

Development and characterization of EST-SSRs for Muscidae (Diptera) and their cross-genera transferability

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EST-SSR (Expressed Sequence Tag-Simple Sequence Repeat) markers were developed and used to examine genetic diversity of species assemblages of ten genera of Muscidae (Diptera), and specifically the genetic diversity of *Phaonia* Robineau species, from ten regions in China. 18 EST-SSR markers with high polymorphism and clear bands were screened out from 216 tested markers, with 219 alleles in total (95–280 bp) with an average of 6.1 alleles per locus. In various regions in China such as Tibet, Sichuan, and Yunnan, Muscidae species assemblages exhibited rich genetic diversity, with the polymorphism information content (PIC) values ranging from 0.831 to 0.934, while Hainan region showed a relatively low genetic diversity (PIC = 0.511). Low gene flow ($N_m = 0.842$) was found among the regions, and the average genetic differentiation coefficient (F_{st}) was 0.238, indicating a high degree of genetic differentiation among the ten surveyed assemblages. Accordingly, the ten genera of Muscidae exhibited high genetic diversity and genetic differentiation.

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1. Introduction

Muscidae is one of the four families of the muscoid grade of Calypttratae (Diptera: Cyclorhapha). The family is mainly distributed in the Palaearctic (Hennig 1955–1964) and Nearctic regions (Carvalho *et al.* 2002), with some species occurring in the Oriental region. Muscidae flies are often very abundant (McAlpine 1981) and some species can be found in urban environments as pest species, being carriers of pathogenic bacteria and thus potentially spreading diseases. However, the vast majority of Muscidae live in natural areas, e.g. mountains, being saprophagous

in decaying organic matter, or ingesting dew or flower nectar (Michelsen 2000).

Because the morphology of the flies in Muscidae is often very similar (e.g. Xue *et al.* 1992), it is frequently difficult to identify the fly species according to their morphological characteristics. Although there are many species whose morphological identification is uncomplicated, the number of variable morphological characteristics is numerous, and some characters are strongly influenced by environmental factors, which could lead to erroneous identification of species (Evenhuis *et al.* 2010). However, identification based on molecular markers, especially the DNA

Table 1. Regions (China) and sample sizes of the ten genera of Muscidae used in the study.

	XT	XN	QK	YY	SE	HW	ST	NC	LQ	JC
<i>Fannia</i>	8		11		23		10			
<i>Hydrotaea</i>							6	13		16
<i>Drymeia</i>		6	5	8	6					
<i>Myospila</i>	2				7	8	11		10	
<i>Spilogona</i>			9	7			8			10
<i>Brontaea</i>	7			8		6			6	11
<i>Lispe</i>	6	6		4		7	9		10	
<i>Coenosia</i>					5			7	6	
<i>Helina</i>	4	6	11		5					13
<i>Phaonia</i>	2	8	4	10	11	7	5	9	7	9

XT: Tian-Mountain of Xinjiang, 43°8'20"N 88°3'17"E, 5,445 m a.s.l.

XN: Nianqingtanggula-Mountain of Xizang, 30°4'2"N 90°6'2"E, 7,117 m a.s.l.

QK: Kunlun-Mountain of Qinhai, 34°47'23"N 99°27'18"E, 6,282 m a.s.l.

YY: Yulongxue-Mountain of Yunnan, 27°3'2"N 100°4'2"E, 5,596 m a.s.l.

SE: Emei-Mountain of Sichuan, 30°16'30"N 103°10'30"E, 3,099 m a.s.l.

HW: Wuzhi-Mountain of Hainan, 18°48'59"N 109°32'03"E, 1,867 m a.s.l.

ST: Taihang-Mountain of Shanxi, 34°34'16"N 110°14'08"E, 2,882 m a.s.l.

NC: Chifeng Neimenggu, 41°17'10"N 116°21'07"E, 2,067 m a.s.l.

LQ: Qian-Mountain of Liaoning, 40°55'30"N 122°49'2"E, 708 m a.s.l.

