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Identifying the Main Mosquito Species in China Based on DNA Barcoding

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Abstract

Mosquitoes are insects of the Diptera, Nematocera, and Culicidae families, some species of which are important disease vectors. Identifying mosquito species based on morphological characteristics is difficult, particularly the identification of specimens collected in the field as part of disease surveillance programs. Because of this difficulty, we constructed DNA barcodes of the cytochrome c oxidase subunit 1, the COI gene, for the more common mosquito species in China, including the major disease vectors. A total of 404 mosquito specimens were collected and assigned to 15 genera and 122 species and subspecies on the basis of morphological characteristics. Individuals of the same species grouped closely together in a Neighborhood-Joining tree based on COI sequence similarity, regardless of collection site. COI gene sequence divergence was approximately 30 times higher for species in the same genus than for members of the same species. Divergence in over 98% of congeneric species ranged from 2.3% to 21.8%, whereas divergence in conspecific individuals ranged from 0% to 1.67%. Cryptic species may be common and a few pseudogenes were detected.

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Introduction

Approximately 41 genera and 3500 species and subspecies of mosquito exist worldwide. Although mosquitoes have been studied more extensively than most other insect groups because of their role as vectors of disease, our taxonomic knowledge of these insects is far from complete. Numerous Chinese taxonomists have worked on mosquito classification since 1932, particularly since Edwards provided the modern mosquito classification system [1]. Feng Lan-Zhou reported 100 Chinese mosquito species in 1938 [2]. This number has since then increased to approximately 390 described species and new species are still being identified, particularly within the genera *Armigeres*, *Heizmannia*, *Topomyia* and *Uranotaenia*.

Some species are vectors of medically important pathogens, such as malaria, Dengue fever and Japanese B encephalitis. Species identification therefore constitutes the first step in the surveillance and control of mosquito-borne diseases. The identification of mosquito species is mainly done on the basis of morphological characteristics. This can be problematic because diagnostic morphological features are often damaged during collection or storage, or are not present in all developmental stages. Moreover, the morphological characteristics used to identify intact adult specimens often vary so little between species that usually only experienced mosquito taxonomists are able to distinguish mosquito species reliably [3].

DNA analysis provides a more accurate way of identifying species and the use of molecular data, in combination to morphological methods, has resolved some long-standing taxonomic questions [4,5]. The increase in the number of available molecular markers has facilitated the accurate identification of mosquito species, particularly within groups of sibling species. For instance, *Anopheles anthropophagus* and *Anopheles sinensis* can be identified more simply, rapidly, and accurately using the ITS2 sequence than on the basis of morphology [6,7].

After Tautz proposed using DNA sequences as the main basis of biological classification in 2002 [8,9] Paul Hebert suggested that sequencing the COI gene could allow DNA barcoding that would facilitate such classification [10–12]. Many studies have since then demonstrated that the COI gene is a valid molecular tool for identifying mosquito species [13,14] and revealing cryptic species [15–18].

Although several studies on the distribution of Chinese mosquito species have been conducted using classical morphology identifying sibling and cryptic species remains problematic. Here we provide an updated classification of nearly one-third of China's mosquito species based on a combination of molecular and morphological methods.

Results

Specimen Collection

A total of 122 mosquito species belonging to 15 genera and three subfamilies were collected from sampling sites in eight Chinese provinces (Figure 1, Table 1). We identified mosquitoes on the basis of diagnostic morphological characteristics of their adult and larval stages and cercopoda [19], and by using molecular methods to distinguish sibling species [6,7].

Figure 1. Map of the study area showing the sampling sites of mosquitoes collected in this study. Site 1: Manzhouli City, Neimeng ProvinceXinjiang; Site 2: Yili, Kazakh Autonomous Prefecture, Xinjiang Province; Site 3: Taiyuan City, Shanxi Province; Site 4: Golmud River, QinghaiQinghai Province; Site 5: Tianmu Mountain, Zhejiang Province; Site 6: Zhenxiong County, Yunnan Province; Site 7: Maolan Natural Reserve, Guizhou Province; Site 8: Ruili City, Yunnan Province; Site 9: Mengla County, Yunnan Province; Site 10: Changjiang County, Hainan Province; Site 11: Limushan Nature Reserve, Hainan Province; Site 12: Mangrove Nature Reserve, Hainan Province.
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Sequence Analysis

Individual species were represented by one to eight individuals giving a total of 404 COI sequences, representing 122 species and subspecies. We identified and excluded 3 pseudogenes from further analyses by only selecting sequences without insertions, deletions and stop codons. COI sequences contain a large number of A+T pairs (average of 69% for all codons), particularly at the third codon position (93.4%) (Table S1). There was, however, no G content in *Orthopodomyia anopheloides* and *Topomyia houghtoni* at the third codon. As in the case of *Drosophila* [20,21], this quite strong bias is apparently caused by the relative abundance of iso-accepting tRNA. All sequences contained less T in the first codon compared to the second. However, the A content of the first codon was higher than that of the second. The average *R*-value (transitions/transversions) was 0.7.

Neighbor-Joining (NJ) Tree

The Neighbor-Joining (NJ) tree method is conceptually related to clustering, but without the assumption of clock-like behavior [22]. COI gene fragments accurately revealed species boundaries and provided a clear phylogenetic signal (Figs. 2 and 3). Most of the major branches on the tree represent distinct taxonomic groups, including all genera and subgenera. Moreover, specimens of the same species always grouped closely together, regardless of collection site, and, except for some specimens from Hainan Island, no obvious geographic differences in sequences within the same species were found.

Combining NJ tree and bootstrap analysis is the most appropriate method for evaluating phylogenetic trees using distance methods [23]. Nodes linking sequences of individuals of the same species had a high bootstrap value (98%–99%) whereas some linking sequences of geographically different individuals had low bootstrap values (6%–99%).

Species Boundaries

All species had a distinct set of COI sequences. Excluding the *Culex mirneticus* subgroup and the species listed in Table 2 (see Discussion section), most (98%) conspecific sequences showed $<2\%$ (range $=0\%$ to 1.67%), whereas $>98\%$ of interspecific divergence was in specimens with $>2\%$ K2P divergence (range $=2.3\%$ to 21.8%). Sequence divergence was even higher among species in different genera, ranging from 10.9% to 21.8% (Fig. 4).

