 2024
Accepted: 26 October 2024
Published: 31 December 2024

ABSTRACT

The deer fly (Diptera, Tabanidae), *Chrysops dispar* Fabricius is a common and widespread pest and vector species transmitting pathogens to animals including economically significant livestock. However, there is only limited information on genetic diversity, which crucial for understanding disease epidemiology. In this study, we examined genetic diversity of *C. dispar* collected from northeastern Thailand and compared with Indian material, from where this species was originally described. A molecular approach was used to screen for trypanosome. High genetic diversity was found within Thai *C. dispar* specimens with maximum 3.10% intraspecific genetic divergence due to the existence of two cryptic genetic lineages. Because these lineages coexist geographically, this indicates some degree of isolation, or the early stage of speciation. Phylogenetic analyses between Thai and Indian *C. dispar* populations revealed that they are genetically clearly distinct with minimum genetic divergence of 2.59%. A molecular species delimitation analysis supported that they belong to different species. Molecular screening of trypanosomes revealed that 20 of 90 specimens were positive and 16 of these were successfully sequenced. Based on sequence similarity, all were belonging to *Trypanosoma theileri* complex detected in cattle, the first report of this parasite in *C. dispar*. Phylogenetic analyses revealed that they belonged to two lineages (TthI and TthII) of this protozoa, corresponding to the occurrence of this parasite found in cattle in Thailand.

Keywords: Deer fly; DNA barcode; insect vector; *Trypanosoma theileri* complex.

INTRODUCTION

The family Tabanidae comprises 4,455 species assigned to 144 genera (Morita *et al*., 2016). Many species are vectors of pathogens transmitted to humans and other animals. The significant pathogens include viruses (e.g. equine infectious virus, bovine leucosis virus, vesicular stomatitis virus), filarial nematodes (e.g. *Loa loa*, *Elaeophora schneideri*, *Dirofilaria roemeri*), protozoa (*Haemoproteus metchinikovi*, *Trypanosoma evansi*, *T. theileri*) and bacteria (e.g. *Bacillus anthracis*, *Anaplasma marginale*) (Baldacchino *et al*., 2014; Mullens, 2019). Even without disease agent transmission, biting by these insects can also have significant impact on livestock by reducing weight gain, milk yield and feed-utilization efficiency which cause economic loss (Hansens, 1979; Mullens, 2019).

The genus *Chrysops* Meigen is commonly known as deer fly and it belongs to the subfamily Tabaninae of the Tabanidae. This genus comprises 286 species (Morita *et al*., 2016) and many are vectors of pathogens causing diseases in humans and other animals. The most significant disease for which deer flies are vectors is loiasis caused by filarial nematode, *Loa loa*, that affect 13 million people in western Africa (Morita *et al*., 2016). The significant vector species are *Chrysops dimidiatus* Wulp and *C. silaceus* Austen (Baldacchino *et al*., 2014; Morita *et al*., 2016; Mullens, 2019).

Chrysops dispar Fabricius is one of the significant vectors of *Trypanosoma evansi* (Krinsky, 1976; Burger & Chainey, 2000). In addition, this deer fly species also transmits the bacterium *Pasteurella multocida*, the causative agent of buffalo sickness (Baldacchino *et al*., 2014). *Chrysops dispar* was originally described from India (Burger & Chainey, 2000) and is among the most widespread species of the genus *Chrysops*, being recorded from Pakistan, India, throughout the Oriental region to eastern China and to the Philippines in the south (Burger & Chainey, 2000). In Thailand, this species is geographically widespread, having been recorded throughout the country (Changbunjong *et al*., 2020; Phetkarl *et al*., 2023; Thinnabut *et al*., 2024).