JC: Changbai-Mountain of Jinlin, 41°41'49"N 127°42'55"E, 2,691 m a.s.l.

fingerprinting technologies, are accurate, repeatable, simple and fast to perform (Posada 2008). The EST-SSR (Expressed Sequence Tag-Simple Sequence Repeats) molecular markers are a relatively new group of DNA-based genetic markers (Schuehli *et al.* 2007). In comparison with morphological characteristics, cytological or biochemical markers, the EST-SSR markers are faster, better and more economical for testing and use in research. The EST-SSR markers take the form of coding DNA sequence, which can be detected in various tissues and various stages of growth or development of e.g. flies. Furthermore, the EST-SSR markers are not subjected to seasonal, environmental or human influenced (subjective) factors (Savage & Wheeler 2004). The enormous numbers of EST-SSR markers cover the entire genome, they are characterized as highly polymorphic, co-dominant and reliable markers, which make it possible to identify homozygous and heterozygous genotypes. In basing on expressed sequence tags, EST-SSRs produce simple sequence repeats, in comparison to the genome SSR (also called microsatellites), and the advantage of the former is their high transferability across species.

Currently, EST-SSR markers are widely used for genomics research, such as genetic mapping, comparative mapping, and analysis of the genetic

diversity, germplasm identification, phylogenetics and evolution.

EST-SSRs are generated by sequencing cDNA, which itself is synthesized from the mRNA molecules in cells and are thus copies of the genes that are being expressed (Renaud *et al.* 2012). mRNA does not contain sequences from the regions between the genes, nor from the non-coding introns that are present within many parts of the genome that are useful in practical research. The expressed genes constitute 3–5% of the whole genome, so a 300–400 bp EST is enough to reflect the gene characteristics.

To date, very few reports exist about genetic diversity of Muscidae species. In this study, EST-SSR primers were developed for Muscidae taxa, which could be useful for generating e.g. DNA fingerprints for identification of Muscidae species. Here EST-SSR markers were used to examine the genetic diversity of ten genera of Muscidae and of *Phaonia* species from ten regions in natural populations in China.

2. Materials and methods

2.1. Fly materials

The research objects of this study were the flies in 10 representative genera of Muscidae from 10



Fig. 1. Map of China showing the ten regions where samples of Muscidae were collected.

provinces in China (Table 1, Fig. 1). All fly samples were collected during more than 8 years. The fly material is preserved in the museum of the Agricultural college of Eastern Liaoning University and Institute of Entomology, Shenyang Normal University.

2.2. DNA extraction and amplification

The flies were crushed with a mortar and pestle, the tissue homogenate was put into an eppendorf tube which had 40 μl 0.25 $\text{mol}\cdot\text{L}^{-1}$ NaOH, held 30 s in boiling water; then 40 μl 0.25 $\text{mol}\cdot\text{L}^{-1}$ HCl and 20 μl 0.5 $\text{mol}\cdot\text{L}^{-1}$ Tris-HCl (pH 8.0, containing 0.25% Nonidet-P-40) was added, held 2 min in boiling water, then the tissue homogenate was used in 25 μl PCR reactions. PCR reactions: 2.5 μl 10 \times reaction buffer, 2 μl 2.5 $\text{mmol}\cdot\text{L}^{-1}$ of each dNTPs, 1U of Taq polymerase, 0.5 μl 10 $\mu\text{mol}\cdot\text{L}^{-1}$ each of forward and reverse primers, 50–100 ng tissue homogenate, 1.5 μl 1.5 $\text{mmol}\cdot\text{L}^{-1}$ MgCl₂, then added double-distilled water up to total 25 μl reaction volume (Kutty *et al.* 2008). The following PCR program was used: the initial hot-start of a denaturation step at 94 °C for 10 min, followed by 35 cycles of a 50 s denaturation

step at 94 °C, a 50 s annealing step at 58–60 °C, and a 1 min extension step at 72 °C, and the final extension step at 72 °C for 10 min before holding the sample at 4 °C.