Transition and transversion distances varied consistently with sequence divergence (Fig. 5). Transition distance was significantly greater than transversion distance when sequence divergence was $<2\%$. However, transversion distances increased slowly with sequence divergence to eventually exceed transition distances at K2P divergence of $\geq 6\%$. Both transition and transversion distances then decreased until K2P divergence reached about 15%. The relationship between the transversion distance, sequence divergence, and morphological characteristics are shown in Tables 2 and 3.

Discussion

Accuracy of COI

The primary function of DNA barcoding is accurate species identification. We found that COI sequence differences among

Table 1. List of mosquito species, collection sites and GenBank accession numbers.

Mosquito species	Collection site	GenBank accession number
An. lindesayi	Site 6, Yunnan	JQ728147; JQ728148;JQ728149
	Site 7, Guizhou	JQ728370
	Site 5, Zhejiang	JQ728076
An. gigas baileyi	Site 6, Yunnan	JQ728161;JQ728162;JQ728163
An. barbirostris	Site 8, Site 9, Yunnan	JQ728025;JQ728220
	Site 10–12, Hainan	JQ728403;JQ728404;JQ728405
An. barbumbrosus	Site 9, Yunnan	JQ728212
An. jamesii	Site 9, Yunnan	JQ728209
An. messeae	Site 1, Neimeng	JQ728113; JQ728114; JQ728115; JQ728116; JQ728077
	Site 2, Xinjiang	JQ728279; JQ728280
An. sinensis	Site 6, Site 8, Site 9, Yunnan	JQ728141;JQ728388;JQ728389; JQ728390; JQ728391;JQ728343; JQ728233
	Site 10–12, Hainan	JQ728409; JQ728410;JQ728411
	Lab	JQ728020
An. yatsushiroensis	Site 3, Shanxi	JQ728372; JQ728373
An. hyrcanus	Site 2, Xinjiang	JQ728293; JQ728294;JQ728295
An. claviger	Site 2, Xinjiang	JQ728274
An. kweiyangensis	Site 6, Yunnan	JQ728386
	Site 5, Zhejiang	JQ728378
An.sawadwongpormi	Site 12, Hainan	JQ728407; JQ728408
An. peditaeniatus	Site 8, Site 9, Yunnan	JQ728088; JQ728089;JQ728090; JQ728342; JQ728230; JQ728231
An. maculatus	Site 9, Yunnan	JQ728164
An. xui	Site 9, Yunnan	JQ728232; JQ728203
An. tessellatus	Site 8, Site 9, Yunnan	JQ728102; JQ728103
	Site 10–12, Hainan	JQ728050; JQ728051; JQ728052; JQ728053;JQ728054
An. kochi	Site 8, Site 9, Yunnan	JQ728307; JQ728242;JQ728243; JQ728290; JQ728291; JQ728292
An. aitkenii	Site 9, Yunnan	JQ728268;JQ728269; JQ728270
An. pseudowillmori	Site 9, Yunnan	JQ728241
An. vagus	Site 8, Site 9, Yunnan	JQ728070; JQ728042
	Site 10–12, Hainan	JQ728305; JQ728045; JQ728044; JQ728043
An. minimus	Site 9, Yunnan	JQ728026; JQ728027; JQ728028; JQ728029
	Site 10, Hainan	JQ728406; JQ728030
An. aconitus	Site 9, Yunnan	JQ728412; JQ728413; JQ728414; JQ728415; JQ728416
	Site 10, Hainan	JQ728306; JQ728417; JQ728418; JQ728419
An. jeyporiensis	Site 9, Yunnan	JQ728235; JQ728236; JQ728218
An. dirus	Site 12, Hainan	JQ728302; JQ728303
An. splendidus	Site 8, Yunnan	JQ728261
Cx. halifaxia	Site 9, Yunnan	JQ728180; JQ728387; JQ728333
	Site 10, Hainan	JQ728073; JQ728074; JQ728075
Cx. brevipalpis	Site 8, Site 9, Yunnan	JQ728158; JQ728159; JQ728160; JQ728336
	Site 7, Guizhou	JQ728358; JQ728359
Cx. foliatus	Site 9, Yunnan	JQ728234
Cx. minor	Site 9, Yunnan	JQ728188; JQ728189
	Site 12, Hainan	JQ728374
Cx. infantulus	Site 8, Yunnan	JQ728267
Cx. malayi	Site 5, Zhejiang	JQ728092
Cx. richei	Site 5, Zhejiang	JQ728091; JQ728265
Cx. peytoni	Site 9,Yunnan	JQ728379; JQ728380
Cx. spiculosus	Site 8, Site 9, Yunnan	JQ728022; JQ728023; JQ728024
Cx. bicornutus	Site 9, Yunnan	JQ728205

Table 1. Cont.