Information of genetic diversity of vector species is crucial for understanding disease epidemiology as well as implementation of efficient control and prevention (Tabachnick & Black, 1995; McCoy, 2008). This information is particularly important for species that are geographically widespread, distributed across different biogeographic regions. Large geographic isolation along with different environmental conditions potentially drives genetic differentiation to the level that the different population may be considered as different species. Therefore, it is necessary to assess genetic differentiation and species status from populations that are

Table 1. Sampling locations, number of specimens, the GenBank accession numbers of the cytochrome c oxidase I (COI) sequences of *Chrysops dispar* and number of specimens used for molecular detection of trypanosomes in this study

Location (code)	Coordinate	Elevation (m)	n for COI (accession no.)	n for trypanosome detection (positive)	Collection date
1. Prangku, Si sa ket (SR)	14.829744, 104.059484	147	42 (PQ198559-PQ198600)	77 (19)	26 Aug 2023
2. Na Dun, Maha Sarakham (MK)	15.696646, 103.225751	157	2 (PQ198601-PQ198602)	2 (0)	2 Sep 2023
3. Selaphum, Roi Et (RE1)	16.182767, 103.887268	154	3 (PQ198603-PQ198605)	3 (0)	9 Sep 2023
4. Phon Thong, Roi Et (RE2)	16.181510, 103.937225	140	3 (PQ198606-PQ198608)	3 (0)	12 Sep 2023
5. Non Sang, Nong Bua Lamphu (NL)	16.823467, 102.568600	194	1 (PQ198609)	2 (0)	23 Sep 2023
6. Waritchaphum, Sakon Nakhon (SK)	17.238843, 103.573124	198	3 (PQ198610-PQ198612)	3 (1)	6 Oct 2023
Total			**54**	**90 (20)**	

substantively isolated geographically because it can be related to the vector-pathogen competency (McCoy, 2008; Powell, 2018).

Genetic studies can also be used to support traditional morphological taxonomy particularly for species with limited morphological diagnostic characters among closely related species. Morphologically, *C. dispar* is similar to some other species such as *C. indianus* Ricardo and *C. flaviventris* Macquart. Intraspecific variations of morphological characteristics have also been reported such as coloration of scutellum and legs (Burger & Chainey, 2000). This can challenge traditional (morphology-based) identification. Furthermore, some diagnostic characters of deer flies such as the antenna and wings can be easily damaged during specimen collection making their morphological identification problematic (Changbunjong *et al*., 2020). Therefore, DNA barcoding is preferable for assisting species identification. A study in India revealed two cryptic genetic lineages of *C. dispar* with very high (6.34%) intraspecific genetic divergence based on the mitochondrial cytochrome c oxidase I (COI) (Banerjee *et al*., 2015). A DNA barcoding study of *C. dispar* in Thailand found relatively high diversity (maximum 2.65%) in *C. dispar* compared to other species of the genus *Chrysops* (Changbunjong *et al*., 2020). In this study, we examined genetic diversity of *C. dispar* collected from cattle pens in the northeastern region of Thailand which had not been studied before. We also used a molecular approach to screen trypanosomes in the *C. dispar*.

MATERIALS AND METHODS

Ethics approval

This study was approved by the Institutional Animal Care and Use Committee of Mahasarakham University (Approval No. IACUC-MSU-53/2023).

Specimen collections and identification

Specimens of *C. dispar* were collected from six locations in Northeastern Thailand between August and October 2023 (Table 1 and Figure 1). The adult specimens of deer flies were collected using a hand-net swept around cattle. Adult flies were also collected by hand directly once the fly had been attracted to cattle. Specimens were preserved in 80% ethanol and stored at -20°C until use. Species were identified using the keys and descriptions of Burger & Chainey (2000). An example of *C. dispar* from northeastern Thailand used in this study was shown in Figure 2.