2.3. EST-SSR primers

All the available Muscidae EST sequences up to January 2016 were downloaded from GenBank/NCBI EST database (National Center for Biotechnology Information <http://www.ncbi.nlm.nih.gov>). For developing EST-SSR primers, the steps were as follows. Using Perl software (www.perl.org) all 21,880 Muscidae EST sequences were assembled in FASTA format. The Blastclust software (www.ncbi.nlm.nih.gov) was used in searching for the redundant sequences in all EST sequences. Then, using the CD-HIT software (<http://weizhongli-lab.org/cd-hit/>), the short (<100 bp) and long sequences (>700 bp) were removed, as also the 5'-hat and 3'-poly tail (A or T) of mRNA by EST-TRIMMER software. The MISA.PL software was used to search, identify and locate SSR sites, and primer 3 was used to develop EST-SSR primers. The designed parameters of the EST-SSR primers were as follows:

length 20–25 bp, annealing temperature (T_m) 56–63 °C, and the content of G+C was 30–70%. Finally, BLAST was used to test these developed primers.

2.4. EST-SSR primers screening

The developed EST-SSR primers were used to PCR amplified 46 DNA samples of different Muscidae genera (Chen 2009). The amplified fragments were separated and analyzed using 6% polyacrylamide gel electrophoresis for 1.5 h (constant power 55 W, DNA marker GsDL0501 from 50 bp to 500 bp). The primers were tested and screened three times, and then, of the screened primers those, which amplified stable, polymorphic and clear bands, were chosen in the final study.

2.5. Data analysis

The different amplified bands (EST-SSR products) that were generated by each primer pair were identified using gel electrophoresis. The EST-SSR molecular markers were recorded in co-dominant format, and therefore they can identify the homozygous and heterozygous genotype. POPGENE version 1.32 was used to analyze the genetic diversity of each polymorphic locus, including expected heterozygosity (H_e), observed heterozygosity (H_o), coefficient of genetic differentiation (F_{st} and G_{st}), gene flow (N_m) [$N_m = 0.25(1 - F_{st})/F_{st}$], percentage of polymorphic loci (PPL), observed number of alleles (N_a), effective number of alleles (N_e) and Shannon's information index (I). The polymorphism information content (PIC) was calculated by the formula:

$$PIC = 1 - \sum_{i=1}^n P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^n 2P_i^2 P_j^2 \quad (1),$$

where P_i and P_j are the frequencies of the i_{th} and j_{th} allele, and n is the number of alleles (Botstein *et al.* 1980). The genetic relationship of *Phaonia* species from the studied ten regions of China was also analyzed using POPGENE version 1.32.

A clustering analysis was used to generate a dendrogram for 46 accessions using the unweighted pair-group method with arithmetic

mean (UPGMA) and Nei's unbiased genetic distance using the NTSYPC 2.10 software package (Nei 1978). A Mantel test was used to examine the relationship between UPGMA results and the co-characterization correlation of the genetic distance matrix.

3. Results

3.1. EST-SSR marker polymorphisms

A total of 216 EST-SSR markers were developed, and after screening three times there were 18 markers which produced polymorphic, stable and clear bands, which were used in the further study (Table 2). The dominant motifs among the 18 EST-SSR markers of the studied Muscidae species were the trinucleotides and the dinucleotides. The different frequencies were 20.7, 74.0, 4.6, 0.3, 0.3 and 0.1% for the di-, tri-, tetra-, penta-, hexa-, and other dinucleotides, respectively. In the trinucleotides, the motifs AAG, AAC, ACC, AGC, ACG had a high frequency whereas in the dinucleotides that was the case with the motifs AT and AC.

The 18 screened EST-SSR markers of the species of ten genera (Table 2) produced a total of 216 alleles, with an average of 6.1 alleles per locus. The PIC value provides an estimate of the discriminatory power of a locus by taking in account the number of alleles, which are expressed, and the relative frequencies of these alleles. In general, PIC values have been used to evaluate the polymorphism information content of each marker. Following Botstein *et al.* (1980) the following scale was used: $PIC > 0.5$ with high polymorphism information, $0.5 \geq PIC \geq 0.25$ moderate, $PIC < 0.25$ low. In this study, the PIC values of the 18 EST-SSR markers ranged from 0.642 to 0.908, with the mean of 0.860 (Table 2), which showed high polymorphism (Angus *et al.* 2011). The values of effective number of alleles (N_e) ranged from 3.267 to 11.765, with the mean of 8.720. The degree of heterozygosity (H_e) reflected the consistency of population genotype; however, the H_o value (0.547) was slightly lower than H_e value (0.878) per locus for the ten genera. The values of Shannon's information index (I) ranged from 1.323 to 2.586, with the mean of 2.259, which showed high polymorphism as well.