Mosquito species	Collection site	GenBank accession number
Cx. fuscocephala	Site 8, Site 9, Yunnan	JQ728383; JQ728338; JQ728339; JQ728237; JQ728354
Cx. hayashii	Site 9, Yunnan	JQ728264; JQ728266
Cx. fuscanus	Site 9, Yunnan	JQ728037
Cx. rubithoracis	Site 9, Yunnan	JQ728155
Cx. infula	Site 9, Yunnan	JQ728199
Cx. nigropunctatus	Site 8, Site 9, Yunnan	JQ728087;JQ728347;JQ728348; JQ728206; JQ728207; JQ728208 JQ728071; JQ728072
	Site 10, Hainan	JQ728298
Cx. pipiens	Site 2, Xinjiang	JQ728284; JQ728285; JQ728286
	Lab	JQ728036; JQ728035
Cx.pipiens quinquefasciatus	Site 6, Site 8–9, Yunnan	JQ728381;JQ728382;JQ728327
	Lab	JQ728021
Cx. pipiens pallens	Lab	JQ728040
Cx. pallidothorax	Site 10, Hainan	JQ728057; JQ728058
Cx. whitmorei	Site 9, Yunnan	JQ728304
Cx.bitaeniorhynchus	Site 8–9, Yunnan	JQ728034; JQ728349; JQ728200
Cx. sitiens	Site 10, Hainan	JQ728396; JQ728397; JQ728398; JQ728399; JQ728400; JQ728401; JQ728402
Cx. mimulus	Site 9, Yunnan	JQ728244; JQ728245; JQ728246; JQ728247
	Site 5, Zhejiang	JQ728082; JQ728083; JQ728084; JQ728085; JQ728086
Cx. mimeticus	Site 9, Yunnan	JQ728150; JQ728151; JQ728152
	Site 5, Zhejiang	JQ728078
Cx. murrelli	Site 5, Zhejiang	JQ728079; JQ728080; JQ728081; JQ728017
Cx. vagans	Site 1, Neimeng	JQ728101
Cx. modestus	Site 1, Neimeng	JQ728108; JQ728109; JQ728110; JQ728111; JQ728112
	Site 3, Shanxi	JQ728375; JQ728376
	Site 2, Xinjiang	JQ728296
Cx. tritaeniorhynchus	Site 6, Site 8–9, Yunnan	JQ728031; JQ728350; JQ728346; JQ728238
	Site 10–12, Hainan	JQ728059; JQ728060;JQ728061; JQ728062
Cx. gelidus	Site 9, Yunnan	JQ728366
Ae. prominens	Site 9, Yunnan	JQ728239;JQ728240;JQ728145; JQ728146
Ae. flavescens	Site 1, Neimeng	JQ728104; JQ728105; JQ728106; JQ728107
Ae. dorsalis	Site 1, Neimeng	JQ728117; JQ728118; JQ728119; JQ728120
	Site 4, Qinghai	JQ728317
	Site 2, Xinjiang	JQ728281; JQ728282; JQ728283
Ae. omorii	Site 9, Yunnan	JQ728272
Ae. fengi	Site 5, Zhejiang	JQ728015
Ae. albolateralis	Site 10, Hainan	JQ728394; JQ728395
	Site 7, Guizhou	JQ728365
	Site 9, Yunnan	JQ728289
Ae. khazani	Site 7, Guizhou	JQ728364
Ae. desmotes	Site 7, Guizhou	JQ728361
Ae. tonkinensis	Site 7, Guizhou	JQ728360
Ae. japonicus	Site 6, Yunnan	JQ728181
	Site 5, Zhejiang	JQ728068; JQ728069
Ae. albolineatus	Site 10, Hainan	JQ728308
Ae. chrysolineatus	Site 9, Yunnan	JQ728271
Ae. formosensis	Site 7, Guizhou	JQ728362; JQ728363
	Site 9, Yunnan	JQ728260; JQ728153
Ae. elsiae	Site 9, Yunnan	JQ728332
	Site 5, Zhejiang	JQ728093; JQ728094

Table 1. Cont.

Mosquito species	Collection site	GenBank accession number
Ae. togoi	Lab	JQ728038; JQ728039
Ae. vexans	Site 11, Hainan	JQ728135; JQ728136; JQ728137; JQ728049
	Site 1, Neimeng	JQ728095; JQ728096;JQ728097; JQ728098; JQ728099
	Site 9, Yunnan	JQ728392; JQ728393
	Site 2, Xinjiang	JQ728287; JQ728288
Ae. kasachstanicus	Site 2, Xinjiang	JQ728276; JQ728277; JQ728278
Ae. aegypti	Site 8, Yunnan	JQ728344; JQ728345
	Lab	JQ728041
Ae. novoniveus	Site 7, Guizhou	JQ728368; JQ728369
Ae. dissimilis	Site 9, Yunnan	JQ728018; JQ728385; JQ728384; JQ728259; JQ728258
Ae. craggi	Site 5, Zhejiang	JQ728142; JQ728143
Ae. niveoides	Site 8, Yunnan	JQ728201
Ae. annandalei	Site 8–9, Yunnan	JQ728202; JQ728227
Ae. subsimilis	Site 8, Yunnan	JQ728226
Ae. aureostriatus kanaranus	Site 9, Yunnan	JQ728225
Ae. gilli	Site 9, Yunnan	JQ728215; JQ728216
Ae. albopictus	Site 10–12, Hainan	JQ728063; JQ728064; JQ728065; JQ728066; JQ728067;JQ728299 JQ728300; JQ728301
	Site 7, Guizhou	JQ728192; JQ728193; JQ728194
	Lab	JQ728019
Ae. subalbopictus	Site 7, Guizhou	JQ728198
Ae. pseudalbopictus	Site 7, Guizhou	JQ728197
Ae. albotaeniatus mikiranus	Site 9, Yunnan	JQ728248; JQ728249; JQ728250; JQ728251; JQ728154
Ae. assamensis	Site 9, Yunnan	JQ728190; JQ728191
	Site 7, Guizhou	JQ728355; JQ728356
Ae. Vittatus	Site 10, Hainan	JQ728328
Ae. mediolineatus	Site 12, Hainan	JQ728297
Ae. malikuli	Site 9, Yunnan	JQ728324; JQ728325; JQ728326
Ae. harveyi	Site 8–9, Yunnan	JQ728211; JQ728351; JQ728352; JQ728353
Ar. flavus	Site 9, Yunnan	JQ728321; JQ728322; JQ728323
Ar. durhami	Site 9, Yunnan	JQ728171; JQ728172; JQ728173; JQ728174; JQ728175; JQ728331
Ar. subalbatus	Site 6, Yunnan	JQ728219
	Lab	JQ728033
Hz. proxima	Site 9, Yunnan	JQ728213; JQ728214
Hz. menglianensis	Site 9, Yunnan	JQ728377
Hz. lii	Site 9, Yunnan	JQ728252; JQ728253
Hz. chengi	Site 9, Yunnan	JQ728255; JQ728257
Hz. reidi	Site 8–9, Yunnan	JQ728182; JQ728183; JQ728184; JQ728254; JQ728256; JQ728217
Ur. nivipleura	Site 9, Yunnan	JQ728221; JQ728222
Ur. macfarlanei	Site 11, Hainan	JQ728128; JQ728129; JQ728130; JQ728131; JQ728132; JQ728133; JQ728134; JQ728016
	Site 2, Xinjiang	JQ728311
Ur. lutescens	Site 9, Yunnan	JQ728165; JQ728335; JQ728334
Ur.bicolor	Site 9, Yunnan	JQ728223; JQ728224
Ur. novobscura	Site 8, Yunnan	JQ728357
Ur. jinhongensis	Site 9, Yunnan	JQ728228; JQ728229
Tx. gravelyi	Site 8–9, Yunnan	JQ728144; JQ728341; JQ728330; JQ728210
Tx. edwardsi	Site 9, Yunnan	JQ728337
Tx. splendens	Site 8, Yunnan	JQ728340; JQ728126; JQ728127
Tx. kempi	Site 8, Yunnan	JQ728329

Table 1. Cont.