Molecular analysis

In total, 90 specimens were used for molecular analysis. All of these were used for molecular detection of *Trypanosoma* and 54 were used for COI sequence study (Table 1). For each individual specimen, only head and thorax were used for DNA

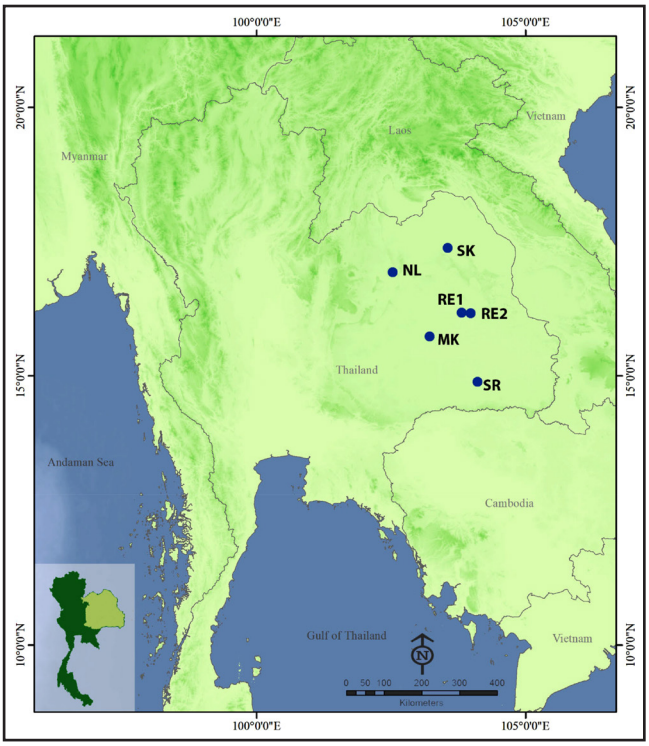

Figure 1. Sampling locations of *Chrysops dispar* from northeastern Thailand used in this study. Details of each sampling sites are given in Table 1.

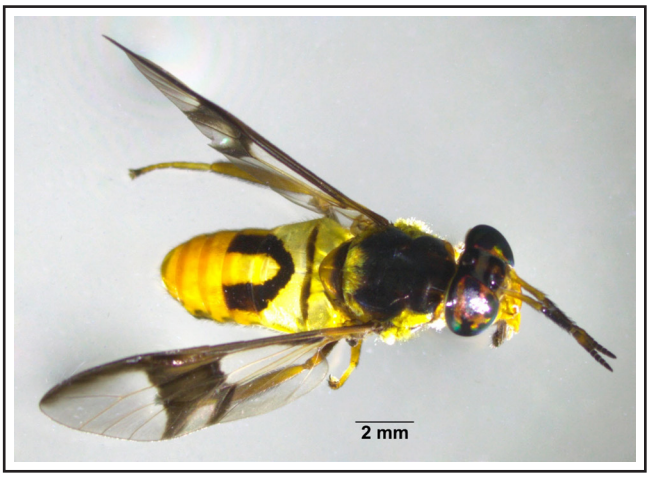

Figure 2. Female of *Chrysops dispar* from northeastern Thailand used in this study.

extraction. DNA was extracted using the GF-1 Nucleic Acid DNA extraction kit (Vivantis Technologies Sdn. Bhd., Malaysia). The cytochrome oxidase I (COI) gene was amplified using the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAG GGTGACAAAAAATCA-3') (Folmer *et al*., 1994). The PCR reaction conditions followed Tangkawanit *et al*. (2018). PCR products were checked with 1% agarose gel electrophoresis and were purified using the PureDireX PCR CleanUp & Gel Extraction kit (Bio-Helix, Taiwan, China) and then sequenced at ATCG Company Limited (Thailand Science Park, Pathumthani, Thailand) using the same primers as for PCR.

For molecular detection of *Trypanosoma*, the nested PCR method of Cox *et al*. (2005) was used to amplify a DNA fragment (approximately 1,000 bp) containing the ITS1/5.8S/ITS2/LSU rRNA region. The primers ITS1 (5'-GATTACGTCCCTGCCATTTG-3') and ITS2 (5'-TTGTTCGCTATCGGTCTTCC-3') (Cox *et al*., 2005) were used in the first round and ITS3 (5'-GGAAGCAAAAGTCGTAACAAGG-3') and ITS4 (5'-TGTTTTCTTTTCCTCCGCTG-3') (Cox *et al*., 2005) were used in the second round.