Table 2. The sequence (5'→3') (F: forward, R: reverse), annealing temperature (T_m), repeat motif and expected product size (bp) of 18 polymorphic EST-SSR markers and their diversity statistic values^s in Muscidae of ten regions in China.

Marker	T_m (°C)	motif	bp	N_a	N_e	H_o	H_e	I	F_{st}	N_m	PIC
MD108, F: CTTTGTTGCTCAGTTGGGGT, R: AAACATTTGTTTTGGGCCTG	60	(AGC) ₆	252	10	6.274	0.551	0.845	2.264	0.158	1.332	0.821
MD110, F: TGTGCGCTACGTTTGAAGAC, R: ATTTTGATGCAGATGAGCCC	60	(AT) ₉	213	9	4.961	0.573	0.803	1.806	0.217	0.902	0.770
MD115, F: CCCATAAAGGGCTGAAAAGA, R: AAAATATCATTACAATCGTCACCA	58	(AG) ₆	270	11	9.195	0.564	0.896	0.292	0.214	0.921	0.881
MD123, F: GGGATCTGATGCAATACGGT, R: TCATCAATTTTCATTGGCTGC	59	(AAG) ₅	251	13	9.985	0.502	0.904	2.414	0.190	1.066	0.891
MD136, F: GAAAGCGAGATGGCAAGAAC, R: CCCGGCACTGTTGTTAAAGT	60	(GGT) ₅	278	12	7.675	0.523	0.874	2.227	0.278	0.649	0.857
MD148, F: AACCAAAAACAAAATGGGGA, R: GCATGACGCATGACTTTTCT	59	(AAT) ₅	234	14	11.338	0.557	0.916	2.519	0.196	1.026	0.904
MD150, F: AAAAGGGAGCTGGAACAGGT, R: GCTCCAGCAAGAACAGGAAG	60	(ATT) ₅	259	14	10.040	0.554	0.905	2.464	0.330	0.508	0.892
MD157, F: ACCAGCCACCATTTATAGCG, R: CGGGTCGTAGGTGAAATGTT	59	(CAA) ₅	217	14	11.109	0.552	0.917	2.535	0.226	0.856	0.905
MD158, F: CACAACCCCTTGAAAAGTCGC, R: CAAGACGAGGTGGAGATGGT	60	(AGT) ₅	277	14	11.547	0.562	0.918	2.530	0.185	1.101	0.906
MD168, F: AGTTTGACGGCGAAAATGAG, R: CTGCGGTGCTGACAATAGAA	60	(GA) ₇	256	14	10.565	0.534	0.910	2.473	0.294	0.600	0.897
MD171, F: AGTTTGACGGCGAAAATGAG, R: CTGCGGTGCTGACAATAGAA	60	(GA) ₁₀	262	14	12.610	0.597	0.925	2.586	0.121	1.816	0.915
MD174, F: TTAATTGCGGACCCAATAGC, R: ATCAAAGTTGCGTCCAGGTC	60	(AAC) ₈	281	14	11.765	0.553	0.920	2.537	0.242	0.783	0.908
MD185, F: ATCCCCTCCCTCCCTAAAT, R: ATGGTTGACCCAAACCGTAA	60	(CCA) ₅	211	5	3.267	0.459	0.697	1.323	0.173	1.195	0.642
MD187, F: AGCTGCAACAGCCCTTAAAA, R: CACGCCACTTTACACCACAC	60	(TTG) ₅	110	13	10.858	0.554	0.913	2.460	0.287	0.621	0.900
MD191, F: TGCATTTGCTTTATGGACTGAC, R: TGGCCAGTCATTACTTTTGG	60	(TA) ₁₂	244	14	11.541	0.557	0.918	2.540	0.105	2.131	0.906
MD195, F: CAACCGCTCAGTGGATACCT, R: TTGGCTTTGGAGGAAAGAAA	59	(ACA) ₆	206	13	10.633	0.544	0.911	2.465	0.188	1.080	0.898
MD198, F: GCAACTACAAACAGCAACGC, R: ACCAGACGACCAAATGGAAG	59	(GCA) ₅	270	12	6.412	0.548	0.848	2.122	0.199	1.006	0.827
MD199, F: GTCGAACCACTCAGGGACAC, R: GAAGCCCTTTTGTGTCAGCAG	60	(TCT) ₅	279	14	11.105	0.560	0.915	2.511	0.295	0.597	0.902
Mean	59		223	12	8.720	0.547	0.878	2.259	0.238	0.842	0.860

