Genetic variation and population dispersal of Yangtze voles
*Microtus fortis calamorum* in the Dongting Lake region

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**Abstract**

To understand genetic variation and population dispersal in the Yangtze vole *Microtus fortis calamorum* distributed in the Dongting Lake region, 144 individuals were collected from six habitat patches. The mitochondrial DNA control region was sequenced and 17 haplotypes were observed. Of the six investigated populations, haplotype and nucleotide diversities of those from larger patches were higher than those from smaller patches. Nonparametric correlation analysis showed that patch size had a positive correlation with haplotype diversity (*r* = 0.943, *P* < 0.01). A neighbour-joining tree of the 17 haplotypes showed no geographic genetic structure among the six populations. Analysis of isolation by distance showed that genetic differentiation among the six populations was not positively related to geographic distance. Analysis of mismatch distribution indicated that the voles had passed through a population expansion. The pattern of haplotype distribution in the Changsha population suggests that the population was established by a founder effect [Current Zoology 58 (2): 211–220, 2012].

**Keywords** *Microtus fortis calamorum*, mitochondrial DNA control region, genetic structure, Dongting Lake, Habitat patch

Dongting Lake (28°44′–29°35′N, 111°53′–113°05′E) is a typical wetland landscape located in Hunan, China. It has experienced a complicated evolution since the Pleistocene and changed from small to large, then from large to small (Tong, 2003). Dongting Lake changed rapidly in the last several hundred years (Fig. 1) because of natural events (floods and sedimentation of the lake) and human activities (anthropogenic infilling, settlements and farming). Not only was the area of the lake drastically reduced from 6,300 km² in 1825 (Zhang et al., 1982; Wang, 1989) to 2,691 km² in 1983 (Wang et al., 1989), but it was also changed into complex river networks and the region around Dongting Lake partitioned into various patches.

Alteration of habitat patches has had a great influence on the Yangtze vole, *Microtus fortis calamorum*, a rodent pest in the Dongting Lake region since the 1950s (Shou, 1962). Many patches have not been suitable habitat for the vole due to urbanization and the expansion of agricultural land, but a few large patches with vast lowlands are optimal habitats. For example, the reed and Amur silvergrass patch, *Carex cinerascens* and *Carex breviculmis* patch are good habitats for the vole, whereas highland and cropland patches are not (Wu et al., 1996; Guo et al., 1997). Many studies have been done on the biology, population dynamics and environmental factors causing vole outbreaks (Chen et al., 1995; Guo et al., 1997; Zou et al., 2000; Wang et al., 2004; Zhang et al., 2007), but to date, reports on genetic structure among regional populations, population dispersal, population demography and potential underlying genetic factors are absent from the literature. In addition, interestingly, the vole was observed in the 1980s and its population threatened the woods in the summer of 2003 (Li et al., 2005) in Changsha, the capital city of Hunan. It was suggested that the voles may have migrated from Dongting Lake along the banks of the Xiangjiang River; however, there was no evidence to support this scenario.

Mitochondrial DNA exhibits several peculiar features such as maternal inheritance, the presence of single-copy orthologous genes, lack of recombination and a high mutation rate (Pesole et al., 1999; Larizza et al., 2002). In particular the mtDNA control region (D-loop) is a noncoding segment and the most variable part of the molecule, which evolves 3–5 times more rapidly than
Alteration of habitat patches has affected the distribution and population dynamics of this vole, but has fragmentation of the Dongting Lake region affected gene flow and lead to genetic differentiation among populations? Further, what is the population history of the vole and was the Changsha population established by a founder event? To resolve these questions we employed mtDNA control region sequence analyses.

1 Materials and Methods

1.1 Sampling

We trapped Yangtze voles from six different habitat patches (P1, P2, P3, P4, P5 and P6, Fig. 2) using clamps baited with sunflower seeds. Patch size (km²) was calculated using GIS (Table 1). We obtained 144 samples and the sample size from each patch was: 24 from P1, 26 from P2, 25 from P3, 20 from P4, 26 from P5 and 23 from P6. Vole populations from P1, P2, P3, P4, P5 and P6 are referred to as Chunfeng, Datonghu, Nanzui, Miluo, Changsha and Junshan populations.