PCR products were checked and purified using the same methods as for the COI gene. Purified PCR products were sequenced at ATCG Company Limited (Thailand Science Park, Pathumthani, Thailand) using the second round PCR primers.

Data analysis

Sequences were checked for quality using the "Edit/View sequencer file" option in MEGA X (Kumar et al., 2018). In total, 54 COI sequences of *C. dispar* were obtained in the present study (accession nos. PQ198559-PQ198612). To compare the COI sequences in this study with those reported in BOLD, additional sequences reported from Thailand (n=9) (accession nos. MN934077–MN934085) (Changbunjong *et al*., 2020), India (n=15) (accession nos. KM111666–68, KM111674–75, KM111677, KM111679–80, KM111682–83, KM111685, KM111708, KM111711, KM111715) (Banerjee *et al*., 2015) plus one (accession no. KM243494) with unknown country of the origin (Morita *et al*., 2016) were retrieved and were included in the data analyses. Intraspecific genetic divergence was calculated using the uncorrected p-distance in TaxonDNA (Meier *et al*., 2006). Genetic relationships between COI sequences of *C. dispar* were inferred using neighbor-joining (NJ) and maximum likelihood (ML) methods in MEGA X (Kumar *et al*., 2018). Branch support was estimated using 1000 bootstrapping replications.

Because there are seven BINs for *C. dispar* recorded in BOLD (BOLD:ACO2623, BOLD:ACO2638, BOLD:ACS3965, BOLD:ACS3966, BOLD:ACS5139, BOLD:ACS7088, BOLD:AEC1929 (https://www.boldsystems.org), we therefore used the identification engine tool in BOLD (https://www.boldsystems.org/index.php/IDS_OpenIdEngine) (Ratnasingham & Hebert, 2013) (accessed on 15 February 2024) to identify BIN of specimens used in this study. In addition, we also used Assemble Species by Automatic Partitioning (ASAP; Puillandre *et al*. 2021) to examine cryptic diversity within *C. dispar*. The ASAP analysis was performed in the web server (https://bioinfo.mnhn.fr/abi/public/asap/#) (accessed 10 August 2024), using the p-distance as a model for genetic distance calculation. The ITS1/5.8s rRNA/ITS2 sequences of trypanosomes were compared with those reported in the NCBI GenBank, using the Basic Local Alignment Search Tool (BLAST).

RESULTS

Genetic diversity of *Chrysops dispar*

Intraspecific genetic divergence varied between 0.00% and 3.10% within specimens obtained in the present study. However, there was relatively high intraspecific genetic divergence on account of one specimen (accession no. PQ198580). If this specimen was omitted, maximum intraspecific genetic divergence was lowered to 2.41%. When sequences from the public database were included, there was much greater diversity with maximum intraspecific genetic divergence of 6.21% amongst Indian specimens. Specimens obtained from present study compared with Indian specimens also showed high genetic divergence with minimum value of 2.59%.

Identification in BOLD revealed that sequences obtained in the present study belonged to BOLD:ACS7088 with five sequences (MN934079, MN934081, MN934083, MN934084, MN934085) from Thailand recorded in Genbank and one sequence (KM243494) from an unknown locality. However, one sequence (PQ198580) had no match BIN in BOLD. Species delimitation using ASAP delimited these specimens into five species (Figure 3). All of specimens in the present study assigned to a single species along with the other sequences reported from Thailand by ASAP analysis. The Indian specimens were treated as representing three species which were different from our specimens.

Phylogenetic tree analyses revealed similar tree topologies, therefore, only the NJ tree is showed (Figure 3) and this revealed clades that were in good agreement with BIN assignment (Figure 3). All of our specimens but one belonged to a clade of specimens of BIN: BOLD:ACS7088. A specimen of the present study formed a clade with four sequences, also from Thailand. These two clades had a minimum genetic divergence of 1.90%. Two other clades that also contained deep divergent within each clade were all specimens from India.