^s) N_a : observed number of alleles, N_e : effective number of alleles, H_o : observed heterozygosity, H_e : expected heterozygosity, I: Shannon's information index, F_{st} : coefficient of genetic differentiation, N_m : gene flow, PIC: polymorphism information content.

3.2. Genetic diversity of genera

The mean value of the percentage of polymorphic loci (PPL) of *Phaonia* species from the ten regions in China was 85.4% (range: 71.4–100%,

Table 3). The Neimenggu (NC) and Jinlin (JC) regions showed the highest PPL (100%), followed by Sichuan (SE) and Qinghai (QK), while for Xinjiang (XT) it was the lowest (71.4%, Table 3). The mean value of PIC of the ten genera was

Table 3. Genetic diversity^a in natural populations of *Phaonia* species from ten regions in China.

Population ^b	No. of Alleles	I	H _o	PPL%	PIC
XT	7	1.384	0.511	71.4	0.710
XN	12	1.614	0.612	83.3	0.989
QK	9	1.489	0.784	90.5	0.793
YY	8	1.397	0.658	77.8	0.910
SE	14	1.809	0.690	92.9	0.924
HW	4	1.124	0.524	75.0	0.627
ST	6	1.320	0.483	83.3	0.511
NC	3	1.001	0.582	100	0.571
LQ	10	1.499	0.691	80.0	0.729
JC	5	1.279	0.625	100	0.876
Mean	8.3	1.391	0.672	85.4	0.764

a) I: Shannon's information index, H_o: observed heterozygosity, PPL%: percentage of polymorphic loci, PIC: polymorphism information content.

b) For abbreviations of populations, see Table 1.

0.764 (range: 0.511–0.989), the Xizang (XN) species assemblage having the highest PIC value (0.989), and the Shanxi (ST) species assemblage the lowest one (0.511). The Sichuan (SE) species assemblage had the highest value of the Shannon's information index (I = 1.809), and the Neimenggu (NC) species assemblage the lowest one (1.168). The mean value of the Shannon's information index of the ten genera was 1.391. The observed heterozygosity (H_o) value ranged from

0.511 to 0.784. The values of H_o and PIC of Qinghai (QK), Liaoning (LQ), Sichuan (SE) and Yunnan (YY) genera were relatively high. Accordingly, the genera in Western China had a high genetic diversity, closely followed by the north-east regions.

Based on the UPGMA Dendrogram, the *Phaonia* genus can be divided into two main branches, the Xinjiang (XT), Hainan (HW), Shanxi (ST), Liaoning (LQ), Neimenggu (NC) and Jinlin (JC) assemblages in northeast part of China clustering into the other branch (Fig. 2, Table 4). The LQ and NC assemblages had a close relationship within this branch, followed by HW and ST, but XT had a larger genetic distance from others. The western regions, Qinghai (XN), Yunnan (YY), Qinghai (QK) and Sichuan (SE), clustered into another branch. XN and YY had a closer relationship within it than the other two (QK and SE).

Nei's (1978) genetic distances among the ten genera of Muscidae were also calculated. The resulting UPGMA dendrogram is shown in Fig. 3. The species of most genera cluster in the branch of their own, except *Lispe* from Xinjiang (XT) clustered with species of *Brontaea*. The degree of congruence of UPGMA results and the Mantel test of co-characterization correlation of genetic distance matrix was excellent with its co-phenetic correlation (r) value being 0.876.