Mosquito species	Collection site	GenBank accession number
Tx. aurifluus	Site 9, Yunnan	JQ728204
Tr. aranoides	Site 8–9, Yunnan	JQ728166; JQ728167; JQ728168; JQ728169; JQ728170; JQ728262; JQ728263
Tr. tarsalis	Site 5, Zhejiang	JQ728014
	Site 7, Guizhou	JQ728371
Tr. similis	Site 7, Guizhou	JQ728367; JQ728320
Ml. jacobsoni	Site 9, Yunnan	JQ728185; JQ728186; JQ728187; JQ728273
Ml. genurostris	Site 10, Hainan	JQ728046
Cq. crassipes	Site 9, Yunnan	JQ728179
	Site 10–11, Hainan	JQ728121; JQ728122; JQ728123; JQ728124; JQ728125; JQ728319
Cq. richiardii	Site 2, Xinjiang	JQ728309; JQ728310
Cs. nipponica	Site 2, Xinjiang	JQ728316
	Site 1, Neimeng	JQ728100
Cs. annulata	Site 2, Xinjiang	JQ728312; JQ728313; JQ728314; JQ728315
Ma. uniformis	Site 8–9, Yunnan	JQ728176; JQ728177; JQ728178
	Site 10–12, Hainan	JQ728055; JQ728056; JQ728047; JQ728048; JQ728318
Mi. luzonensis	Site 9, Yunnan	JQ728156; JQ728157
Or. anopheloides	Site 12, Hainan	JQ728138; JQ728139; JQ728140
To. houghtoni	Site 9, Yunnan	JQ728195; JQ728196; JQ728275

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congeneric mosquito species were approximately 30 times higher than the average differences within species. Moreover, more than 98% of COI fragments had clear interspecific boundaries, a result consistent with the results of other authors [13]. The average conspecific K2P divergence in this study, 0.39%, is similar to values reported for fish species in Australia [24] and slightly higher than those reported for North American birds (0.27%) [25] and moths (0.25%) [10]. It is slightly less than the K2P divergence value reported for Canadian mosquitoes (0.55%) [13].

Transversion Distance and Speciation

Mitochondrial DNA (mtDNA) functions as a molecular clock in that transversions accumulate in a linear fashion over time [26,27]. Comparison of the molecular and morphological data indicates that the number of transversions may raise to about 7 value without apparent or detectable changes in morphology. (Fig. 5). Transition distance was significantly greater than transversion distance when sequence divergence was below 2% at which level there were almost no morphological differences between specimens. At higher levels of sequence divergence transversion distances slowly increased, eventually exceeding transition distances when sequence divergence reached 6%. Morphological differences were undetectable when sequence divergence was about 2% but were distinct when this reached 6%. Transversion distances increased steadily at sequence divergence levels of 6% to 15% at which level plesiomorphy also first became evident. Plesiomorphy stabilized at sequence divergence of 15%. In addition, the vast majority of intraspecific distances occurred between sequence divergence levels of 6% and 15% whereas most intergeneric distances occurred from 15% to 20% (Fig. 4). Very few intraspecific, and no intergeneric, distances occurred between sequence divergence levels of 2% and 6%.

We found that transversion distances indicated a clear boundary between species. The transversion distance between most species was $<1.1\%$ at sequences divergence values of less than 2%. There were, however, some exceptions; although the transversion distance between two plesiomorphous species was usually $<1.1\%$ (Table 3), some species with anomalous intraspecific COI sequences divergences $>2\%$ (Table 2) had intraspecific transversion distances $>1.1\%$. This suggests the presence of cryptic species, which, if confirmed, in turn suggests that transversion distances may be a useful supplement to barcoding information in species identification**.** Further research on the use of transversion as an additional index of taxonomic similarity is recommended.

Molecular Data Versus Morphology

Sequence divergence values of 14% to 16% were indicative of either interspecific or intergeneric differences. There are two possible reasons for this; temporary substitution saturation of the COI fragment and the limitations of morphological identification.

We found some cases of high intraspecific sequence divergence among *Aedes dorsalis*, *Aedes vexans*, *Culex modestus*, *Tripteroides aranoides*, and *T*oxorhynchites *splendens* (Table 2). Although the degree of niche separation within these species remains unclear, this result suggests the existence of cryptic species. We also detected intraspecific sequence divergence slightly greater than the 2% threshold within *Coquillettidia crassipes* and *Anopheles sinensis* (Table 2). Although no morphological differences within these species were observed, differences in feeding habits and habitat have been documented within *Anopheles sinensis* populations[19]. This, togeth-