Molecular detection of *Trypanosoma*

Molecular detection of *Trypanosoma* found that 20 from 90 specimens (prevalence = 22%) were positives (Table 1). However, only 16 were successfully sequenced (accession nos. PQ206242–PQ206257). Comparisons of the sequences obtained from *C. dispar* revealed that all were belong to the *Trypanosoma theileri* complex with e"97% sequence similarity (Table 2). All top hit sequences were *T. theileri* complex detected in cattle (Table 2). Phylogenetic analyses revealed that *T. theileri* complex found in *C. dispar* belonged to two different lineages (TthI and TthII) of this parasite (Figure 4). The majority (13 of 16) of the *T. theileri* sequences found in *C. dispar* from Thailand belonged to lineage TthI.

DISCUSSION

Geographically widespread species facing diverse environmental conditions are more likely to be a complex of species (Adler & McCreadie, 1997). Cryptic genetic diversity has been reported even within Indian populations of *C. dispar*. Very high (6.34%) maximum intraspecific genetic divergence corresponded with two deep divergent lineages that were proposed as cryptic species (Banerjee *et al*., 2015). Comparisons of Thai specimens in this study and those reported by Changbunjong *et al*. (2020) with Indian *C. dispar* revealed that they are different with minimum genetic distance of 2.59%. This level of genetic differentiation falls within the range of intraspecific genetic divergence of Tabanidae (Banerjee *et al*., 2015; Changbunjong *et al*., 2018, 2020; Votýpka *et al*., 2019) in which interspecific genetic divergence varies between 4.29%–13.47% (Banerjee *et al*., 2015; Changbunjong *et al*., 2020). However, very low (1.5%) or no interspecific genetic divergence has also been reported for some species of Tabanidae (Cywinska *et al*., 2010; Votýpka *et al*., 2019; Changbunjong *et al*., 2018). The ASAP and BIN assignment in BOLD treated Thai and Indian specimens of *C. dispar* as different species. Because *C. dispar* was described from India, although the exact type locality is unknown (Burger & Chainey, 2000), specimens recorded from India are more likely to be true *C. dispar*. Morphologically, *C. dispar* in Thailand (Figure 2) agrees well with descriptions provided by Burger & Chainey (2000). However, we have found some morphological variations compared to the descriptions. According to Burger & Chainey (2000), the scutellum of *C. dispar* is yellow-brown or brown, sometimes darkened anteriorly. The scutellum of the