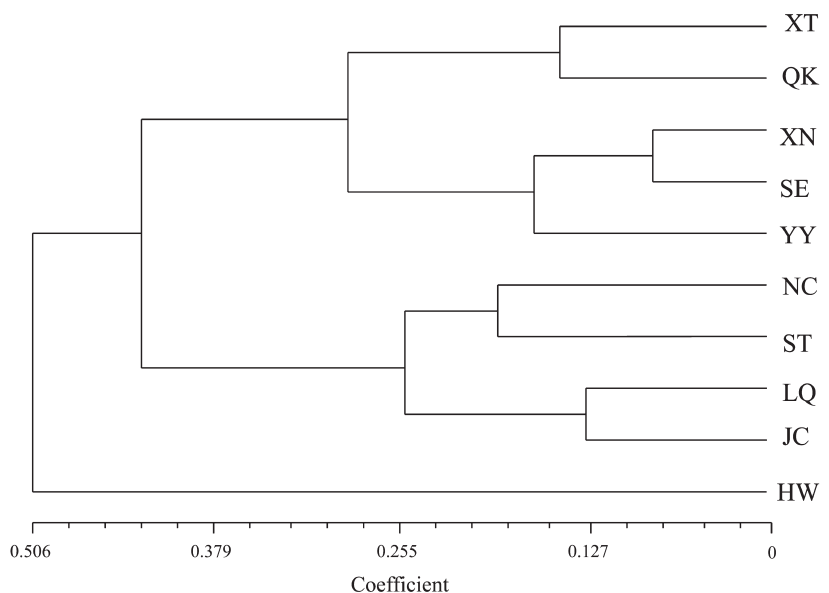


Fig. 2. UPGMA dendrogram of *Phaonia* species among ten regions in China (Table 1).

Table 4. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) for *Phaonia* species from ten regions of China (for abbreviations of the regions, see Table 1).

Region	XT	XN	QK	YY	SE	HW	SE	NC	LQ	JC
XT	*****	0.8383	0.8302	0.8214	0.7616	0.8034	0.8392	0.8326	0.8755	0.8192
XN	0.1764	*****	0.8528	0.8748	0.8224	0.7503	0.7826	0.8030	0.7833	0.7863
QK	0.1861	0.1593	*****	0.8464	0.8420	0.7644	0.7952	0.7958	0.8069	0.7843
YY	0.1968	0.1338	0.1667	*****	0.8531	0.8041	0.8345	0.8029	0.7976	0.7602
SE	0.2723	0.1955	0.1720	0.1589	*****	0.7655	0.8101	0.7659	0.7805	0.7059
HW	0.2190	0.2873	0.2687	0.2181	0.2673	*****	0.9320	0.8872	0.8973	0.8389
ST	0.1753	0.2451	0.2291	0.1809	0.2106	0.0704	*****	0.9193	0.9403	0.8793
NC	0.1832	0.2194	0.2285	0.2195	0.2667	0.1197	0.0841	*****	0.9201	0.9235
LQ	0.1330	0.2442	0.2146	0.2262	0.2478	0.1083	0.0615	0.0832	*****	0.8884
JC	0.1994	0.2404	0.2430	0.2742	0.3483	0.1756	0.1287	0.0796	0.1183	*****

