Phylogeny of certain members of Hyrcanus group (Diptera: Culicidae) in China based on mitochondrial genome fragments

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Abstract

Background: Species of the Anopheles hyrcanus group are widely distributed in Palearctic and Oriental regions and some of them are important malaria vectors. The cryptic species of An. hyrcanus group was almost impossible to identify based only on their morphology. The phylogenetic relationship of An. hyrcanus group was also not clear.

Methods: Five members of An. hyrcanus group were identified by rDNA ITS2 sequencing as An. yatsushiroensis, An. belenrae, An. kleini, An. lesteri and An. sineroides. The mitochondrial genome fragments were sequenced and annotated using the mitochondrial genome of An. sinensis as reference. Based on the four segments and Joint Data sequences of these species, and other four anopheline species downloaded from GenBank, intraspecific as well as interspecific genetic distances were calculated and the phylogenetic trees were reconstructed by the methods of neighbor joining, maximum parsimony, minimum evolution and maximum likelihood.

Findings: Four parts of mitochondrial genomes, which were partial fragments COI + tRNA + COII (F5), ATP6 + COIII(F7 + F8), ND1(F19) and tRNA (F21), were obtained. All fragments were connected as one sequence (referred as Joint Data), which had a total length of 3393 bp. All fragment sequences were highly conservative within species, with the maximum p distance (0.026) calculated by F19 of An. belenrae. The pairwise interspecific p distance calculated by each fragment showed minor or even no difference among An. sinensis, An. kleini and An. belenrae. However, interspecific p distances calculated by the Joint Data sequence ranged from 0.004 (An. belenrae vs An. kleini) to 0.089 (An. sineroides vs An. minimus), and the p distances of the six members of An. hyrcanus group were all less than 0.029. The phylogenetic tree showed two major clades: all subgenus Anopheles species (including six members of An. hyrcanus group, An. atroparvus and An. quadrimaculatus A) and subgenus Cellia (including An. dirus and An. minimus). The An. hyrcanus group was divided into two clusters as ((An. lesteri, An. sineroides) An. yatsushiroensis) and ((An. belenrae, An. sinensis) An. kleini).

Conclusions: The An. hyrcanus group in this study could be divided into two clusters, in one of which An. belenrae, An. sinensis and An. kleini were most closely related. More molecular markers would make greater contribution to phylogenetic analysis.

Keywords: Anopheles hyrcanus group, Mitochondrial genome fragment, Phylogenetic relationship
Background

*Anopheles hyrcanus* group belongs to the subgenus *Anopheles*, genus *Anopheles*. It is widely distributed in Palearctic and Oriental regions, including 25 species with valid reported mosquito species [1]. There were 22 species of *An. hyrcanus* group distributed in China, including three unnamed ones [2]. Some cryptic species of *An. hyrcanus* group have similar morphological characteristics, making it difficult to identify them based only on their morphology [3]. Moreover, quite a few hybridized individuals were found in field [4, 5] and reestablished phylogenetic trees of *An. hyrcanus* group showed disparity according to various molecular markers [3, 6, 7]. These facts illustrated the complex phylogenetic relationships within *An. hyrcanus* group.

Mitochondrial genome strictly followed maternal inheritance in structure as well as in evolution, with abundant information for genetic and phylogenetic population studies. The mitochondrial genome of *Anopheles* mosquitoes consisted of 13 protein-coding genes, 22 transfer RNA (tRNA) genes, two ribosomal RNA (rRNA) genes and an AT-rich control region [8–10]. At present, certain genes of mitochondrial genome were employed to analyze the interspecific or intraspecific differences. For instance, COI sequence was used as DNA barcoding to distinguish mosquito species [11, 12], while genes such as COI [13], COII [14], ND5 [15] and control region [16] were utilized to detect the genetic population structure of Hycanus group members.

So far, we have reported the complete mitochondrial genome of *An. sinensis* in Hycanus group [9]. In this study, we sequenced and analyzed mitochondrial genome fragments of *An. hyrcanus* group members in China in order to reestablish the phylogenetic relationships and determine the taxonomic status of cryptic species. Moreover, we discussed the contribution of mitochondrial genome fragments in phylogenetic study.

