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Genetic diversity and population structure of Stomoxys calcitrans (Diptera: Muscidae) inferred from cox1 sequences in Tumed Left Banner, Inner Mongolia

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Abstract

Background *Stomoxys calcitrans*, the stable fly, is a blood-feeding muscid that causes direct production losses and mechanically transmits livestock pathogens. Despite its economic importance in northern China's dairy belt, its mitochondrial genetic diversity, population structure, and demographic history remain undescribed at fine spatial scales, leaving farm-level and area-wide control decisions without a genetic basis.

Methods Partial mitochondrial *cox1* sequences (658 bp) were obtained from 155 specimens collected on nine cattle farms in Tumed Left Banner, Inner Mongolia. Sequences were identified against GenBank and BOLD and analysed using maximum-likelihood phylogeny, nucleotide composition, diversity indices, pairwise F_{ST}, AMOVA, neutrality tests (Tajima's *D* and Fu's F_s), mismatch distribution, and statistical parsimony networks.

Results All 155 sequences fell within the *S. calcitrans* clade. The regional sample contained 27 segregating sites and 19 haplotypes, with a haplotype diversity of 0.648, a nucleotide diversity of 0.010, and an A+T content of 68.25%. Pairwise F_{ST} revealed limited differentiation (32 of 36 comparisons non-significant), and AMOVA attributed 96.66% of the variation to within-population differences (global F_{ST} = 0.033). Tajima's *D* was significantly negative only for Farm 1 (−2.278, p = 0.001). Haplotype networks revealed two central shared haplotypes connecting the local population to reference sequences from Asia, Europe, Africa and North America.

Conclusions The nine farms form a single gene pool in which dispersal, assisted by livestock and forage movement, overrides local drift. Farm 1 nevertheless retains a detectable recent demographic signal. These results support area-wide, synchronised control rather than isolated farm-level treatments, and provide the first mitochondrial genetic baseline for *S. calcitrans* in northern China.

1 Background

Stomoxys calcitrans (Linnaeus, 1758), commonly known as the stable fly, is a haematophagous dipteran of the family Muscidae that infects livestock worldwide. Stable flies cause direct economic losses through blood-feeding-induced stress, reduced weight gain, and decreased milk production, with annual losses in the United States exceeding USD 2 billion at densities as low as five flies per leg [1]. The fly also serves as mechanical vectors for multiple livestock pathogens. These include viruses (Lumpy skin disease virus and African swine fever virus) [2, 3], bacteria (*Anaplasma marginale* and *Staphylococcus aureus*) [4, 5], and protozoa (*Trypanosoma evansi* and *Besnoitia besnoiti*) [6, 7]. What makes *S. calcitrans* unusually difficult to manage is not any single trait but combination: larvae develop in scattered, often concealed substrates such as wet hay, bedding and manure-soil interfaces [8, 9]; populations respond quickly to insecticide pressure, with knockdown-resistance (*kdr*) alleles recurring across continents [10]; and adults routinely move several kilometres between farms [11]. Control programmes that ignore this

dispersal scale tend to fail, and integrated pest management frameworks must therefore be built on knowledge of how flies actually move between and among farms.

Population genetic analyses of vector insects provide critical insights into their dispersal history, demographic trajectories, and adaptive potential. Genetic diversity indices and haplotype distributions can serve as proxies for historical gene flow and population connectivity, thereby informing whether localized interventions or regionally coordinated control strategies are warranted [12]. Mitochondrial cytochrome *c* oxidase subunit I (*cox1*) is widely used in insect population genetics owing to its maternal inheritance, absence of recombination, and intermediate substitution rate, properties that make it well suited to resolving both deep phylogeographic splits and recent demographic fluctuations in Diptera. Early mitochondrial work on *S. calcitrans* used *cox1* and 16S to date the arrival of stable flies in the New World to within the past 500 years, a Palaearctic origin that implies long-distance transport by livestock [13]. Global surveys have since defined the broad genetic architecture of the species. Dsouli-Aymes et al. sampled five zoogeographical regions with mitochondrial and nuclear markers, recovering two deeply divergent lineages: an Oriental clade isolated since roughly the mid-Pleistocene and a cosmopolitan clade spanning Afrotropical, Palaearctic, Nearctic, Neotropical, and Oceanian populations [14]. They further detected signatures of Late Pleistocene–Early Holocene expansion in the Oriental, Afrotropical, and Palaearctic regions [14]. Subsequent analyses have refined this picture. Tsai et al. placed the principal divergence of the species at 0.32 — 0.47 Mya, identified the Ethiopian region as its most probable origin, and showed that regional populations tend to retain their own genetic signatures even where long-distance movement is suspected [15]. A complementary line of evidence comes from genome-wide markers: amplified fragment length polymorphism surveys recovered only limited genetic variability across the species' global range, a pattern taken to reflect extensive contemporary gene flow [16]. At finer geographic scales, molecular characterization of three *Stomoxys* species in Central Thailand recovered high *cox1* haplotype diversity and evidence of cryptic structuring within the Oriental lineage, consistent with post-bottleneck expansion [17]. Against this background, the literature on Asian *S. calcitrans* remains thin. Much of it has aimed at species identification through barcoding or at the molecular basis of insecticide resistance [10], and fine-scale descriptions of *cox1* diversity, haplotype connectivity and demographic history are still rare for synanthropic muscids in Asia.

Inner Mongolia is home to the largest dairy and beef industrial clusters in China. These farms maintain large, permanent cattle herds and generate year-round accumulations of manure and waste forage, the substrates on which stable flies depend. *S. calcitrans* has been recorded from livestock systems across Inner Mongolia, including camel-rearing areas of the steppe [18] and commercial cattle farms [19]. These records establish that the species is resident rather than sporadic in the region, yet no study has described how its mitochondrial diversity is partitioned among farms or whether those farms exchange flies. Without such data, it is impossible to tell whether outbreaks are driven by local reproduction or by immigration from neighbouring operations—a distinction that determines whether control should be mounted farm by farm or coordinated across the landscape.