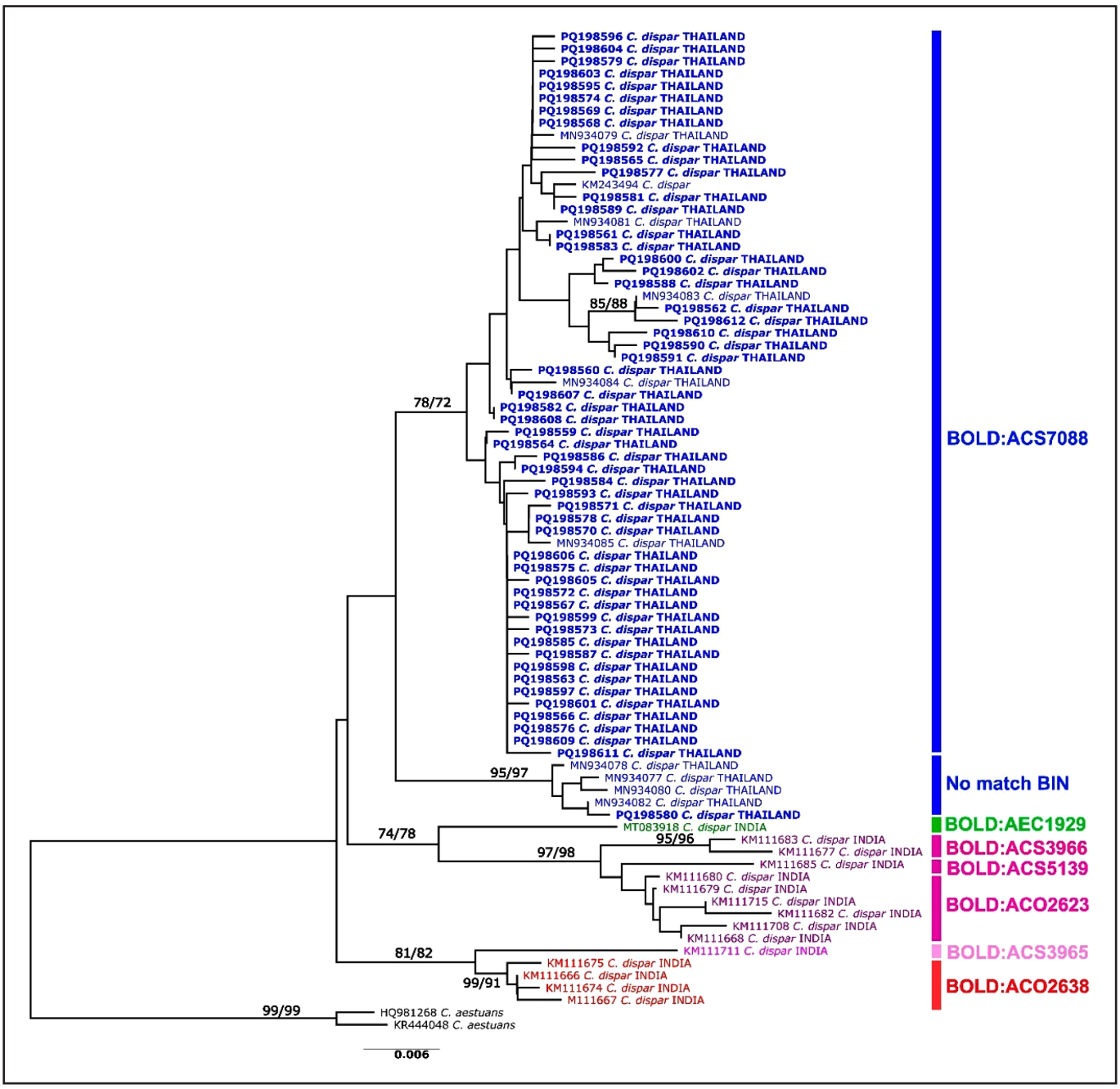

Figure 3. Neighbor-joining tree of *Chrysops dispar* based on 54 COI sequences obtained in this study (bold) and 25 from GenBank. Bootstrap values for NJ and ML analyses are shown above the branch. Color indicates species delimited by ASAP and vertical bars indicate the BIN assignment.

specimens used in this study was yellow-brown with 1/3 to 1/2 darkened anteriorly. In addition, the inverted v-shaped marking of all specimens used in this study extends posteriorly onto only tergite 3, similar to *C. indianus* (Burger & Chainey, 2000), but those of *C. dispar* often extend to tergite 4. However, the wing crossband of Thai *C. dispar* is divided posteriorly resembling the descriptions of this species and different from *C. indianus* in which it is not divided on the margin (Burger & Chainey, 2000) (Figure 2). Further investigation using additional genetic markers such as those from nuclear genes and in-depth morphological characteristic are needed to test the species status of *C. dispar* from Thailand.

Cryptic genetic diversity was also revealed within Thai specimens of *C. dispar*. Previous study in Thailand revealed much lower (1.83%) maximum intraspecific genetic divergence despite specimens being collected from geographically widespread localities within the country (Changbunjong *et al*., 2020). Our specimens were collected from six locations, all in the northeastern region, showed greater genetic divergence with maximum intraspecific genetic divergence of 3.10%. This relatively high genetic diversity is due to inclusion of a divergent haplotype that belongs to a different genetic clade from other members obtained in the present study. This divergent haplotype was clustered with four specimens, also from Thailand, reported by Changbunjong *et al*. (2020), with genetic divergence between these two Thai *C. dispar* clade of 1.90%. The two divergent clades within Thai *C. dispar* were not geographically associated. A specimen that was genetically different from all others obtained in the present study was from a location (SR) where 42 specimens were included in this study. Similarly, three specimens from the same location (Kanchanaburi) reported by Changbunjong *et al*. (2020) also belong to different clades, one in BOLD:ACS:7088 clade with our majority specimens and two in another clade with our single divergent haplotype. The existence of genetically divergent lineages within a population suggests the possibility of reproductive isolation (Hausdorf & Hennig, 2020). The relatively low (1.90%)