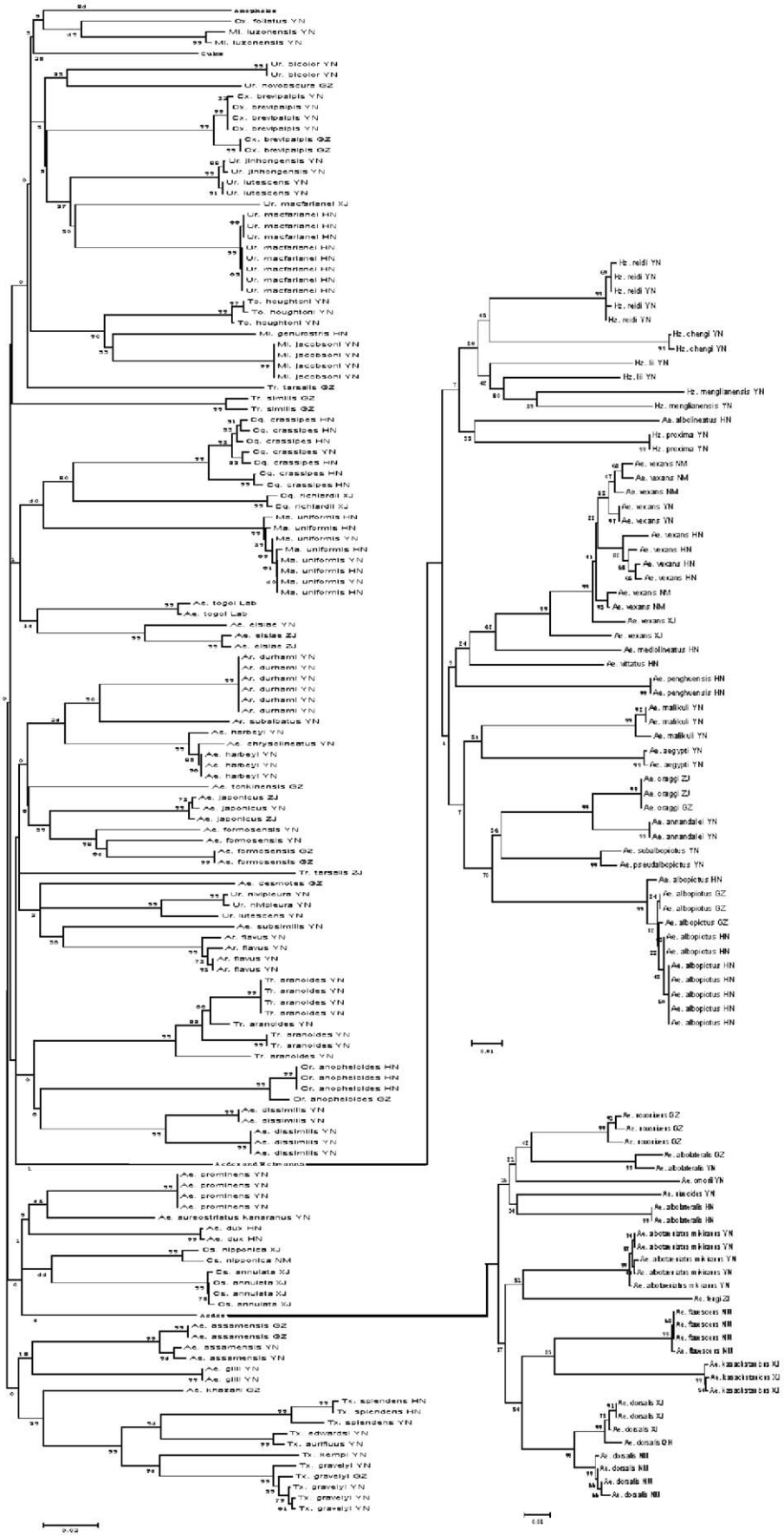
Anopheles
Cx. foliatus YN
Mi. luzonensis YN
Mi. luzonensis YN
Culex
Ur. bicolor YN
Ur. bicolor YN
Ur. novescurs GZ
Cx. brevipalpis YN
Cx. brevipalpis YN
Cx. brevipalpis YN
Cx. brevipalpis YN
Cx. brevipalpis GZ
Cx. brevipalpis GZ
Ur. jinhongensis YN
Ur. jinhongensis YN
Ur. lutescens YN
Ur. lutescens YN
Ur. macfarlanei XJ
Ur. macfarlanei HN
Ur. macfarlanei HN
Ur. macfarlanei HN
Ur. macfarlanei HN
Ur. macfarlanei HN
Ur. macfarlanei HN
Ur. macfarlanei HN
Ur. macfarlanei HN
To. houghtoni YN
To. houghtoni YN
To. houghtoni YN
Mi. genurostris HN
Mi. jacobsoni YN
Mi. jacobsoni YN
Mi. jacobsoni YN
Mi. jacobsoni YN
Tr. tarsalis GZ
Tr. similis GZ
Tr. similis GZ
Cq. crassipes HN
Cq. crassipes HN
Cq. crassipes HN
Cq. crassipes YN
Cq. crassipes HN
Cq. crassipes HN
Cq. crassipes HN
Cq. richiardii XJ
Cq. richiardii XJ
Ma. uniformis HN
Ma. uniformis HN
Ma. uniformis YN
Ma. uniformis YN
Ma. uniformis HN
Ma. uniformis YN
Ma. uniformis HN
Ma. uniformis HN
Ae. togoi Lab
Ae. togoi Lab
Ae. elsiae YN
Ae. elsiae ZJ
Ae. elsiae ZJ
Ar. durhami YN
Ar. durhami YN
Ar. durhami YN
Ar. durhami YN
Ar. durhami YN
Ar. durhami YN
Ar. subalbatus YN
As. hanryi YN
As. chrysolineatus YN
As. harbeyi YN
As. harbeyi YN
As. harbeyi YN
Ae. tonkinensis GZ
Ae. japonicus ZJ
Ae. japonicus YN
Ae. japonicus ZJ
As. formosensis YN
Ae. formosensis YN
As. formosensis GZ
Ae. formosensis GZ
Tr. tarsalis ZJ
As. desmotes GZ
Ur. nivipleura YN
Ur. nivipleura YN
Ur. lutescens YN
As. subsimilis YN
Ar. flavus YN
Ar. flavus YN
Ar. flavus YN
Ar. flavus YN
Tr. aranoides YN
Tr. aranoides YN
Tr. aranoides YN
Tr. aranoides YN
Tr. aranoides YN
Tr. aranoides YN
Tr. aranoides YN
Tr. aranoides YN
Or. anopheloides HN
Or. anopheloides HN
Or. anopheloides HN
Or. anopheloides GZ
Ae. dissimilis YN
Ae. dissimilis YN
Ae. dissimilis YN
Ae. dissimilis YN
Ae. dissimilis YN
Aedes and Armigeres
Ae. prominens YN
Ae. prominens YN
Ae. prominens YN
Ae. prominens YN
As. aurostriatus kanaranus YN
As. dux HN
As. dux HN
Cs. nipponica XJ
Cs. nipponica NM
Cs. annulata XJ
Cs. annulata XJ
Cs. annulata XJ
Cs. annulata XJ
Aedes
Ae. assamensis GZ
Ae. assamensis GZ
Ae. assamensis YN
Ae. assamensis YN
Ae. gilli YN
Ae. gilli YN
As. khazari GZ
Tx. splendens HN
Tx. splendens HN
Tx. splendens YN
Tx. edwardsi YN
Tx. aurifluus YN
Tx. kempi YN
Tx. gravelyi YN
Tx. gravelyi GZ
Tx. gravelyi YN
Tx. gravelyi YN
Tx. gravelyi YN
0.02
Hz. neidi YN
Hz. neidi YN
Hz. neidi YN
Hz. neidi YN
Hz. neidi YN
Hz. changi YN
Hz. changi YN
Hz. li YN
Hz. li YN
Hz. menglianensis YN
Hz. menglianensis YN
Ae. albolineatus HN
Hz. proxima YN
Hz. proxima YN
Ae. vexans NM
Ae. vexans NM
Ae. vexans NM
Ae. vexans YN
Ae. vexans YN
Ae. vexans HN
Ae. vexans HN
Ae. vexans HN
Ae. vexans HN
Ae. vexans NM
Ae. vexans NM
Ae. vexans NM
Ae. vexans XJ
Ae. vexans XJ
Ae. medilineatus HN
Ae. nitidus HN
Ae. penghuensis HN
Ae. penghuensis HN
Ae. malikuli YN
Ae. malikuli YN
Ae. malikuli YN
Ae. aegypti YN
Ae. aegypti YN
Ae. caggi ZJ
Ae. caggi ZJ
Ae. caggi GZ
Ae. annandalei YN
Ae. annandalei YN
Ae. subalbopictus YN
Ae. pseudalbopictus YN
Ae. albopictus HN
Ae. albopictus GZ
Ae. albopictus GZ
Ae. albopictus GZ
Ae. albopictus GZ
Ae. albopictus HN
Ae. albopictus HN
Ae. albopictus HN
Ae. albopictus HN
Ae. albopictus HN
Ae. albopictus HN
Ae. albopictus HN
0.01
Ae. scutellaris GZ
Ae. scutellaris GZ
Ae. scutellaris GZ
Ae. albotaeniatus GZ
Ae. albotaeniatus YN
Ae. arabiensis YN
Ae. riparius YN
Ae. albotaeniatus HN
Ae. albotaeniatus HN
Ae. albotaeniatus mikiamus YN
Ae. albotaeniatus mikiamus YN
Ae. albotaeniatus mikiamus YN
Ae. albotaeniatus mikiamus YN
Ae. albotaeniatus mikiamus YN
Ae. fengi ZJ
Ae. flavescens NM
Ae. flavescens NM
Ae. flavescens NM
Ae. flavescens NM
Ae. kazachstanicus XJ
Ae. kazachstanicus XJ
Ae. kazachstanicus XJ
Ae. dorsalis XJ
Ae. dorsalis XJ
Ae. dorsalis XJ
Ae. dorsalis QH
Ae. dorsalis NM
Ae. dorsalis NM
Ae. dorsalis NM
Ae. dorsalis NM
0.01