Materials and methods

Ethics statement

No permits were required for the described field studies. Adult mosquito collection in chicken farms and livestock pens was agreed by the owners at each location.

Mosquito collection and species identification

With the consent of the owners, mini light traps (MYFS-HJY-1, Houji Shenzhen, China) were set up in chicken farms and livestock pens between 6:30 pm and 7:30 am. Then the captured *Anopheles* specimens were collected by the mini light traps and manually by entomological aspirators in the evening and killed by freezing before being individually transferred to laboratory in centrifuge tubes for further analysis (Table 1). In the light of the taxonomic key by Lu et al. [17], the samples were morphologically identified as members of *An. hyrcanus* group.

The member species of *An. hyrcanus* group was further identified by molecular markers with rDNA ITS2 sequences. Single mosquito genomic DNA was extracted using DNAzol (Life Technologies, USA) following the manufacturer’s instructions. DNA pellet was dissolved in 80 μl H2O. The rDNA ITS2 fragment was amplified according to the method by Lin et al. and Ma et al. [18, 19]. An ABI 3730 (Boshang Biotech Co., Ltd. Shanghai, China) was applied to purify and sequence the PCR products. Finally, the sequences were Blast aligned in Genebank on the NCBI website to determine the species [18, 19].

Amplification and sequencing of mitochondrial genome fragments

Some fragments of the mitochondrial genome were amplified referring to the universal primers designed for mitochondrial genome of Diptera [20] (Table 2, Additional file 7: Figure S1). The length range of the

| Species             | Collection sites          | Date     | Number of samples |
|---------------------|---------------------------|----------|-------------------|
| *Anopheles yatsushiroensis* | Wuxiang, Shanxi          | June 2017 | 8                 |
|                     | Taian, Shandong           | July 2017 | 3                 |
|                     | Xingcheng, Liaoning       | August 2008 | 2             |
| *An. belenrae*      | Jining, Shandong          | July 2017 | 2                 |
|                     | Donggang, Liaoning        | July 2018 | 2                 |
| *An. kleini*        | Wuxiang, Shanxi           | June 2017 | 11                |
|                     | Tongliao, Inner Mongolia  | August 2018 | 3            |
| *An. lesteri*       | Jiangsu, Lab colony       | September 2018 | 6          |
| *An. sineroides*    | Kuandian, Liaoning        | August 2018 | 1              |
amplified product was from 500 bp to 1200 bp and the overlapping length between adjacent sequences was between 50 bp and 485 bp. The range of percentage of GC was from 40 to 60%, while the annealing temperature was either 45 °C to 47 °C. PCR reaction was run in a 25 μL mixture containing 1 μL DNA template, 0.2 μmol/L primers and other PCR reagents (Aidlab Biotechnologies, China). PCR thermal cycling included a 2 min initial denaturation at 94 °C, followed by 30–35 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C/47 °C for 30 s, elongation at 72 °C for 1 min, and a final extension for 8 min at 72 °C. The PCR products were purified and sequenced by an ABI 3730 machine.

Phylogenetic analysis

The sequences were compared using DNASTAR 7.1 (https://www.dnastar.com/software/lasergene/) [21] and annotation as well as splicing was completed referring to the mitochondrial genome of An. sinensis (GenBank accession No. KT218684.1) [9]. The intraspecific differences (p distance) were calculated by MEGA 7.0 (https://mega.software.informer.com/7.0/) [22] before further analysis was performed. A congruence length test for JD was conducted using consensus sequence as the species-specific identity.