Table 2. Top BLAST hit results of *Trypanosoma* detected in *Chrysops dispar* from Thailand based on the ITS1/5.8S/ITS2 sequences

GenBank accession nos. of trypanosome from *C. dispar*	Top BLAST (% similarity)	GenBank accession nos.	Host/Country	Reference
PQ206242	*T. theileri* strain: Esashi 9 (98%)	AB569249	Bos Taurus/ Japan	Hatama *et al*. (2007)
PQ206245	*T. theileri* strain Kor_M_41 (97%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206250	*T. theileri* strain Kor_M_41 (97%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206248	*T. theileri* strain Kor_M_41 (97%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206243	*T. theileri* strain Kor_M_41 (98%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206244	*T. theileri* strain Kor_M_41 (98%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206249	*T. theileri* strain Kor_M_41 (98%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206251	*T. theileri* strain Kor_M_41 (98%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206252	*T. theileri* strain Kor_M_41 (97%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206253	*T. theileri* isolate TthQ71 (97%)	OQ341211	Cattle/ Ecuador	Chávez-Larrea *et al*. (2023)
PQ206254	*T. theileri* strain Kor_M_41 (97%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206247	*T. theileri* strain Kor_M_41 (98%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206255	*T. theileri* strain Kor_M_41 (98%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206246	*T. theileri* strain Kor_M_41 (98%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206256	*T. theileri* strain Kor_M_41 (97%)	PP188045	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)
PQ206257	*T. theileri* strain 77 (97%)	OR973752	Cattle/ Côte d'Ivoire	Ekra *et al*. (2024)