Figure 2. NJ phylogenetic tree based on Kimura two-parameter genetic distances of COI gene sequences of mosquitoes prevalent in China. Sequence analysis was conducted using MEGA version 4.0 software with 1000 replications. Most major branches on the tree represent recognized groups, including all genera and subgenera except *Anopheles* and *Culex* which comprise separate subtrees and are shown in detail in Fig.3.
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er with the >2% level of COI sequence divergence, suggests the presence of cryptic species [28]. Some cases of low interspecific sequence divergence were found among some pairs of species (Table 3), including *Aedes craggi* and *Aedes annandalei*, as well as *Culex spiculosus* and *Culex minor*. Although there is no evidence of niche separation between these species, slight morphological differences were observed. This suggests that the taxonomic status of these species should be re-confirmed. Although few doubt that mtDNA barcodes are a valuable molecular tool for matching unidentified specimens to described taxa, there has been relatively little use of barcodes to delimit species [29]. More research on rDNA, morphology, biogeography and ethology are required to improve the applicability of barcoding to species-level taxonomy.

Culex neomimulus was previously classified as *C*ulex *mimulus* in the *C*ulex *mirneticus* group [30]. Although our COI data supports the previous view, we found that anomalous COI sequence divergence values were relatively common in the *C*ulex *mirneticus* group with some morphologically distinct specimens having similar barcodes. This could be due to infection with the *Wolbachia* bacteria. The maternally inherited *Wolbachia* bacteria causes a loss of haplotype diversity in populations by inducing a selective sweep of the initially infected individual's haplotype through a population. We detected *Wolbachia* infection in *Culex mimulus* so it's possible that this may also occur in this species. Although Smith et.al concluded that the presence of *Wolbachia* DNA in total genomic extracts is unlikely to compromise the accuracy of the DNA barcode library, this is a complex problem that requires further investigation [31].

Pseudogenes

The presence of pseudogenes can affect the accuracy of barcoding identification but, since their incidence was <1%, their influence on our data was presumably small. The distinctive characteristics of the COI gene (no insertions, deletions and stop codons) allowed pseudogenes to be easily identified and excluded from the sequences we obtained. Although the leakage of paternal mtDNA may influence the results of barcoding this phenomenon is only occasionally (<0.004%) found in higher animals.

A total of three pseudogenes were detected. For instance, one of the samples of *A*edes *dissimilis* collected from the same area exhibited high interspecific sequence (3.74%) and transversion divergence (3.00%). A total of 12 different protein sequence sites were observed, which is very rare in the Culicidae. The substitution rate at nucleotide codons 1, 2, and 3 was 1:2:2, very different to the average of 5:1:18. We also amplified the pseudogenes of *Uranotaenia lutescens* and *Culex halifaxia*, which have insertions and deletions, respectively. The sequence divergence between pseudogenes and COI fragments in *Culex halifaxia* was 10.93% and the substitution rate at nucleotide codons 1, 2, and 3 was 5:4:11. The divergence time formula of mtDNA and pseudogenes [32] suggests that the nuclear transfer event occurred 500 million years ago in *Culex halifaxia* and 170 million in *Aedes dissimilis*. We found an insertion site at 54 bp in the sequence of *Uranotaenia lutescens*, with a substitution rate at nucleotide codons 1, 2, and 3 of 7:1:18. Two different protein sequence sites were also observed. These abnormal phenomena disappeared when the inserted site was deleted manually. Therefore, these anomalous sequences likely caused by the frameshift mutations of PCR.

Overall, DNA-based species identification systems depend on the ability to distinguish intraspecific from interspecific variation. This analysis of 404 COI sequences from 15 mosquito genera and 122 species and subspecies indicates that >98% of specimens formed distinctive clusters and that barcode divergence was relatively large between these groupings. Although it has limitations, DNA barcode technology has several advantages over traditional taxonomic methods as a tool for species identification. For example, it is unaffected by morphological variation between different life cycle stages. Another benefit is that it allows the homogenization, or calibration, of the taxonomic units identified in different areas. DNA barcode technology generally produces accurate results thereby greatly reducing the need for experienced taxonomists.