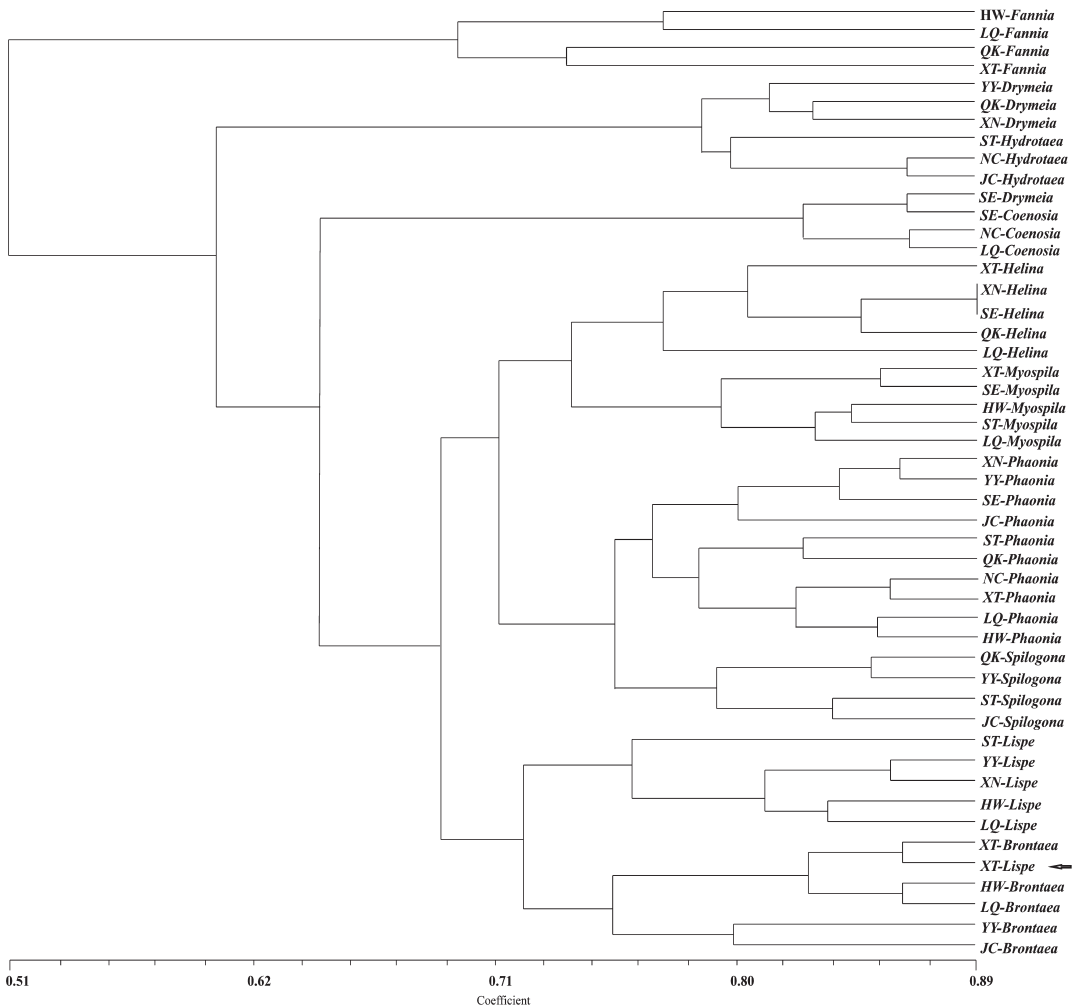


Fig. 3. Dendrogram of 46 species assemblages of Muscidae of ten regions in China (Table 1) based on EST-SSR data (UPGMA). The single *Lispe* in the *Brontaea* branch has been indicated by an arrow.

The species and the number of them in each region were different and the genetic differentiation coefficient (F_{st}) was also different. The mean F_{st} value of the 18 loci for the ten species assemblages was 0.238. The mean value of gene flow (N_m) was 0.842, when the EST-SSR molecular markers were used to detect the genetic differentiation of the studied Muscidae species. The mean F_{st} value of the 36 EST-SSR molecular markers for the ten species assemblages was relatively high, 0.238. This is consistent with the generally low values of gene flow (mean $N_m=0.842$). However, the low F_{st} (0.121) and the high N_m (1.816) (Table 2) values also existed, showing low differentiation in some markers.

4. Discussion

Transferability of the chosen 18 EST-SSR markers was examined for ten different species assemblages, which successfully amplified stable and clear bands. Generally, successful cross-genera amplification of the EST-SSRs usually occurs in the related species within a genus, but a high cross-genera amplification demonstrated for the EST-SSR markers developed in this study demonstrate a high transferability being applicable to ten different species assemblages, thus being potentially useful in comparative genomics. To conclude, in this study, we developed and characterized 18 new EST-SSR markers for Muscidae species aiming to enrich the set of SSR markers for this fly family. Most of the markers were polymorphic, i.e. more than one allele could be detected in the set of ten assemblage's species. Accordingly, the results demonstrated that these markers are applicable in the surveyed species assemblages and potentially in other Muscidae species, too.