We present the first mitochondrial genetic survey of *S. calcitrans* in northern China, based on partial *cox1* sequences from 155 specimens collected on nine cattle farms in Tumed Left Banner. Specifically, we (1) establish a regional baseline of nucleotide composition and diversity by characterising nucleotide diversity (π) and haplotype richness within and among farms; (2) test for population structure and genetic connectivity using pairwise F_{ST}, analysis of molecular variance (AMOVA), and statistical parsimony haplotype networks; (3) infer historical demographic dynamics from neutrality tests (Tajima's D and Fu's F_s) and mismatch-distribution analysis. Together these analyses provide the first genetic baseline for *S. calcitrans* in northern China and supply the empirical basis for deciding whether stable fly management in the region should be organised as farm-level treatments or as an area-wide integrated programme.

2 Materials and methods

2.1 Study area and sample collection

Specimens of *Stomoxys calcitrans* were collected from nine dairy farms in Tumed Left Banner, Hohhot, Inner Mongolia, between August and October 2025. Flies were swept from dairy cattle sheds and the surrounding area with sweep nets between 06:00 and 18:00 on two consecutive days. A total of 155 specimens were obtained. They were killed by freezing at − 20°C and placed separately in 1.5 mL microcentrifuge tubes, which were then transported to the Parasitic Diseases Research Group, Institute of Veterinary Medicine, Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences. There, preliminary species identification was carried out on morphological characters [20]. The number of specimens from each farm was Farm 1, n = 18; Farm 2, n = 9; Farm 3, n = 18; Farm 4, n = 15; Farm 5, n = 23; Farm 6, n = 19; Farm 7, n = 15; Farm 8, n = 18; Farm 9, n = 20.

2.2 DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from two to three legs of each *S. calcitrans* specimen using a Tissue Genomic DNA Extraction Kit (DP304; Tiangen Biotech, China) following the manufacturer's instructions. Each DNA sample was eluted in 50 µL and kept at − 20°C until PCR analysis.

The mitochondrial *cox1* gene was amplified as a ~ 658 bp fragment with the primers LepF1 (5'–ATTCAACCAATCATAAAGATATTGG–3') and LepR1 (5'–TAAACTTCTGGATGTCCAAAAAATCA–3') [21]. Each 50 µL reaction contained 25 µL KOD FLEX™ PCR Master Mix (Code No. KMX-101; TOYOBO Co., Ltd., Osaka, Japan), 1.5 µL of each primer (10 µM), 4 µL of template DNA, and 18 µL ddH_2O. Cycling began with denaturation at 94°C for 2 min, followed by 30 cycles of 98°C for 10 s, 50°C for 10 s, and 68°C for 15 s, and ended with a final extension at 68°C for 5 min. Positive and negative controls were run with every batch.

PCR products were checked on 1% agarose gels stained with Super Red Nucleic Acid Stain (biosharp, Beijing Labgic Technology Co., Ltd., China). Band sizes were confirmed on a UV Gel Imaging Analysis System (Model BG-gdsAUTO320; Beijing Baygene Biotech Co., Ltd., China). Products showing a single

band of about 658 bp were sent to Sangon Biotech (Shanghai) Co., Ltd. for purification and Sanger sequencing.

2.3 Sequence assembly and species identification

Chromatograms of the *cox1* gene were inspected and edited in SnapGene (version 8.2.2; Dotmatics, San Diego, USA; https://www.snapgene.com). Forward and reverse reads were assembled into consensus sequences for downstream analysis. The *cox1* sequences were compared against GenBank using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and against the Barcode of Life Data System (BOLD; http://www.boldsystems.org) to confirm species identity. All *S. calcitrans cox1* sequences generated here were deposited in GenBank under accession numbers QB012740–QB012757 (Table 1).

Table 1
GenBank accession numbers of the *cox1* sequences of *Stomoxys calcitrans* obtained in this study.

Farms	Study sites	n	GenBank accession numbers
Farm 1	Tumd Left Banner	18	QB012740 — QB012757
Farm 2	Tumd Left Banner	9	QB012758 — QB012766
Farm 3	Tumd Left Banner	18	QB012767 — QB012784
Farm 4	Tumd Left Banner	15	QB012785 — QB012799
Farm 5	Tumd Left Banner	23	QB012800 — QB012822
Farm 6	Tumd Left Banner	19	QB012823 — QB012841
Farm 7	Tumd Left Banner	15	QB012842 — QB012856
Farm 8	Tumd Left Banner	18	QB012857 — QB012874
Farm 9	Tumd Left Banner	20	QB012875 — QB012894

Phylogenetic analysis used the maximum likelihood method with the T92 + G model in MEGA 11, and node support was assessed with 1 000 bootstrap replicates. *Musca domestica* (PV460693) and *Lucilia sericata* (PZ111940) served as the outgroup. Reference sequences of *S. calcitrans* (KC960711, KM497259, PV460660, OR742413, PX285788 and PP587241) and four closely related species were retrieved from GenBank, namely *Stomoxys sitiens* (PV664325 and PP587233), *Stomoxys bengalensis* (OP975956 and OP975952), *Stomoxys indicus* (KC960735 and KC960734) and *Stomoxys niger* (PV664320). The tree was visualized in iTOL (https://itol.embl.de) [22].

2.4 Nucleotide composition and genetic diversity analysis

Nucleotide composition (A, T, C and G percentages) was calculated for all *S. calcitrans cox1* sequences from Tumed Left Banner, Inner Mongolia, using BioDataTools (www.biodatatools.top). Genetic diversity indices were calculated in DnaSP version 6.12.03 [23] using the *cox1* sequences. These indices were the

number of segregating sites (S), nucleotide diversity (π), number of haplotypes (h), haplotype diversity (H_d), and average number of nucleotide differences (k).