In summary, this study provides the first COI barcodes for mosquitoes in China and provides further evidence of the effectiveness of DNA barcoding in identifying recognized species. An insufficient number of specimens prevented in-depth investigation of sibling species complexes but we plan to address this area in the future. Care must be taken to exclude pseudogenes from COI databases to ensure the accuracy of molecular identification. COI databases also need to include specimens of the same species collected from different geographical locations in order to determine the extent of intraspecific variation. A complete evaluation of the effectiveness of DNA barcoding for the Culicidae can be achieved through multinational research.

Materials and Methods

Ethics Statement

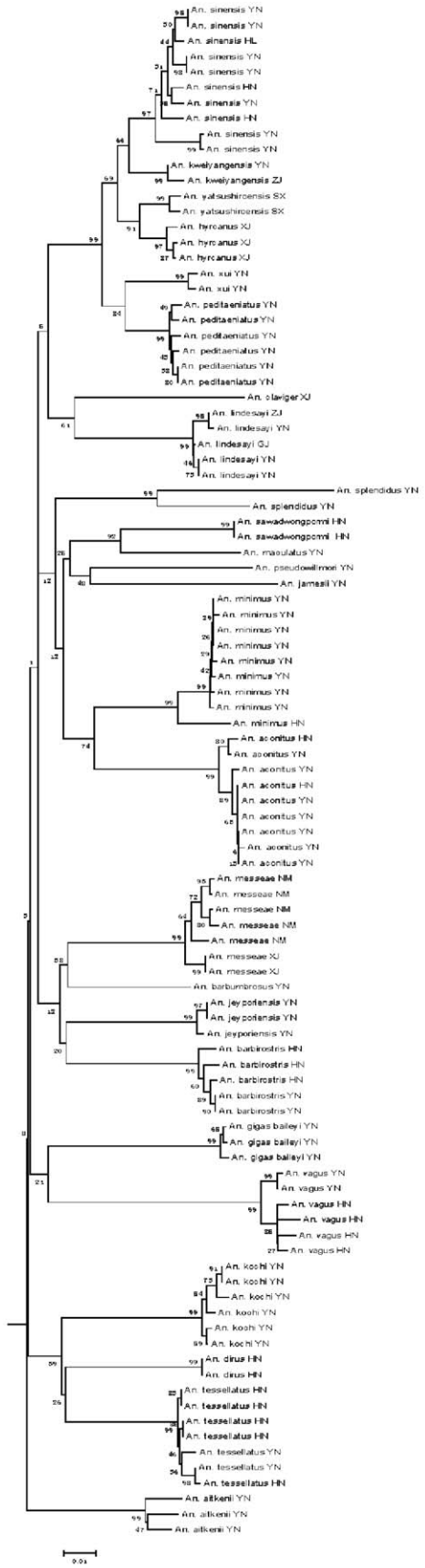
No specific permits were required for this study. All experiments were conducted within state-owned land in China. Therefore, the local ethics committee deemed that approval was unnecessary.

Mosquito Collections

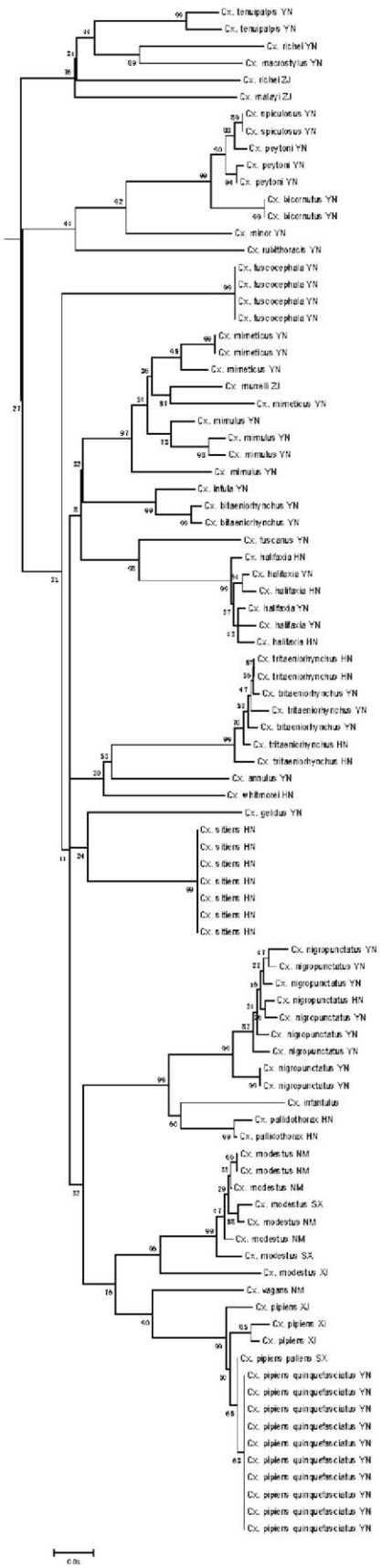
Mosquito specimens used for constructing DNA barcodes were collected from different Chinese Provinces in 2009 and 2010. Details on specimens collected are provided on Fig. 1 and Table. 1. Larval and adult mosquitoes were collected in the field. Adults were sampled with CO_2-baited miniature light traps. Larvae were reared individually and associated larval and pupal skins were mounted. All specimens were identified using standard taxonomic keys [19].

Target Gene Preparation

Total DNA (100 μL to 150 μL) was extracted from each specimen using the Universal Genomic DNA Extration Kit (Invitrogen). PCR was performed to amplify the 5′ COI region of mtDNA using the following cycle: An initial denaturation of 1 min (94°C) followed by five cycles of 94°C for 40 s (denaturation), 45°C for 40 s (annealing), and 72°C for 1 min (extension); 30 cycles of 94°C for 40 s (denaturation), 51°C for 40 s (annealing), 72°C for 1 min (extension) and a final extension at 72°C for 5 min. PCR cocktails were made as follows: A 50 μL solution comprised of 0.3 μL Taq DNA polymerase (5 U/μL), 5 μL of 10×PCR buffer, 5 μL of 2 mmol/L dNTP, 2 μL of 10 μmol/L each of the forward and reverse primers, 5 μL of template DNA and sufficient ddH_2O to make up to 50 μL. The



Subtree: Anopheles



Subtree: Culex

Figure 3. Two distinct sub-trees comprised of *Anopheles* and *Culex* in the NJ phylogenetic tree (Fig. 2).
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Table 2. Intraspecific K2P distance, transversion distance, and morphological characteristics of some mosquitoes.