1.2 Tissue sampling and DNA extraction

Animals were killed and muscle tissue from the legs of each individual was sampled and immediately preserved in absolute ethanol, and finally stored at -20°C in the laboratory. We extracted genomic DNA from a small section of the muscles for all individuals follow-
Table 1  The variable sites of haplotypes, haplotype diversity (h), nucleotide diversity (π), and haplotype distribution of Microtus fortis calamorum based on the mtDNA control region sequence, sampling number (n) and patch size (s, km²).

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>The variable sites</th>
<th>Chunfeng</th>
<th>Changsha</th>
<th>Datonghu</th>
<th>Junshan</th>
<th>Nanzui</th>
<th>Miluo</th>
</tr>
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<tr>
<td>112335556666666677</td>
<td>h = 0.736</td>
<td>n = 24</td>
<td>n = 26</td>
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<td>66990254890091123355949</td>
<td>π = 1.68%</td>
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<td>n = 26</td>
<td>n = 26</td>
<td>n = 25</td>
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<tr>
<td>3835865622301934557912</td>
<td>S = 53.67</td>
<td>n = 24</td>
<td>n = 26</td>
<td>n = 26</td>
<td>n = 26</td>
<td>n = 25</td>
<td>n = 20</td>
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<th>Haplotype</th>
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<th>Changsha</th>
<th>Datonghu</th>
<th>Junshan</th>
<th>Nanzui</th>
<th>Miluo</th>
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<tbody>
<tr>
<td>H1</td>
<td>AATTTTGCTCTAAGTTGTCTC</td>
<td>9</td>
<td>6</td>
<td>8</td>
<td>7</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>H2</td>
<td>.. . A . . . . . . . . . . T . . .</td>
<td>12</td>
<td>12</td>
<td>20</td>
<td>11</td>
<td></td>
<td></td>
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<tr>
<td>H3</td>
<td>.. . A . . . . . . . . . . T . . .</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>.. . A . . . . . . . . . . C . . .</td>
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<td></td>
<td></td>
<td></td>
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<td>.. . CA . . . . . . . . . . T . . .</td>
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<td></td>
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<td>H6</td>
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<tr>
<td>H8</td>
<td>.. . . . . . . . . . . . . . . . GGG . . .</td>
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<td></td>
</tr>
<tr>
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<td>.. . . . . . . . . . . . . . . . G . . .</td>
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</table>

Fig. 2  Sampling localities of Microtus fortis calamorum

1.3 PCR and Sequencing

A 1200 bp fragment of mtDNA was amplified using primers L15135: 5’-GAGGACAACCAGTGTGAATACC and H91: 5’-ATAAGGCCAGGACCAACCT-3’ (Yang, 2007). The fragment includes partial sequence of cytochrome b gene, the complete sequence of tRNA-Phr, control region, tRNA-Pro gene, tRNA-Phe gene, and the standard phenol-chloroform method (Sambrook et al., 1989). The remaining muscle tissues were stored at −20°C.
gene and partial sequence of 12 S RNA. Polymerase chain reaction (PCR) amplification was carried out using DNA thermal cyclers (Eppendorf) with 25 μl of the reaction mixture containing 0.25 mM dNTP, 0.4 mM of each primer, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 1.25 units Taq DNA polymerase (Tiangen Biotech 2× Taq PCR MasterMix), and ~200 ng of DNA template. The PCR protocol was an initial 4 min denaturation at 94°C followed by 35 cycles of 40 s at 94°C, 50 s at 56°C, 1 min at 72°C and a final extension for 10 min at 72°C before cooling to 22°C. PCR products were separated by electrophoresis in a 1% agarose gel in 1× TAE buffer and stained with ethidium bromide. For each sample, after electrophoresis, a band was observed at ~1200 bp. This band was excised from the gel and DNA was purified from the agarose with a DNA purification kit (Tiangen, Beijing, China) following the manufacturer’s instructions.