The complete mitochondrial genome of An. sinensis (KT218684.1), An. dirus A (JX219731.1), An. atroparvus (KT382817.1), An. quadrimaculatus (AL04272.1) and An. minimus (KT895423.1) were downloaded from GenBank database. Mitochondrial genome fragment sequences from a total of 10 species were aligned using MEGA 7.0, including An. yatsushiroensis, An. belenrae, An. kleini, An. lesteri and An. sineroides, in addition to the aforementioned five species downloaded from GenBank. The variable bases, parsimony information bases and nucleotide composition were analyzed by MEGA 7.0. In light of the Joint Data (JD) and the separated segments (F5 (COI + tRNA + COII), F7 + F8 (ATP6 + COIII), F19 (ND1) and F21 (lrRNA)), phylogenetic trees were constructed by the methods of neighbor joining (NJ), maximum parsimony (MP) and minimum evolution (ME), respectively. On the other hand, maximum likelihood (ML) tree was reconstructed by PhyML 3.0 (http://phylogeny.lirmm.fr/phylo.cgi/one_task.cgi?task_type=phyml) [23]. The best-fit nucleotide substitution model was obtained by Modeltest 3.7 (http://evomics.org/resources/software/molecular-evolution-software/modeltest/) [24] before ML tree construction. Moreover, bootstrap values for 1000 replicates of all trees were calculated. A congruence length test for JD was performed before analysis [25].

Results and discussion

Sequence characters of mitochondrial genome fragments

Five mitochondrial genome fragments out of the five members in An. hyrcanus group were obtained. Fragment F5 (893 bp in length) comprised segmental COI, full-length tRNA-Leu plus segmental COII (MK690504–MK690508). Due to a partial overlapping found in Fragment F7 and F8, they were connected (denoted as F7 + F8) for further analysis. Its length was 1407 bp and included segmental ATP6 and segmental COIII (MK825734–MK825738). F19, 583 bp in length, was segmental ND1 (MK825739–MK825748). F21 was segmental 16 lrRNA (MK825744–MK825748) with 510 bp in length. As the combined sequence of all fragments, JD had an aggregate length of 3393 bp.

Intraspecific differences

The five fragments’ sequences of mitochondrial genome were aligned among the five members of An. hyrcanus group in this study. The averages of the nucleotide composition as well as the numbers of conserved and variable bases are shown in Additional file 2: Table S1. All fragments’ sequences were highly conservative within species, with the maximum p distance (0.026) calculated by F19 of An. belenrae (Table 3). The reported intraspecific differences of mitochondrial genomes in An. hyrcanus group were as follows: An. lesteri (COII: h = 0.000–0.005; Cyt B: h = 0.000–0.005) [14], An. sinensis (2019) 8:91 (http://evomics.org/resources/software/molecular-evolution-software/modeltest/) [24] before ML tree construction. Moreover, bootstrap values for 1000 replicates of all trees were calculated. A congruence length test for JD was performed before analysis [25].

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| Table 2 Primers for amplification of mitochondrial genome fragments |
|---------------------------------------------------------------|
| Amplication fragments | Primer name | Sequence (5′ → 3′) | Annealing temperature (°C) |
|-----------------------|-------------|---------------------|---------------------------|
| F5: COI + tRNA + COII | 5-F2637     | AGCAGGWTTRTYCAYTGAT | 45                        |
|                       | 5-R3590     | CTCCCTAACGAGGWGKATGT | 47                        |
| F7: ATP6 + COIII      | 7-F4076     | ATTTCTGACTYGYACCCYTCTC | 47                        |
|                       | 7-R4929     | TCTCGAWACATATCCGTACTCAT | 47                        |
| F8: ATP6 + COIII      | 8-F4518     | CGACCWGGACWTTAGCAGWGT | 47                        |
|                       | 8-R5523     | TAYCCTCTCATACARTAAAT | 47                        |
| F19: ND1              | 19-F11982   | AAAGCAAAWCCYCYCCTTC | 47                        |
|                       | 19-R12558   | ATATCCAACTCAGTAAAGGG | 47                        |
| F21: lrRNA            | 21-F12834   | TTACRCCGGTITGAACTCAG | 47                        |
|                       | 21-R13356   | WTAAAGTCTACCTGCCCAC | 47                        |

The sequences were compared using DNAStar 7.1 (https://www.dnastar.com/software/lasergene/) [21] and annotation as well as splicing was completed referring to the mitochondrial genome of An. sinensis (GenBank accession No. KT218684.1) [9]. The intraspecific differences (p distance) were calculated by MEGA 7.0 (https://mega.software.informer.com/7.0/) [22] before further analysis was performed. A congruence length test for JD was conducted using consensus sequence as the species-specific identity.