Species	K2P distance (%)	Transversion distance (%)	Variation in morphological characters
A. dorsalis	2.98	1.11	stripe shape and color of metascutellum
A. vexans	4.71	1.86	mesopleuron and urotergite
T. aranoides	5.72	1.29	stable
T. splendens	2.79	1.29	stable
C. modestus	4.71	1.67	larvae chest hair and male terminalia
C. crassipes	3.57	0.37	stable
A. sinensis	2.61	0.18	stable

doi:10.1371/journal.pone.0047051.t002

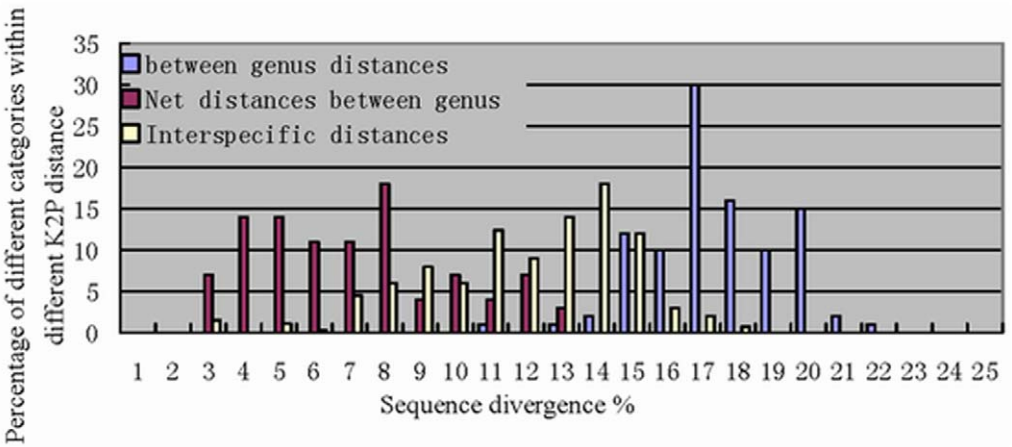

Figure 4. Pairwise comparisons between COI sequences among mosquito species separated into three categories; interspecific distances, between gene distances and net distances between genera. All sequences were grouped with MEGA software, each group includes all species of a particular genus.
doi:10.1371/journal.pone.0047051.g004

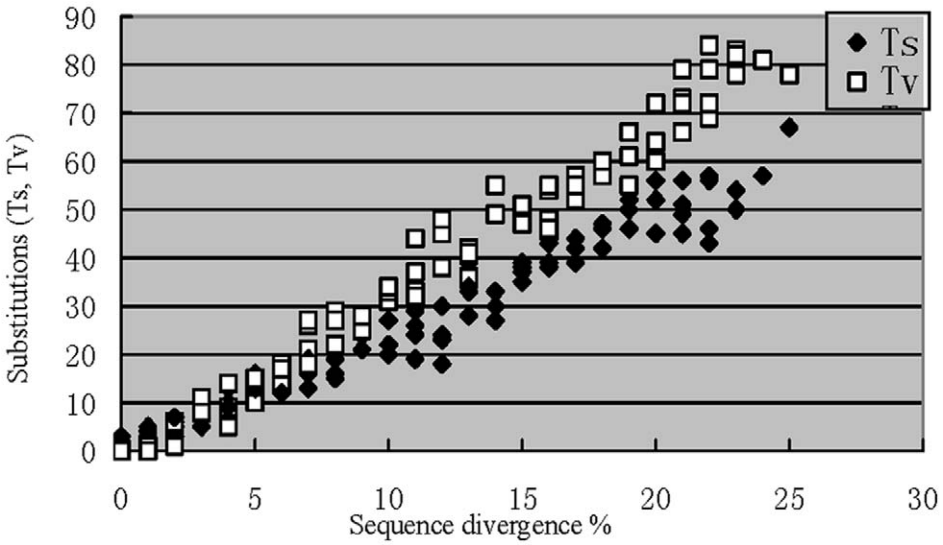

Figure 5. The numbers of COI transitions (ts) and transversions (tv) plotted against sequence divergence.
doi:10.1371/journal.pone.0047051.g005

Table 3. Interspecific K2P distance, transversion distance, and morphological characters of some mosquitoes.

Species	K2P distance (%)	Transversion distance (%)	Variation in morphological characters
Ae. craggi and *Ae. annandalei*	2.99	0.37	male terminalia
Cx. minor and *Cx. spiculosus*	1.86	0.37	male antenna and terminalia

doi:10.1371/journal.pone.0047051.t003

primer pairs LCO1490 and HCO2198 [33] were used to amplify a 650 bp fragment of COI. The amplified fragments were run on a 1% agarose gel to check the integrity of the fragments after which the PCR product was purified with a normal PCR purification kit (Tiangen). Both reads (forward as well as reverse primer) were done.

Data Analysis

DNA sequences were aligned using Clustal X [34]. Sequence analysis and Ts/Tv calculation was conducted using MEGA version 4.0 software [14]. Sequence divergence and Ts, Tv distance among individuals was quantified using the Kimura two-parameter distance model [35]. An NJ tree of K2P distances was created to provide a graphic representation of the clustering pattern among different species [36].

Supporting Information

Table S1 Sequence divergence and nucleotide composition for the mosquito genera. The frequencies of nucleotides in sequence are presented as the total average values for all Condon positions and for each condon position separately with the accuracy to tenths of a percent. (*) Figures in brackets are the number of mosquito species used to estimates of sequence divergence for the genus
(PDF)

Author Contributions

Conceived and designed the experiments: GW TYZ. Performed the experiments: GW ZZ. Analyzed the data: GW. Contributed reagents/materials/analysis tools: GW YDD CXL XXG Z. Wang YMZ DX MDL HDZ XJZ Z. Wu. Wrote the paper: GW.

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