The purified mtDNA fragments were directly sequenced using an ABI3730XL automatic sequencer (Invitrogen Biotechnology Limited Company, Shanghai, China). Because there are special structures (poly G or C) in the control region of the vole there are overlapping peaks in the sequence. Each sample was sequenced twice utilizing the PCR primers and one newly designed internal primer (W1F: AATCTCGCCGAGTGGTATC) in order to avoid base call errors.

1.4 DNA sequence alignment and analysis

We aligned nucleotide sequences using Clustal X Version 1.83 (Thompson et al., 1997) and further refined them manually. To avoid possible base call errors for both ends of the fragment we only utilized the complete control region in subsequent analyses. Gaps resulting from the alignment were excluded from population genetic analysis. Nucleotide polymorphic sites in the mtDNA control region were explored using MEGA Version 3.1 (Kumar et al., 2004), in which genetic distances within and between populations were computed. We constructed a neighbour-joining tree (Saitou and Nei, 1987) based on haplotypes according to Kimura’s 2-parameter model (Kimura, 1980), with M. rossiaemeridionalis and M. kikuchii (GenBank accession numbers: DQ015676 and NC003041) as outgroups. Haplotype (h) and nucleotide diversities (τ; Nei, 1987) were determined by DnaSP 4.0 (Rozas et al., 2003). To determine the population genetic structure within and among populations we performed an analysis of molecular variance (AMOVA, Excoffier et al., 1992) using Arlequin Version 3.0 (Excoffier et al., 2005). Fixation indices ($F_{st}$, Excoffier et al., 1992) were calculated to assess overall genetic divergence and between paired populations. Estimates of gene flow ($Nm$) were derived using the equation: $Nm = [(1/F_{st})-1]/2$ (Slatkin, 1987; Hudson et al., 1992). The statistical significance of the total and pairwise fixation indices was estimated by comparing the observed distribution with a null distribution generated by 10,000 permutations.

We conducted nonparametric correlation analysis between patch size and population genetic diversity using SPSS 16.0 (SPSS Inc., Chicago, USA). In order to test for isolation by distance (IBD) we performed a linear regression of estimates of $F_{st}/(1-F_{st})$ between all pairs of populations against the logarithms of interpopulation geographical distances following Rousset (1997) and Ramirez and Haakonsen (1999). The significance of regression was tested by the Mantel test, performing 5,000 randomizations for the analysis. All of these were finished by using the isolation by distance web service version 3.15 (www.bio.sdsu.edu/pub/andy/IBD.html). Additionally, we constructed a median-joining haplotype network (Bandelt et al., 1999) using Network 4.2.0.1 (http:// www.fluxus-engineering.com) to determine the spatial distribution of haplotypes. We characterized the historical demography of the vole population by examining the observed mismatch distribution (Slatkin and Hudson, 1991) of pairwise differences between sequences, as implemented in Arlequin Version 3.0 (Excoffier et al. 2005) and DnaSP 4.0 (Rozas et al. 2003). Populations that have passed through demographic expansions and genetic bottlenecks are predicted to have a unimodal wave in samples, whereas bimodal or multimodal distributions of sharp peaks are often found in populations that have been constant over time (Rogers and Harpending, 1992; Schneider and Excoffier, 1999). To test the goodness-of-fit of distributions with the expected distributions using a model of population expansion we calculated the sum of squared deviations (SSD) and raggedness index ($r$). To test whether the sequences conformed to the expectations of neutrality of evolution, we used Tajima’s D statistics (Tajima, 1989) and Fu’s $F_s$ (Fu, 1997) as two tests of neutrality in DnaSP 4.0 (Rozas et al., 2003).

2 Results

2.1 Molecular characteristics of the vole mitochondrial control region

We obtained the entire sequence (914–917 bp) of the mtDNA control region, which defined 17 haplotypes among 144 samples (Table 1). We have deposited all
sequences in GenBank with accession numbers FJ597650-FJ597731 and GU474450-GU474511. After alignment, we adopted a 912 bp sequence of the mtDNA control region to analyze genetic variation in the vole. Analysis of the 912 bp sequence of the mitochondrial control region revealed 23 polymorphic sites, consisting of 11 singleton variable sites and 12 parsimony informative sites.