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than that among the species within the same subgenus Cellia between subgenus An. sinensis fragments showed minor difference among fragment had no interspecific resolution. Moreover, other An. kleini genome showed that F21 sequence was completely conserved fragments (F5, F7 + F8, F19, F21) of mitochondrial genome among the 10 anopheline species in this study Table 4.

The alignment information of the mitochondrial genome fragments among the 10 anopheline species in this study (Additional file 3: Table S2, Additional file 4: Table S3, Additional file 5: Table S4 and Additional file 6: Table S5).

The interspecific p distances calculated by the JD sequence ranged from 0.004 (An. belenrae vs An. kleini) to 0.089 (An. sineroides vs An. minimus), and the p distances of the 6 members of An. hyrcanus group were all less than 0.029 (Table 5).

COI gene was the most common mitochondrial genome fragment for mosquito identification and genetic relationship analysis [7, 12, 13, 29–34]. In addition, the vast majority of intraspecific distances in 122 mosquito species (15 genera) were at levels from 6 to 15% [12]. On the basis of mtDNA COI sequence, the average intraspecific K2P distance of Hyrcanus group 17 species was 0.008 (range: 0.002–0.017) [7]. However, compared with the intraspecific distance, the interspecific distance among cryptic species was smaller, such as An. sinensis vs. An. belenrae (0.009) [7], An. liangshanensis vs An. kunmingensis (0.002), and An. yatsushiroensis vs An. jullianensis (0.003) [35]. The F21 fragments were even the same in both An. kleini and An. belenrae. Therefore, only partial mitochondrial genome fragment sequences were insufficient to accurately explain the phylogenetic relationship within An. hyrcanus group, especially among cryptic species.

**Interspecific differences**

The four different mitochondrial genome fragments and the JD sequences of the five members of An. hyrcanus group in this study, together with the five anopheline species downloaded from the GenBank were analyzed using MEGA 7.0. The ranges of the variable and parsimony information bases were from 46 (F21) to 636 (JD) respectively, and the averages of GC content varied from 24.6 to 27.4% (Table 4).

The pairwise interspecific p distance based on the four fragments (F5, F7 + F8, F19, F21) of mitochondrial genome showed that F21 sequence was completely conserved between An. kleini and An. belenrae, suggesting that F21 fragment had no interspecific resolution. Moreover, other fragments showed minor difference among An. sinensis, An. kleini and An. belenrae. Meanwhile, the p distance between subgenus Cellia and Anopheles species was greater than that among the species within the same subgenus (Additional file 3: Table S2, Additional file 4: Table S3, Additional file 5: Table S4 and Additional file 6: Table S5).

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**Phylogenetic analysis**

The phylogenetic trees were reconstructed using four methods based on the three independent fragments (except F21). All topological structures of the phylogenetic trees based on F7 + F8 and F19 were consistent and split up into two clades. One clade consisted of An. dirus and An. hyrcanus group six species, while the

| Species       | F5     | F7     | F8     | F19    | F21    |
|---------------|--------|--------|--------|--------|--------|
| An. belenrae  | 0.008  | 0.015  | 0.004  | 0.018  | 0.004  |
| An. kleini    | 0.008  | 0.015  | 0.002  | 0.000  | 0.000  |
| An. lesteri   | 0.000  | 0.003  | 0.000  | 0.012  | 0.000  |
| An. yatsushiroensis | 0.010 | N      | 0.009  | N      | N      |

The mean precedes the range presented in parentheses. N denotes the absence of data.

**Table 3** Interspecific p distances of mitochondrial genome fragments of Anopheles hyrcanus group members

**Table 4** The alignment information of the mitochondrial genome fragments among the 10 anopheline species in this study
other included the rest three species (Additional file 8: Figure S2). The topology based on F5 and JD was also consistent. There were two major clades, one of which included all nine species (An. hyrcanus group six species, An. atroparvus and An. quadrimaculatus A) that belonged to subgenus Anopheles, and the other contained An. dirus and An. minimus that belonged to subgenus Cellia (Fig. 1).