2.5 Population genetic structure and molecular variance

Pairwise genetic differentiation among the nine populations was quantified as the fixation index (F_{ST}) in DnaSP v6.12.03 using the *cox1* sequences. Partitioning of genetic variation was then examined by analysis of molecular variance (AMOVA) in Arlequin v3.5.2.2 [24]. A standard AMOVA was first applied to the nine farms treated as a single group of populations. A hierarchical AMOVA followed, with Farm 1 as Group A and Farms 2 to 9 as Group B, to test whether the between group component was significant. Significance of all variance components and *F*-statistics was assessed from 1000 permutations.

2.6 Neutrality tests and mismatch distribution

Tajima's *D* and Fu's F_s were calculated in DnaSP v6.12.03 to test for departure from neutrality. Mismatch distributions were then generated in Arlequin v3.5.2.2 under a sudden expansion model. Goodness of fit between the observed and the expected distribution was assessed with the sum of squared deviations (SSD) and Harpending's raggedness index (*r*), and significance was obtained from 1000 parametric bootstrap replicates.

2.7 Haplotype network

Haplotype relationships were visualised with a statistical parsimony network constructed in PopART v1.7 [25], following the method of Templeton et al. [26]. The 658 bp *cox1* alignment was used as it was, with the default epsilon of 0. Two datasets were analysed. The local dataset contained the 155 sequences from the nine farms. The global dataset combined these 155 sequences with 46 reference sequences from GenBank, giving 201 sequences in total. Connections between haplotypes were retained at the 95% parsimony confidence limit, and every sequence in both datasets was joined into a single connected network under this limit.

3 Results

3.1 Molecular identification and phylogenetic analysis

A total of 155 *cox1* sequences of *Stomoxys calcitrans*, each 658 bp in length, were obtained and used for molecular analysis. Comparison against the GenBank and BOLD reference libraries showed that all sequences shared greater than 99% similarity with *S. calcitrans* reference sequences. The molecular results were consistent with the morphological identification of all specimens.

To further verify that the samples were *S. calcitrans*, a circular maximum likelihood tree was constructed from the partial *cox1* sequences (Fig. 1). Reference sequences of *S. calcitrans* were included, together with four closely related congeners (*S. bengalensis*, *S. sitiens*, *S. niger* and *S. indicus*) and two dipteran outgroups, *Musca domestica* and *Lucilia sericata*. The sequences from this study (pure red stars) fell

inside the *S. calcitrans* clade, shown as a yellow green range, and were mixed among the conspecific references instead of grouping with any congener. Each of the four congeners formed its own branch, and the two outgroups branched basally, outside the *Stomoxys* ingroup. No sequence from this study appeared outside the *S. calcitrans* clade. The tree therefore placed specimens from the nine cattle farms in Tumed Left Banner within *S. calcitrans*, and separated this species from its congeners on the partial *cox1* fragment used here.

3.2 Nucleotide composition and genetic diversity

The nucleotide composition of the *cox1* sequences from the nine farms in Tumed Left Banner is summarised in Table 2. All farms showed an AT bias. Thymine was the most frequent base, ranging from 39.52% on Farm 1 to 39.70% on Farm 4, with a mean of 39.61%. Adenine followed, between 28.54% and 28.76%, with a mean of 28.64%. Cytosine and guanine were less frequent, averaging 15.37% (range 15.26% to 15.50%) and 16.38% (range 16.31% to 16.44%). The A + T content averaged 68.25% against 31.75% for G + C. Differences among farms were small. The largest range for any base was 0.18 percentage points for thymine, and no farm departed from the overall pattern.

Table 2
Nucleotide composition (%) of *cox1* sequences of *Stomoxys calcitrans* from Tumed Left Banner, Inner Mongolia.

Farms	**% Average nucleotide composition**			
	Thymine (T)	**Cytosine (C)**	**Adenine (A)**	**Guanine (G)**
Farm 1	39.52	15.50	28.54	16.44
Farm 2	39.67	15.26	28.76	16.31
Farm 3	39.62	15.39	28.56	16.43
Farm 4	39.70	15.27	28.67	16.36
Farm 5	39.58	15.44	28.58	16.40
Farm 6	39.68	15.26	28.72	16.34
Farm 7	39.61	15.37	28.65	16.37
Farm 8	39.55	15.44	28.60	16.41
Farm 9	39.64	15.29	28.73	16.33
Average	39.61	15.37	28.64	16.38

Genetic diversity indices based on the *cox1* sequences are summarised in Table 3. The 155 sequences contained 27 segregating sites and 19 haplotypes, giving a haplotype diversity of 0.648 ± 0.040, a nucleotide diversity of 0.010 ± 0.001 and an average of 6.235 nucleotide differences. Haplotype diversity at the farm level ranged from 0.562 to 0.889, and the highest value was recorded on Farm 2. Nucleotide

diversity ranged from 0.004 ± 0.002 on Farm 1 to 0.014 ± 0.002 on Farm 2, and k from 2.523 on Farm 1 to 9.056 on Farm 2. Farm 1 and Farm 8 had the lowest haplotype diversity, 0.569 and 0.562, and their standard deviations of 0.138 and 0.134 overlapped. Farm 1 also had the lowest nucleotide diversity and the lowest *k*, although it carried seven haplotypes, the joint highest count among the nine farms.

Table 3
Genetic diversity based on *cox1* sequences of *Stomoxys calcitrans* from Tumed Left Banner, Inner Mongolia.

