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Phylogeny of the African and Asian *Phortica* (Drosophilidae) deduced from nuclear and mitochondrial DNA sequences

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ABSTRACT

Phylogenetic relationships of 26 *Phortica* species were investigated based on DNA sequence data of two mitochondrial (*ND2*, *COI*) and one nuclear (*28S rRNA*) genes. Five monophyletic groups were recovered in the genus *Phortica*, of which three were established as new subgenera, *Alloparadisa*, *Ashima,* and *Shangrila*. The subgenus *Allophortica* was suggested as the most basal lineage in *Phortica*, followed by the lineage of *P. helva* + *P. sobodo* + *P. varipes*. The remaining *Phortica* species, most of Oriental distribution, formed a monophyletic group, and were subdivided into three lineages (i.e., the subgenera *Ashima*, *Phortica,* and *Shangrila*). The subgenera *Shangrila* and *Phortica* were suggested as sister taxa, and four clades were recovered in the subgenus *Ashima*. The result of reconstruction of ancestral distribution and estimation of divergence times indicates that, the ancestor of the genus *Phortica* restricted to Africa, its initial diversification was dated back to ca. 23 Mya (coinciding with the Oligocene/Miocene boundary); sympatric speciation and an Africa-to-Asia dispersal was proposed to account for the current distribution of *Allophortica* and the rest *Phortica*; most of the rest diversification of *Phortica* occurred in southern China, and the divergence between the African clade and its Oriental counterpart was suggested as a result of vicariance following a dispersal of their ancestral species from southern China to Africa.

1. Introduction

The genus *Phortica* was established by Schiner (1862) with the type species *Drosophila variegata* Fallén. It is one of the largest genera in the subfamily Steganinae, and consists of 122 described species (Chen et al., 2007; Brake and Bächli, 2008; Cheng et al., 2008; Prigent and Chen, 2008; He et al., 2009a). Flies of this genus feed on the tree sap fluxes (Cheng et al., 2008). Most (ca. 110) of *Phortica* species were recorded from East and Southeast Asia and Africa, with the remainder found in Europe, the Mediterranean region, North and Central America, Australia and the Pacific area. The habitats of *Phortica* are less than 2700 m in elevation.

Wheeler (1952) treated *Phortica* as a subgenus in the genus *Amiota* Loew. Máca (2003) elevated *Phortica* to generic rank, established in this genus the subgenus *Allophortica* for two African and one European species. Based on some plesiomorphic characters specific to *Allophortica*, Máca (2003) considered this subgenus as the first branching one within the genus *Phortica*. Most *Phortica* species share the following autapomorphic characters: ocellar triangle with a pair of small setae below ocellar setae (Fig. 1A); proclinate orbital seta nearer to inner vertical seta than to ptilinal fissure (Fig. 1A); subscutellum protruded (Fig. 1C); palpus with a hollow sense organ except for the subgenera *Allophortica* and *Sinophthalmus* (Fig. 1B).

The phylogenetic structure of the genus *Phortica* is presently not well established. A cladistic analysis by Grimaldi (1990) proposed sister clade status of *Sinophthalmus* and the genus *Apenthecia* Tsacas. Otranto et al. (2008) reconstructed the relationship between eight genera including 13 species in the subfamily Steganinae using DNA sequences of the mitochondrial *COI* gene. Their result suggested that the genus *Phortica*, which was represented in this study by three species in the *P. variegata* species complex, is monophyletic and distinct from the genus *Amiota*.

The present study employed one nuclear (*28S*, 28S ribosomal RNA) and two mitochondrial (*ND2*, NADH dehydrogenase, subunit 2; *COI*, cytochrome *c* oxidase, subunit I) genes to reconstruct the phylogenetic relationships of 26 species in the genus *Phortica* (representing all of the major lineages in this genus except for *Sinophthalmus*). The obtained phylogeny was used to discuss the