The 6 species of An. hyrcanus group were divided into two clusters: ((An. lesteri, An. sineroides) An. yatsushiroensis) and ((An. belenrae, An. sinensis) An. dirus A, ATR An. atroparvus, QUA An. quadrimaculatus, MIN An. minimus). The optimal nucleotide substitution models for F5 and JD were GTR + G and GTR + G + I, respectively. The test result of congruence length for JD showed there was not a congruence length data (P = 0.01). The bootstrap values of ML tree were almost above 47% (Fig. 1).

In An. hyrcanus group, An. sinensis had a very close genetic relationship with An. belenrae and An. kleini. Their adults shared such similar morphology that there were not enough taxonomic characteristics to distinguish them. Anopheles belenrae and An. kleini were reported in 2005 [36], and natural hybridization between An. sinensis and An. kleini were found later [4, 5], indicating possible gene introgression in sympatric population and incomplete reproductive isolation between these two species as well as ongoing speciation. It was known that rDNA ITS2 sequences were the ideal molecular marker to distinguish cryptic species. However, the length of rDNA ITS2 in different mosquito species varied greatly, which led to alignment difficulty. Therefore, it was impossible to reconstruct different mosquito species simultaneously. Partial sequences of mitochondrial genome fragments were not able to provide adequate resolution for cryptic species. However, if mitochondrial genome sequences could be sufficiently informative, such as the employment of joint data connection, the phylogenetic tree of cryptic and distant genetic species could be reconstructed simultaneously.

Conclusions

Anopheles hyrcanus group is widely distributed in Paleartctic and Oriental regions and some of them are important local malaria vectors. The cryptic species of An. hyrcanus group was almost impossible to identify based only on their morphology. In this study, the phylogenetic tree was established by creating mitochondrial genome fragments sequences (JD). It had two major clades, one of which included all Subgenus

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**Table 5** The pairwise interspecific d distances of mtDNA fragments of the 10 mosquito species calculated by Joint Data

|     | YAT | BEL | KLE | LES | SINE | SIN | DIR | ATR | QUA |
|-----|-----|-----|-----|-----|------|-----|-----|-----|-----|
| BEL | 0.020 |     |     |     |      |     |     |     |     |
| KLE | 0.020 | 0.004 |     |     |      |     |     |     |     |
| LES | 0.026 | 0.025 | 0.023 |     |      |     |     |     |     |
| SINE| 0.026 | 0.028 | 0.025 | 0.024 |      |     |     |     |     |
| SIN | 0.020 | 0.005 | 0.005 | 0.027 | 0.029 |     |     |     |     |
| DIR | 0.076 | 0.074 | 0.076 | 0.081 | 0.085 | 0.073 |     |     |     |
| ATR | 0.078 | 0.074 | 0.075 | 0.081 | 0.082 | 0.074 | 0.090 |     |     |
| QUA | 0.074 | 0.071 | 0.071 | 0.074 | 0.078 | 0.070 | 0.086 | 0.063 |     |
| MIN | 0.083 | 0.078 | 0.079 | 0.082 | 0.089 | 0.078 | 0.084 | 0.085 | 0.082 |

YAT An. yatsushiroensis, BEL An. belenrae, KLE An. kleini, LES An. lesteri, SINE An. sineroides, SIN An. sinensis, DIR An. dirus A, ATR An. atroparvus, QUA An. quadrimaculatus, MIN An. minimus
Anopheles species (An. hyrcanus group six species, An. atroparvus and An. quadrimaculatus A) and the other was comprised of An. dirus and An. minimus that belonged to subgenus Cellia. Anopheles hyrcanus group was divided into two clusters as ((An. lesteri, An. sineroides) An. yatsushiroensis) and ((An. belenrae, An. sinensis) An. kleini)). More molecular markers would make greater contribution to phylogenetic analysis.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request, and sequences are available in GenBank.

Ethics approval and consent to participate
This study was carried out in strict accordance with the National Natural Science Foundation of China ethical guidelines for biomedical research involving living animals and human subjects.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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