2.2 Different levels of interpopulation variation

Total nucleotide diversity was 0.0021 and haplotype diversity was 0.707. The haplotype and nucleotide diversities of populations in larger patches were higher than that of those in smaller patches (Table 1). There was a positive correlation ($r = 0.943, P < 0.01$) between patch sizes and haplotype diversities of populations.

2.3 Population genetic structure and haplotype analysis

As the AMOVA of the six investigated populations shows, most molecular variance was observed within populations (85.37%), and variance among populations were 14.63% and significant ($F_{st} = 0.146, P < 0.001$) (Table 2). Pairwise $F_{st}$ indicated significant differentiation ($P < 0.01$) between Changsha and the other five populations (Table 3). Pairwise comparisons between Nanzui and each of the five populations (Chunfeng, Changsha, Datonghu, Junshan and Miluo) were significant ($P < 0.05$). The gene flow estimate between the Changsha and Nanzui populations was the lowest ($N_m = 0.841$) among all gene flow estimates.

As shown in Fig. 3, analysis of genetic distance [$F_{st}/(1-F_{st})$] and the logarithm of geographic distance did not yield a positive correlation, and the Mantel test also supported this result with nonsignificant $r^2$ values ($r^2 = 0.47, P > 0.05$). After excluding the Changsha population, we reexamined IBD with the dataset. The result was consistent with the previous one (Mantel test, $r^2 = 0.287, P > 0.05$). It appears that genetic differentiation in the studied populations was not positively related to physical distance.

Based on Kimura’s 2-parameter genetic distances, a neighbour-joining tree of the 17 haplotypes (Fig. 4) was constructed to gain insight into the evolutionary genetics of the voles. The tree showed no geographic genetic structure among the six populations because of promiscuously distributed haplotypes. The median-joining network (Fig. 5) not only showed no geographic structure, but showed H1 and H2 as the predominant haplotype. The short branch lengths and starlike distribution suggested that divergence among the six populations

<table>
<thead>
<tr>
<th>Structure tested</th>
<th>Variance</th>
<th>Sum of Variance</th>
<th>% total</th>
<th>$F_{st}$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among populations</td>
<td>0.143</td>
<td>21.35</td>
<td>14.63</td>
<td>0.146</td>
<td>0.000</td>
</tr>
<tr>
<td>Within populations</td>
<td>0.836</td>
<td>115.35</td>
<td>85.37</td>
<td></td>
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</tbody>
</table>

Fig. 3 Differentiation among *Microtus fortis calamorum* populations. Estimates of $F_{st}/(1-F_{st})$ of all pairs of populations are plotted against logarithms of interpopulation geographical distances

<table>
<thead>
<tr>
<th>Population</th>
<th>Chunfeng</th>
<th>Changsha</th>
<th>Datonghu</th>
<th>Junshan</th>
<th>Nanzui</th>
<th>Miluo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chunfeng</td>
<td>1.105</td>
<td></td>
<td>11.063</td>
<td></td>
<td>3.870</td>
<td></td>
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<tr>
<td>Changsha</td>
<td></td>
<td>0.312(0.000)</td>
<td>1.498</td>
<td>1.115</td>
<td>0.841</td>
<td>0.974</td>
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<tr>
<td>Datonghu</td>
<td>0.043(0.108)</td>
<td>0.250(0.000)</td>
<td></td>
<td>25.514</td>
<td>2.928</td>
<td>20.785</td>
</tr>
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<td>Junshan</td>
<td>-0.019(0.628)</td>
<td>0.310(0.000)</td>
<td></td>
<td>0.019(0.219)</td>
<td>5.016</td>
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<tr>
<td>Nanzui</td>
<td>0.114(0.013)</td>
<td>0.373(0.000)</td>
<td>0.146(0.003)</td>
<td>0.091(0.039)</td>
<td></td>
<td>4.037</td>
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<tr>
<td>Miluo</td>
<td>-0.031(0.838)</td>
<td>0.339(0.000)</td>
<td>0.023(0.218)</td>
<td>-0.040(0.999)</td>
<td>0.110(0.043)</td>
<td></td>
</tr>
</tbody>
</table>

- * could not be estimated because of negative $F_{st}$ values
occurred recently. Interestingly, the Changsha population not only lacked H2, but also lacked unique haplotypes. In addition, the Changsha population shared H4 only with the Chunfeng population (Fig. 5). The pattern of haplotype distribution in the Changsha population suggests that the population may have been established by a founder effect.