Farms	**n**	**S**	**π (± SD)**	**h**	**H_d (± SD)**	**k**
Farm 1	18	20	0.004 ± 0.002	7	0.569 ± 0.138	2.523
Farm 2	9	19	0.014 ± 0.002	6	0.889 ± 0.091	9.056
Farm 3	18	19	0.009 ± 0.002	7	0.739 ± 0.099	6.248
Farm 4	15	18	0.013 ± 0.002	5	0.790 ± 0.073	8.267
Farm 5	23	18	0.007 ± 0.002	6	0.569 ± 0.114	4.759
Farm 6	19	18	0.012 ± 0.001	5	0.731 ± 0.080	7.988
Farm 7	15	17	0.010 ± 0.002	5	0.638 ± 0.129	6.381
Farm 8	18	17	0.007 ± 0.003	6	0.562 ± 0.134	4.523
Farm 9	20	16	0.011 ± 0.001	4	0.574 ± 0.090	7.274
Total	155	27	0.010 ± 0.001	19	0.648 ± 0.040	6.235

Note: n, number of sequences; S, number of segregating sites; π, nucleotide diversity; h, number of haplotypes; H_d, haplotype diversity; k, average nucleotide differences.

3.3 Genetic differentiation and population structure

Pairwise F_{ST} values estimated with the Tajima-Nei distance model are given in Table 4. Of the 36 pairwise comparisons among the nine farms, 32 were not significant, with values from 0.000 to 0.086, whereas the four significant comparisons ranged from 0.213 to 0.279. Farm 1 differed from four farms, with F_{ST} of 0.279 against Farm 2, 0.228 against Farm 6, 0.214 against Farm 4 and 0.213 against Farm 9, all significant at $p < 0.05$. No other comparison was significant. Among the remaining pairs, most values were zero, including all comparisons among Farms 3, 5 and 7, and those between Farm 2 and Farms 4, 6, 7 and 9.

Table 4
Pairwise F_{ST} among nine *Stomoxys calcitrans* populations from Tumed Left Banner based on *cox1* sequences.

Farm 1	**Farm 1**	**Farm 2**	**Farm 3**	**Farm 4**	**Farm 5**	**Farm 6**	**Farm 7**	**Farm 8**	**Farm 9**
	-								
Farm 2	0.279*	-							
Farm 3	0.053	0.028	-						
Farm 4	0.214*	0.000	0.004	-					
Farm 5	0.006	0.010	0.000	0.066	-				
Farm 6	0.228*	0.000	0.032	0.000	0.086	-			
Farm 7	0.080	0.000	0.000	0.000	0.000	0.000	-		
Farm 8	0.000	0.093	0.000	0.065	0.000	0.083	0.000	-	
Farm 9	0.213*	0.000	0.033	0.000	0.070	0.000	0.000	0.066	-

Note: Values are pairwise F_{ST} estimated with the Tajima-Nei distance model from 10 000 permutations. Asterisks indicate significance at $p < 0.05$. Negative values are statistically equivalent to zero and are reported as 0.00000.

The AMOVA results are given in Table 5. The nine populations comprised 155 sequences and carried a total variance of 3.220. Of this total, 96.66% was distributed within populations and 3.34% among populations, the among-population component contributing 0.108 (8 degrees of freedom) and that within populations a variance of 3.112 with 146 degrees of freedom. The overall F_{ST} was 0.033, and the permutation test was not significant ($p > 0.05$). Because the global test could not resolve whether the small among population component was shared evenly across all farms, a hierarchical AMOVA was next applied with Farm 1 separated from the remaining eight farms.

Table 5
Analysis of molecular variance of *Stomoxys calcitrans* populations based on *cox1* sequences.

Source of variation	d.f.	Sum of squares	Variance components	% of variation	F_{ST}	*p*-value
Among populations	8	39.641	0.108	3.34	0.033	> 0.05
Within populations	146	454.412	3.112	96.66		
Total	154	494.053	3.220	100.00		

The hierarchical AMOVA grouped Farm 1 as Group A (n = 18) and Farms 2 to 9 as Group B (n = 137), and the results are given in Table 6. Among groups variation accounted for 8.39% of the total variance (V_a = 0.289, F_{CT} = 0.084, p = 0.120), variation among populations within groups for 1.13% (V_b = 0.039, F_{SC} = 0.012, p = 0.348), and variation within populations for 90.47% (V_c = 3.112). The overall F_{ST}, computed as the sum of the among group and within group components over the total variance, was 0.095 (p = 0.116). All probability values were obtained from 1000 permutations. None of the three fixation indices reached significance, and the among-group component remained the larger of the two higher-level components

Table 6
Hierarchical AMOVA of *Stomoxys calcitrans*, with populations grouped as Group A and Group B.

Source of variation	d.f.	Sum of squares	Variance components	% of variation	*F*-statistic	*p*-value
Among groups	1	13.000	0.289 (V_a)	8.390	F_{CT} = 0.084	0.120
Among populations within groups	7	26.419	0.039 (V_b)	1.130	F_{SC} = 0.012	0.348
Within populations	146	454.388	3.112 (V_c)	90.470		
Total	154	493.806	3.440		F_{ST} = 0.095	0.116

Note: Group A comprised individuals from Farm 1; Group B comprised the pooled individuals from Farms 2 to 9. Variance components were designated as V_a (among groups), V_b (among populations within groups), and V_c (within populations). Fixation indices were computed as $F_{CT} = Va/(V_a+V_b+V_c)$, $F_{SC} = V_b/(V_b+V_c)$, and $F_{ST} = (V_a+V_b)/(V_a+V_b+V_c)$. *p*-values were estimated from 1000 permutations.

3.4 Neutrality tests and mismatch distribution

Neutrality tests and the corresponding diversity indices are given in Table 7. Tajima's *D* was negative and significant for Group A (− 2.278, p = 0.001), but positive and not significant for Group B (0.634, p = 0.790, n = 137) and for the pooled sample (0.521, p = 0.756, n = 155). Fu's F_s was positive and not significant in

all three groupings, at 0.187 for Group A (p = 0.560, n = 18), 1.610 for Group B (p = 0.740) and 1.127 for the pooled sample (p = 0.702). Across the nine farms the mean Tajima's D was − 0.374 ± 1.649 and the mean Fu's F_s was 0.975 ± 0.723. The mismatch distributions of the three groupings were unimodal, and neither the sum of squared deviations (SSD) nor Harpending's raggedness index (r) departed from the expansion model, with p values of 0.490, 0.157 and 0.193 for SSD and 0.325, 0.335 and 0.344 for r in Group A, Group B and the pooled sample (Table 8). Significance was confined to Tajima's D of Farm 1, and all other statistics were non-significant.