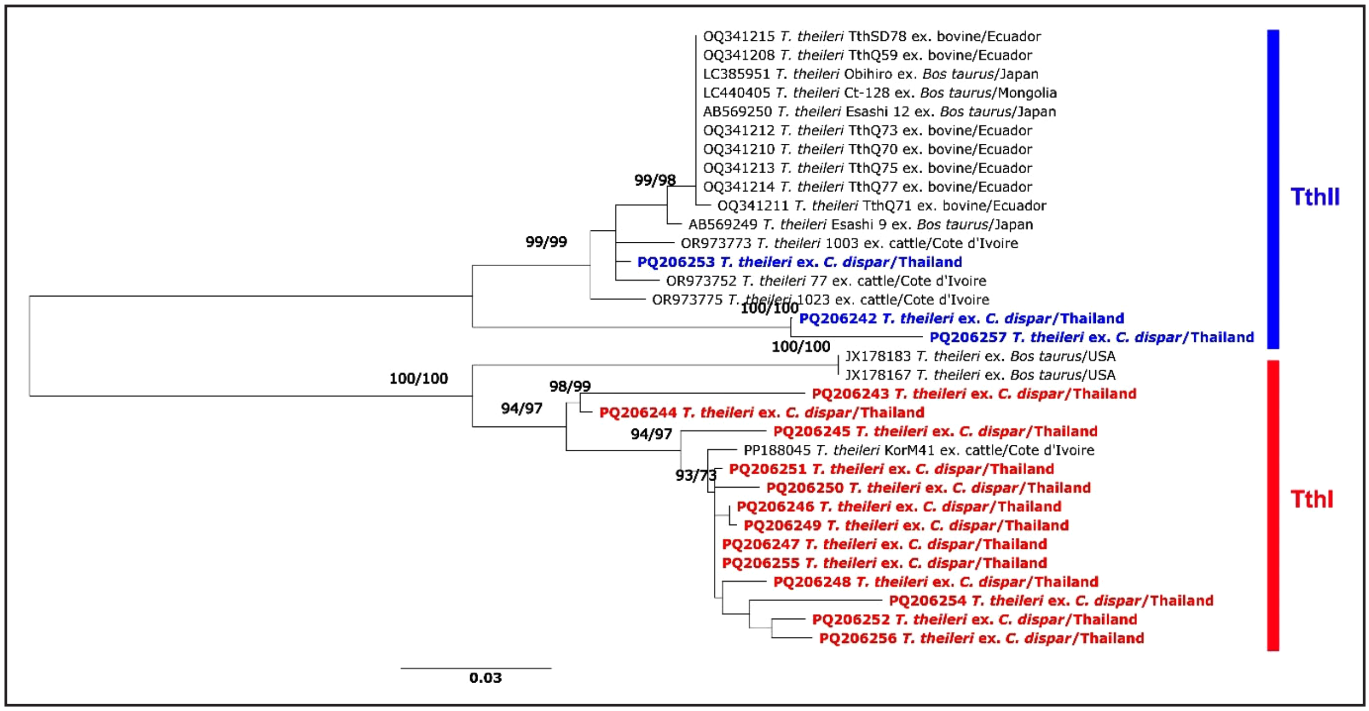

Figure 4. Maximum likelihood tree inferred from the ITS1/5.8S rRNA/ITS2 sequences of *Trypanosoma theileri* detected in *Chrysops dispar* (red) in this study and closely related strains reported in GenBank. Bootstrap values for ML and NJ analyses are shown above the branch. Names of vertebrate hosts or invertebrate vectors and country of origin are listed following the GenBank accession numbers.

sequence divergences that fall within the range of intraspecific variation of Tabanidae (Banerjee *et al*., 2015; Changbunjong *et al*., 2018, 2020; Votýpka *et al*., 2019) possibly indicate an early stage of isolation.

Tabanid flies can transmit *Trypanosoma* biologically and mechanically (Baldacchino *et al*., 2014). In this study we found that *C. dispar* in Thailand can be a vector of *Trypanosoma theileri* complex. Among 90 specimens screening for this protozoan, 20 were positive (prevalence = 22%) and 16 were successfully for sequenced. All of these 16 sequences were belong to the *T. theileri* complex detected in cattle. Phylogenetic analysis indicated that both lineages (TthI and TthII) of *T. theileri* complex (Rodrigues *et al*., 2010) were found in this deer fly species although the lineage TthI is more common (13 of 16). This finding agrees with a recent study that found both lineages of *T. theileri* complex in cow from Thailand (Arnuphapprasert *et al*., 2024). Many species of the family Tabanidae including those of the genus *Chrysops* are vectors of *T. theileri* complex (Votýpka *et al*., 2019; Turɒinaviɒiene *et al*., 2024). However, there is no report that *C. dispar*, a common and geographically widespread species is a vector. Therefore, our finding in this study is the first record of this deer fly species as a vector of *T. theileri* complex.

In conclusion, we found relatively high genetic diversity within Thai *C. dispar* corresponding with the two genetically divergent lineages. Because members of these lineages occur in the same population, geographic or ecological factors were not barriers to gene flow explaining this genetic differentiation. Instead, it is more likely that they possibly represent two morphologically similar species or populations in the process of an early stage of isolation. Further study using additional genetic markers from nuclear loci

and morphological examination will be required to test these hypotheses. We also found in this study that *C. dispar* is a vector of *T. theileri* complex which is the first report for this deer fly species. There are several other pathogens that this biting fly might be a vector for but this possibility has not yet been examined. Therefore, further investigation is necessary to screen other disease-causing agents for which this common and abundant deer fly species could be a vector.

ACKNOWLEDGEMENTS

This study was financially supported by Mahasarakham University (Grant no. 6708011).

We would like to thank Adrian Plant for comment and suggestion and English improvement of the earlier version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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