In addition, the markers developed in this study for the research of genetic diversity of *Phaonia* species revealed that these *Phaonia* species had high genetic diversity and displayed genetic differentiation of natural species assemblages. The genetic diversity of the western regions in China, namely Xizang (XN), Sichuan (SE) and Yunnan (YY), was generally higher (PIC 0.900) than in Shanxi (ST) (PIC=0.511).

Muscidae flies are major pest species and are

thus economically important. In China, previous studies on flies have been about the morphology and biology, while only few have reported about molecular biology, especially molecular markers. The development and improvement of informative molecular markers will further expand and deepen the knowledge on genetic diversity of Muscidae.

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References

- Angus, R. B., Dellow, J., Winder, C. & Credland, P. F. 2011: Karyotype differences among four species of *Callosobruchus* Pic (Coleoptera: Bruchidae). — *Journal of Stored Products Research* 47(2): 76–81.
- Botstein, D., White, R. L., Skolnick, M. & Davis, R. W. 1980: Construction of a genetic linkage map in man using restriction fragment length polymorphisms. — *American Journal of Human Genetics* 32(3): 314–331.
- Carvalho, C. J. B. de, Couri, M. S., Pont, A. C., Pamplona, D. & Lopes, S. M. 2002: A catalogue of the Muscidae (Diptera) of the Neotropical Region. — *Zootaxa* 860: 1–282.
- Chen, X. M. 2009: The morphology, biology and genetic differentiation research of *Musca domestica* in different geographic populations. — *Huazhong Agricultural University* 1: 68–73. [In Chinese.]
- Evenhuis, N. L., Pape, T., Pont, A. C. & Thompson, F. C. 2010: Flying after Linnaeus: Diptera names since *Systema Naturae* (1758). — In: Polaszek, A. (ed.), *Systema Naturae* 250: 75–82, The Linnaean Ark. CRC Press. 321 pp.
- Hennig, W. 1955–1964: Family Muscidae. — In: Lindner, E. (ed.), *Die Fliegen der Palaearktischen Region*, 63b. Schweizerbart, Stuttgart. 1110 pp.
- Kutty, S. N., Pape, T., Pont, A., Wiegmann, B. M. & Meier, R. 2008: The Muscoidea (Diptera: Calypttratae) are paraphyletic: evidence from four mitochondrial and four nuclear genes. — *Molecular Phylogenetics and Evolution* 49: 639–652.
- McAlpine J. F. 1981: Morphology and terminology – adults. — In: McAlpine, J. F., Peterson, B. V., Shewell, G. E., Teskey, H. J., Vockeroth, J. R. & Wood, D. M.

- (eds), Manual of Nearctic Diptera. Volume 1. — Agriculture Canada. Monograph 27: 9–63.
- Michelsen, V. 2000: Oldest authentic record of a fossil calyptrate flies (Diptera): a species of Anthomyiidae from early Coenozoic Baltic amber. — *Studia Dipterologica* 7: 11–18.
- Nei, M. 1978: Estimation of average heterozygosity and genetic distance from a small number of individuals. — *Genetics* 89: 583–590.
- Posada, D. 2008: JModel Test: phylogenetic model averaging. — *Molecular Biology Evolution* 25: 1253 – 1256.
- Renaud, A., Savage, J. & Adamowicz, S. 2012: DNA barcoding of Northern Nearctic Muscidae (Diptera) reveals high correspondence between morphological and molecular species limits. — *BMC Ecology* 12: 24–38.
- Savage, J. & Wheeler, T. A. 2004: Phylogeny of the Azeliini (Diptera: Muscidae). — *Studia Dipterologica* 11: 259–299.
- Schuehli, G. S., Carvalho, C. J. B. de & Wiegmann, B. M. 2007: Molecular phylogenetics of the Muscidae (Diptera: Calyptratae): New ideas in a congruence context. — *Invertebrate Systematics* 21(3): 263–278.
- Xue, W. Q., Zhang, C. T., Wang, M. F. & Zhou, Z. K. 1992: Study on the genus *Drymeia* of China (Diptera: Muscidae). — A paper for the 19th international congress of Entomology, Shenyang Normal University. 16 pp.