Table 7
Neutrality tests based on *cox1* sequences of *Stomoxys calcitrans* populations.

Neutrality tests	***V*alue**	**Group A**	**Group B**	**All Farms**	**Mean ± SD**
Tajima's D test	Tajima's D	−2.278	0.634	0.521	−0.374 ± 1.649
	p-values	0.001	0.790	0.756	0.516 ± 0.446
Fu's F_s test	Fu's F_s	0.187	1.610	1.127	0.975 ± 0.723
	p-values	0.560	0.740	0.702	0.667 ± 0.095

Note: Group A comprised individuals from Farm 1; Group B comprised the pooled individuals from Farms 2 to 9. Tajima's D and Fu's F_s were calculated to test for departure from neutrality. p-values were obtained from 10 000 coalescent simulations. Negative values of both statistics indicate an excess of low frequency variants.

Table 8
Mismatch distribution analysis of *Stomoxys calcitrans* populations.

Statistics	**Group A**	**Group B**	**All Farms**	**Mean ± SD**
SSD	0.039	0.092	0.083	0.072 ± 0.028
p-values (SSD)	0.490	0.157	0.193	0.280 ± 0.183
r	0.214	0.135	0.134	0.161 ± 0.046
p-values (r)	0.325	0.335	0.344	0.335 ± 0.010

Note: Group A comprised individuals from Farm 1; Group B comprised the pooled individuals from Farms 2 to 9. SSD, sum of squared deviations; r, Harpending's raggedness index. p-values were estimated from 1000 permutations. Non-significant SSD and r values indicate that the observed distribution does not depart from the sudden expansion model.

3.5 Haplotype networks of local and global populations

The statistical parsimony network of the local populations is shown in Fig. 2. It contained 19 haplotypes. Hap_1 and Hap_10 occupied the centre with the largest circles, each divided into sectors of several colours, and both were shared by most of the nine farms. The other haplotypes were peripheral, each present in one or a few farms and separated from the centre by one or more mutational steps. A single sequence from Farm 3 and another from Farm 2 formed private haplotypes. Farm 1 contributed a sector

to Hap_1 and also carried haplotypes absent from the other farms. Low frequency haplotypes radiated from Hap_1 and Hap_10, giving the network a star-like shape.

The global network including reference sequences from other regions is shown in Fig. 3. It contained 20 haplotypes. Hap_1 was the largest node with 117 sequences, 109 from this study and eight from reference populations, and Hap_2 was the second with 53, 39 local sequences and 14 references. Both nodes therefore consisted mainly of sequences from this study. Hap_1 also contained single sequences from Thailand, Canada, Kenya and China, whereas Hap_2 contained five sequences from South Africa, seven from Thailand, two from India and two from China. Five haplotypes were formed only by local sequences. Three further haplotypes were each represented by a single Thai sequence, and the four Gabonese sequences were split between two haplotypes. The Spanish and Portuguese sequences shared one haplotype with five Canadian sequences, the remaining Canadian sequences formed two further haplotypes, and the eight Indian sequences were distributed across five haplotypes.

4 Discussion

Stomoxys calcitrans is a blood-feeding pest of cattle and other livestock whose bites reduce weight gain and milk yield and which mechanically transmits livestock pathogens between herds [1, 27]. In Inner Mongolia the species has received little molecular attention, and the identity of flies caught around dairy units has relied on morphology alone. Morphological identification of Stomoxyini relies on a small number of adult characters, and these overlap between species. Dsouli et al. noted that *S. niger niger* and *S. niger bilineatus* differ only in tibial and tarsal colouration, yet show 8.2% *cox1* divergence [28]. All 155 sequences obtained here fell inside the *S. calcitrans* clade and none grouped with *S. bengalensis*, *S. sitiens*, *S. niger* or *S. indicus*, four species whose ranges overlap with that of the stable fly in Asia and Africa [28, 29]. Only *S. calcitrans* was therefore present across the nine farms, which removes a confounder that would otherwise have inflated every diversity estimate in the dataset. The accessions QB012740 to QB012894 extend the Chinese reference library, which until now has been represented by sequences from Hubei and Guangdong.

The AT content of 68.25%, together with the low proportion of cytosine, matches other muscid mitochondrial genomes and the *cox1* region in particular [28]. No double peaks, internal stop codons or unusual amino acid replacements were detected, and the alignment contained no premature stop codons. Numts are widespread in Diptera, and when they are amplified alongside the target region they produce divergent paralogous copies that are scored as extra haplotypes and raise both haplotype diversity and the number of segregating sites [14]. The 19 haplotypes and 27 polymorphic sites are therefore likely to reflect genuine mitochondrial variation rather than a mixture of orthologous and paralogous copies. This matters because rare variants form the basis of both the diversity indices and the neutrality tests that follow.

Haplotype diversity of 0.648 sits between the 0.773 reported for Thailand and the 0.455 reported for Spain, while nucleotide diversity of 0.010 substantially exceeds both the Thai value of 0.008 and the

Spanish value of 0.001 [30]. The two measures therefore point in different directions. Haplotype diversity reflects how many haplotypes are present and how evenly they are represented, and 19 haplotypes among 155 sequences is a lower proportion than the four haplotypes found among 12 Thai flies. Nucleotide diversity weighs the sequence distances between those haplotypes, and the 27 segregating sites recovered here span more variation than the 16 sites found in Thailand and far more than the four found in Spain [30]. The regional population therefore consists of many closely related haplotypes rather than a few divergent ones.