2.4 Historical demography

Both Tajima’s D and Fu’s $F_{S}$ negative values supported the hypothesis that the whole sample had passed through a population expansion (Table 4). In addition, the observed mismatch distribution of the whole sample was nearly unimodal, although there was an additional weak peak (Fig. 6a). Both sum of squared deviation (SSD) and Harpending’s raggedness index ($r$) suggested goodness of fit between the observed and the expected distributions, which further supports population expansion. Among the six populations, the observed mismatch distributions of Chunfeng yielded a unimodal pattern with one peak (Fig. 6b), suggesting that the Chunfeng population has experienced a population expansion. The model of population expansion was also supported by non-significant SSD and $r$ values (Table 4). In contrast, the five other populations have been under demographic equilibrium because the shapes of the mismatch distributions were all ragged and multimodal or bimodal (Fig. 6c–g).
Table 4  Statistical tests of neutrality and demographic parameter estimates for *Microtus fortis calamorum* populations and their associated *P*-value (in parenthesis)

<table>
<thead>
<tr>
<th>Goodness of fit tests</th>
<th>Chunfeng</th>
<th>Changsha</th>
<th>Datonghu</th>
<th>Junshan</th>
<th>Nanzui</th>
<th>Miluo</th>
<th>Whole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tajima’s <em>D</em></td>
<td>−0.58(0.34)</td>
<td>1.00(0.84)</td>
<td>0.14(0.61)</td>
<td>−0.88(0.22)</td>
<td>−1.13(0.13)</td>
<td>−0.47(0.37)</td>
<td>−1.53(0.04)</td>
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<tr>
<td><em>F</em>&lt;sub&gt;s&lt;/sub&gt;</td>
<td>−1.69(0.14)</td>
<td>3.53(0.94)</td>
<td>2.85(0.90)</td>
<td>−0.34(0.45)</td>
<td>−1.42(0.12)</td>
<td>2.17(0.88)</td>
<td>−5.87(0.04)</td>
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Demographic parameters

<table>
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<tr>
<th></th>
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<th>Nanzui</th>
<th>Miluo</th>
<th>Whole</th>
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<tr>
<td>SSD</td>
<td>0.017(0.31)</td>
<td>0.18(0.047)</td>
<td>0.086(0.19)</td>
<td>0.105(0.09)</td>
<td>0.038(0.20)</td>
<td>0.168(0.09)</td>
<td>0.014(0.41)</td>
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<tr>
<td><em>r</em></td>
<td>0.06(0.61)</td>
<td>0.67(0.34)</td>
<td>0.28(0.07)</td>
<td>0.39(0.03)</td>
<td>0.33(0.50)</td>
<td>0.63(0.06)</td>
<td>0.05(0.65)</td>
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Fig. 6  Mismatch distribution for each population of *Microtus fortis calamorum*. (a), (b), (c), (d), (e), (f) and (g) represent mismatch distributions for the whole sample set, the Chunfeng, Changsha, Datonghu, Junshan, Nanzui and Miluo populations, respectively. Dashed lines represent the observed distributions and solid lines represent expected ones (under the population growth model).
3 Discussion