The comparison with Thailand and Spain also marks a difference in spatial scale. Those two populations were separated by intercontinental distance and differed by an F_{ST} of 0.755, with three quarters of the variation distributed between them [30]. The nine farms studied here lie within one banner and gave an F_{ST} of 0.033, with 96.66% of the variation within populations. Structure on the scale of a zoogeographical realm and the absence of structure within a farming landscape are not contradictory. Analyses based on mitochondrial and nuclear sequence data, on amplified fragment length polymorphisms and on microsatellites place the deepest split of the species between the Oriental lineage and all other regions, while finding little differentiation within realms [14, 16, 29, 31]. The local haplotypes fell among the non Oriental references and no private lineage was recovered, which is what a population connected to the wider Palaearctic pool would look like.

The nine farms behaved as a single gene pool. Thirty two of the 36 pairwise comparisons were not significant, only 3.34% of the variance lay among populations, and the global F_{ST} of 0.033 did not differ from zero. Homogeneity of this order has been reported from other local surveys of the species, where samples were taken within a few kilometres of one another [16, 31]. Three processes can produce it. Adults fly well and move several kilometres, with mark release recapture studies documenting dispersal at that scale [32, 33]. Longer movements follow livestock and forage transport, which is frequent in a dairy belt where animals and feed move between units within a season [32]. Finally, larval development takes place in accumulated feed residue and manure, substrates that are abundant on working farms and are removed or renewed during routine cleaning [34]. Repeated removal of these substrates and their recolonisation from neighbouring farms erases the effect of drift within any one unit, which is consistent with 109 of the 155 local sequences occupying a single node of the global network and with the scarcity of private variants.

Farm 1 is the one site where regional homogeneity breaks down. Its haplotype diversity of 0.569 sits at the bottom of the range, Tajima's D of − 2.278 is significant at $p = 0.001$, and its F_{ST} with Farms 2, 4, 6 and 9 ranges from 0.213 to 0.279. Two features point to a recent event rather than long isolation. The population carries little variation yet what it carries is unevenly represented, with an excess of rare variants, and no private lineage was recovered, so the farm has not been diverging in place for long. A founder event involving a few maternal lineages, and a contraction from insecticide pressure or substrate removal, both fit this pattern, and a single mitochondrial locus cannot separate them. Of the neutrality statistics, only Tajima's D supports the interpretation, since Fu's F_s of 0.187 was positive and not significant and the unimodal mismatch distribution with non-significant SSD and raggedness also

occurs in stable populations[35]. The negative *D* could equally arise from selection at a linked site, which the *cox1* fragment alone cannot distinguish from a demographic reduction.

The global network places the local haplotypes within a broader geographical context. Hap_1 and Hap_2, the two largest nodes, connected the local sequences to references from Thailand, Spain, Portugal, South Africa, Gabon, Kenya, Canada, India, Hubei and Guangdong. Hap_1 held 117 sequences (109 local) and Hap_2 held 53 (39 local) alongside references from Thailand, South Africa, India and China. The two central nodes therefore link the Inner Mongolian population to Asian, African and North American sequences rather than defining a lineage of its own. Sharing of identical *cox1* haplotypes between regions this far apart is hard to reconcile with long isolation, and is better explained by transport of livestock and forage over historical time, with the New World populations derived from the Palaearctic after European contact [13]. The branches separating the two Gabonese haplotypes from the central nodes carried many mutational steps, in contrast to the short branches linking the local haplotypes to Hap_1 and Hap_2, so the Gabonese lineages appear to have diverged in relative isolation while the local variants have had little time to accumulate differences. A deep regional lineage such as this also means that a local sample of 155 sequences cannot recover the full history of the species, and that reference coverage from Africa remains too thin to place the Chinese populations precisely.

The genetic continuity of the regional population has three implications for control. A programme confined to one farm is likely to be offset by immigration from neighbouring units, so measures applied across a dairy belt and synchronised in time should hold better than isolated interventions. The same continuity means that resistance alleles, once present, can spread through the landscape. Knockdown resistance mutations in the voltage-gated sodium channel are now common in stable fly populations elsewhere and can be screened with a single amplicon sequencing assay [36], which would allow their arrival and spread to be followed across these farms. Finally, the differentiation of Farm 1 suggests that removing larval development sites from stored feed, silage residue and manure may leave a detectable imprint on the genetic composition of the local population, and thus offer an objective measure of whether control is working [34].

Two constraints shape what these data can show. First, a single mitochondrial locus is maternally inherited, so the estimates describe female mediated gene flow only, and they carry no information on recombination or on male dispersal. Second, sampling covered nine farms in one banner during one period, so neither the wider plateau nor seasonal turnover of populations could be addressed. A third limitation concerns the reference panel, which is uneven, with ten Canadian, eight Indian and ten Thai sequences against single sequences from several other regions, and sparse sampling is known to distort the placement of rare haplotypes in parsimony networks. Nuclear markers such as microsatellites or genome-wide single nucleotide polymorphisms, together with sampling across several banners and repeated over seasons, would allow the Farm 1 signal to be tested over a longer period [37].

5 Conclusions

Stable fly populations in Tumed Left Banner form a single gene pool in which dispersal, assisted by livestock and forage movement, overrides local drift, yet events at the scale of a single farm remain detectable. Farm 1 carries a recent demographic signature that shows in its haplotype composition, in Tajima's *D* and in its differentiation from neighbouring farms, which means that on farm management leaves a record that can be read. Because the local haplotypes belong to a worldwide pool rather than to a regional lineage, control is better coordinated across a production area than applied farm by farm.

Abbreviations

kdr
Knockdown-resistance
cox1
Cytochrome *c* oxidase subunit I
Mya
Million years ago
BOLD
Barcode of Life Data System
S
The number of segregating sites
π
Nucleotide diversity
h
Number of haplotypes
H_d
Haplotype diversity
k
Average number of nucleotide differences
F_{ST}
Fixation index for genetic differentiation among subpopulations relative to the total (subscripts S and T:subpopulation, total)
F_{SC}
Fixation index for differentiation among subpopulations within groups (subscript C:group)
F_{CT}
Fixation index for differentiation among groups (subscripts C and T:group, total)
AMOVA
Analysis of molecular variance
SSD
Sum of squared deviations
r
Harpending's raggedness index.

Declarations

Ethics approval and consent to participate

All research protocols used for insect experimentation in this study were approved by the Animal Ethics Committee of the Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences (Ethics Approval Number: 2024 – 00111) before the initiation of the research.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Author Contribution

Penglong Wang: Methodology, Software, Writing – original draft. Feifei Zhao: Methodology, Writing – original draft. Junyan Li: Resources, Supervision. Xiaoyue Du and Jianting Xu: Investigation. Xiaoping Luo: Validation. Wa Gao: Data curation. Qi Liu: Visualization and Funding acquisition. Fengying Bao: Supervision. Le Pei: Project administration. Qi Mei: Funding acquisition and Writing – review & editing. Xiaoguang Xiao: Writing – review & editing. Xiaona Shi: Funding acquisition. All authors read and approved the final manuscript.

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Figures

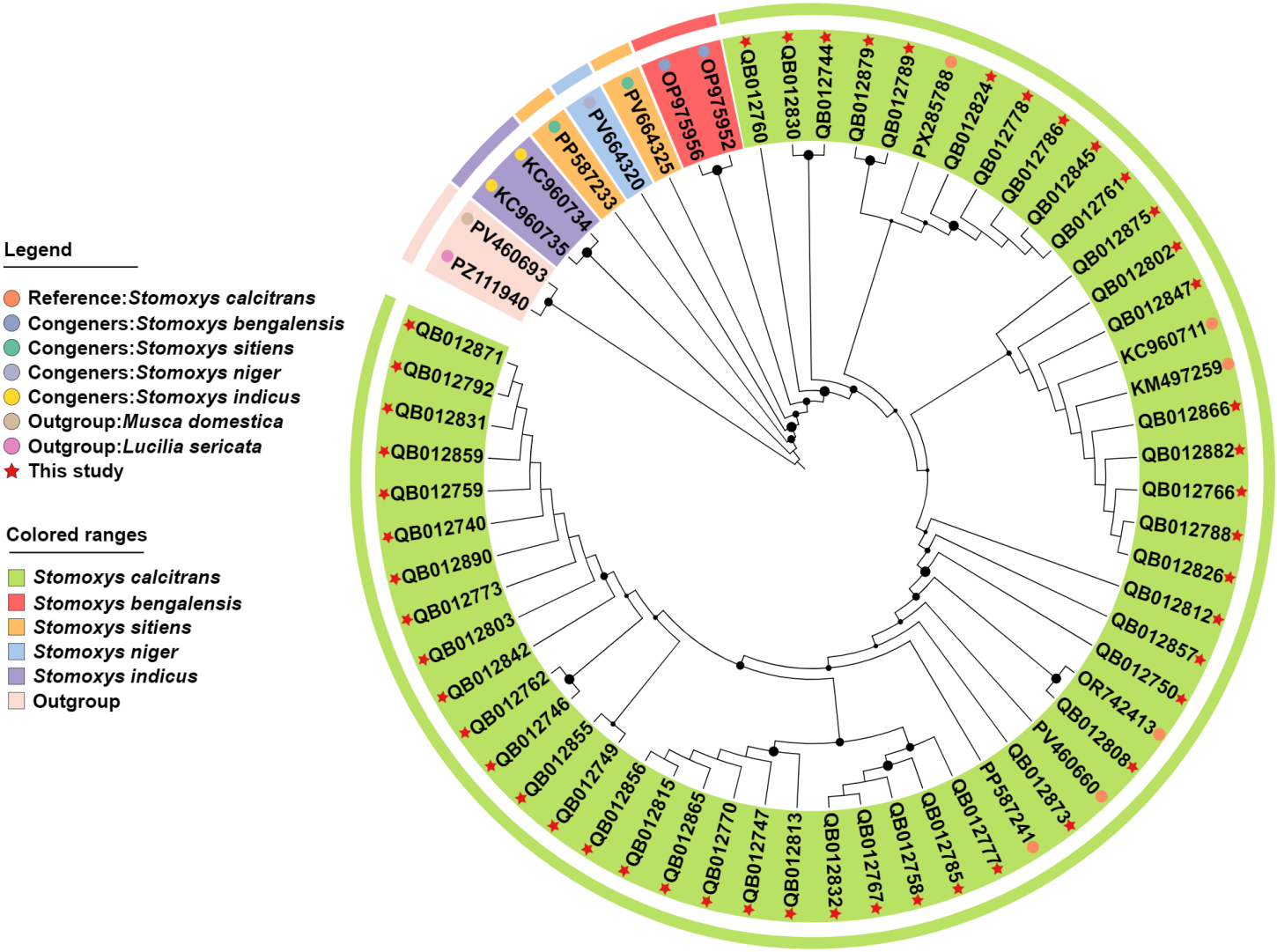


Figure 1

Circular maximum likelihood tree of *Stomoxys calcitrans* and related species based on *cox1* sequences.

Note: The tree includes reference sequences of *S. calcitrans*, four congeners (*S. bengalensis*, *S. sitiens*, *S. niger* and *S. indicus*) and two outgroups (*Musca domestica* and *Lucilia sericata*). Coloured ranges indicate

species clades, yellow-green for *S. calcitrans*, coral for *S. bengalensis*, warm orange for *S. sitiens*, sky blue for *S. niger*, periwinkle for *S. indicus* and pale peach for the outgroups. Pure red stars mark the sequences obtained in this study, all of which fall within the *S. calcitrans* clade.