3.1 Genetic variation

Habitat fragmentation in landscapes have effects on species’ genetic variation and population demography (Gaines et al., 1997; Bender et al., 1998; Gibbs, 2001; Pergams et al., 2003). Dongting Lake has been changing since the Pleistocene. In the modern age the most notable features of Dongting Lake are the drastic reduction in area, complex river networks and various habitat patches. Patch size has been shown to have potential effects on various populations. Here, when compared to smaller patches (P3, P4, P5), larger patches (P1, P2, P6) are associated with higher genetic diversity (Table 1). Nonparametric correlation analysis indicated that patch size had a positive correlation ($r = 0.943, P < 0.01$) with haplotype diversity of populations. Many studies have demonstrated that both isolated populations and island populations have low genetic variation and were a greater risk than mainland or large island populations (Frankham, 1997; Hinten et al., 2003; Wroblewska et al., 2003; Neumann et al., 2004; Ohnishi et al., 2007). Frankham (1996) demonstrated that genetic variation within species was positively related to island size and to population size, and that island populations have less genetic variation than mainland populations.

3.2 Population genetic structure

We found no evidence of geographic genetic structure (Fig.4). Possible explanations may be as follows: The AMOVA showed that the variation occurred mainly within populations, not among populations (Table 2). Haplotypes from different populations were promiscuously distributed (Fig.4). H1 and H2 were the common and widespread haplotypes in the vole population (Fig.5). There was no obvious clustering of haplotypes of the studied populations. In addition, pairwise $F_{st}$ between any two of the four populations (Chunfeng, Datonghu, Junshan and Miluo) were not significant. Correspondingly, estimates of gene flow between any two of the four populations were very high (Table 3). It seems that there was no genetic differentiation among the four populations because of closer physical distance. Interestingly, there was genetic differentiation between Nanzui and Datongtu despite the closer physical distance. A lack of an isolation-by-distance relationship (Fig. 3) among these populations suggests that genetic differentiation was associated with the latest changes to the Dongting Lake. Wright’s theory of isolated distance has been proved by many studies (Goossens et al., 2001, Floyd et al., 2005, Trizio et al., 2005), but genetic barriers such as the anthropogenic obstructions (roads and clear cuts), may exist even among geographically close populations (Selonen and Hanski 2004, Selonen et al., 2005, Trizio et al., 2005, Lampila et al., 2009). Local landscape factors conducted by the anthropogenic obstructions affect gene flow and determine differentiation (Trizio et al., 2005). The Yangtze vole prefers lower lowland and remains far away from areas of human activity (Guo et al., 1997). Human activities such as extensive road networks and the expansion of agricultural land and urbanization have exacerbated the fragmentation of the Dongting Lake region. Fragmentation caused by anthropogenic obstructions has affected gene flow in these voles and led to population differentiation, despite the closer physical distance.

3.3 Population dispersal and historical demography

The Changsha population lacked unique haplotypes and shared H4 (Table 1, Fig. 5) with the Chunfeng population. The pattern of haplotype distribution in the Changsha population can be explained by genetic drift. Colonizing populations are usually composed of a subset of the genetic diversity present in the source population and spatial expansion can lead to bottleneck and founder effects (Provan and Bennett, 2008). For example, the deer mouse, *Peromyscus maniculatus*, has undergone likely colonization events from the mainland to islands (Ashley and Wills, 1987). Similarly, the Changsha population was composed of a subset of the genetic diversity present in the Dongting Lake populations. Although there was genetic differentiation between the Changsha and Chunfeng populations, gene flow estimation showed 1.1 migrants per generation (Table 3). This situation indicates there is lower dispersal between the Chunfeng population and the Changsha population. Taking an ecological view, the Xiangjiang River is a part of the river network and could be the path by which some voles migrated from Dongting Lake. Our results suggest that the Changsha population could have been established by spatial expansion of the voles distributed around Dongting Lake. On the other hand, Tajima’s $D$ and Fu’s $F_{S}$ negative value (Table 4) and mismatch distribution (Fig. 6a, Fig. 6b) of the vole population indicate that the voles experienced population expansion. Due to the peak in the mismatch distribution close to the Y axis, population expansion likely occurred more recently. The pattern of haplotype distribution in the Changsha population and population expansion of the Chunfeng population reminded of us that population expansion of the vole may be related to events related to
the Changsha population. To better understand this complex connection more work on these animals is needed.

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