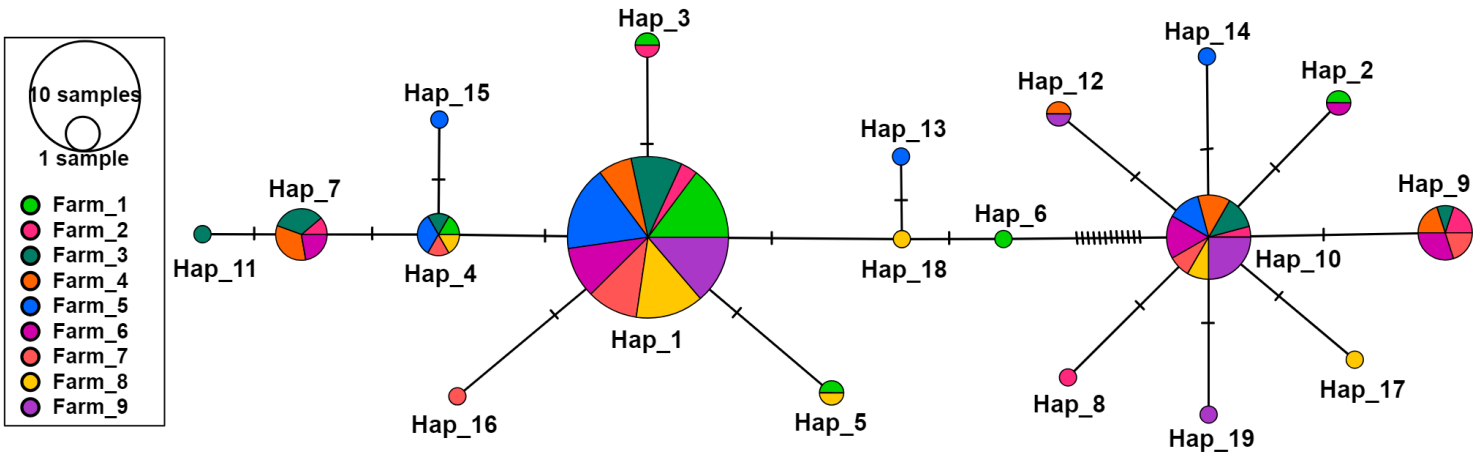


Figure 2

Haplotype network of *Stomoxys calcitrans* based on *cox1* sequences from Tumed Left Banner.

Note: Circle size is proportional to haplotype frequency (scale, 1 and 10 sequences). Colours denote farms, with each of the nine farms assigned a distinct colour. Tick marks on the connecting lines represent single nucleotide changes. Two haplotypes, Hap_1 and Hap_10, occupy the centre of the network and are shared by most of the nine farms. The remaining haplotypes are rare and each is confined to one or a few farms. Haplotype codes are specific to this figure and are not comparable with those in Figure 3.

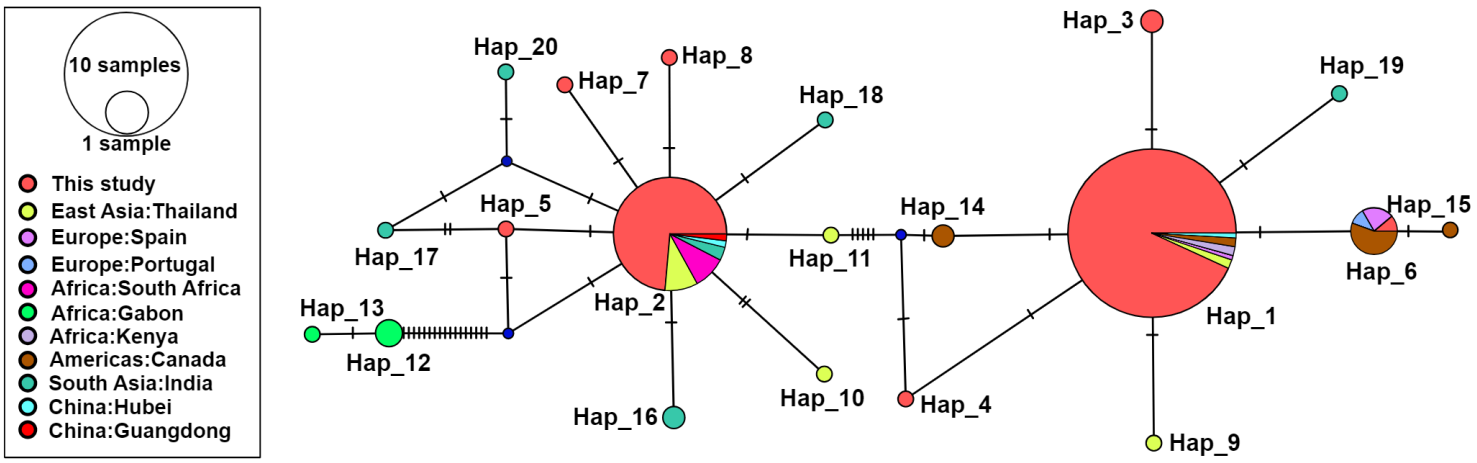


Figure 3

Haplotype network of *Stomoxys calcitrans* from *cox1* (this study and 46 reference sequences).

Note: Circle size is proportional to haplotype frequency (scale, 1 and 10 sequences). Colours denote geographic origin. Coral red marks sequences from this study, and other colours denote Asia (Thailand,

India, Hubei and Guangdong), Europe (Spain and Portugal), Africa (South Africa, Gabon and Kenya) and the Americas (Canada). Tick marks on the connecting lines represent single nucleotide changes. Hap_1 and Hap_2 are the two largest nodes, and both consist mainly of sequences from this study with smaller contributions from other regions. The remaining haplotypes are region specific. Haplotype codes were assigned independently from those in Figure 2 and do not refer to the same sequences.