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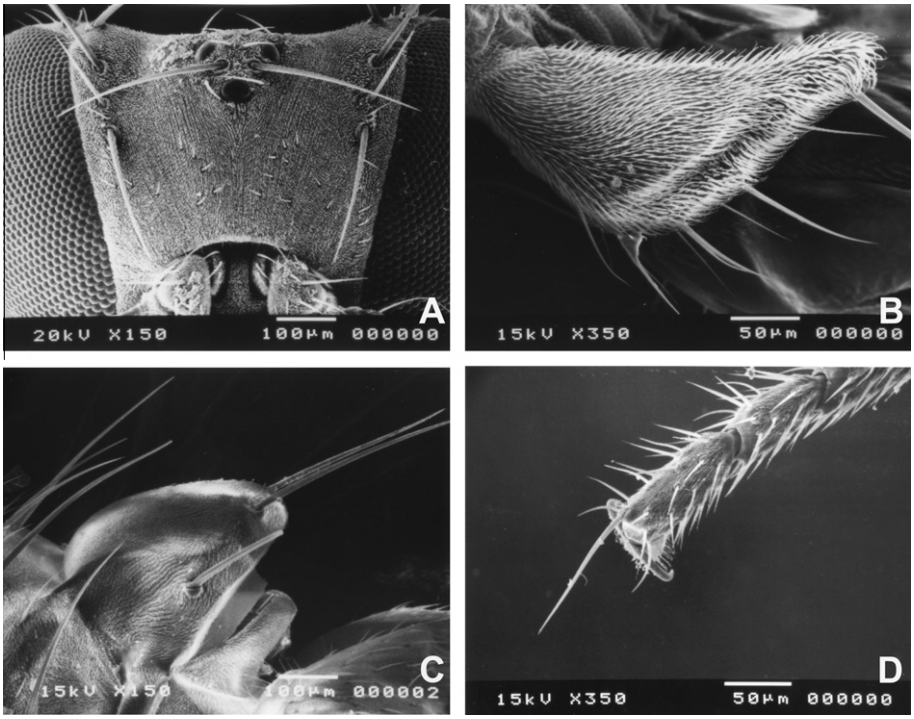

Fig. 1. Autapomorphical characters of the genus *Phortica*: (A) ocellar tubercle and front of *Phortica variegata* Fallén; (B) palpus of *Phortica kappa* Máca; (C) subscutellum of *Phortica pappi* Tsacas and Okada; (D) fore-leg of *Phortica foliiseta* Duda.

evolutionary history of the genus *Phortica*. Based on the result of our phylogenetic reconstruction, the genus *Phortica* was taxonomically revised. Moreover, for the sake of practicality in taxonomy, necessary subdivision of this large taxon was done based on the phylogenetic relationships, thus leading to the establishment of three new subgenera.

2. Materials and methods

2.1. Taxon sampling, DNA extraction, PCR and sequencing

A total of 26 *Phortica* species were employed in our phylogenetic analysis. Three species, each from the genera *Apenthecia*, *Apsiphortica* and *Amiota*, were used as outgroups (Table 1). The sampled *Phortica* species comes from the previous *P. foliiseta*, *P. hani*, *P. magna* and *P. omega* species complexes and the *P. varipes* species group.

Flies were preserved in 75% ethanol when collected in field, and then identified in laboratory. Before extracting DNA, flies were treated in TE buffer for about 12 h to remove ethanol, and then a small portion of each individual fly was used for DNA extraction following the phenol/chloroform method of Gloor and Engels (1992) with slight modifications. The primer pairs used for amplifying DNA fragments from the *ND2*, *COI* and *28S* target genes are listed in Table 2. The PCR cycle program comprised an initial 3 min of predenaturation at 94 °C, 35 cycles of amplification (45 s of denaturation at 94 °C, 45 s of annealing at the optimal temperature, *ND2* (54.5 °C), *COI* (54 °C), *28S* (56 °C), and 1 min of extension at 72 °C), and 5 min of post-extension at 72 °C. The purified amplicons were either sequenced directly or as plasmid inserts (pMD18-T; Takara, China) after cloning and replication in *Escherichia coli* TG-1 as the host. DNA sequences were determined using BigDye terminator chemistry (PE applied Biosystems) and an ABI 3700 sequencer.

2.2. Sequence alignment and characterization

The obtained sequences were edited with the SeqMan module of the DNAStar package (http://www.dnastar.com/) and submitted to GenBank. The GenBank accession numbers of the newly determined sequences and those obtained from GenBank and included into our analysis are shown in Table 1. Sequences were aligned using ClustalW (Thompson et al., 1994) and edited manually using the MEGA 4.0 package (Tamura et al., 2007). Homogeneity of the base composition among taxa was tested using the Chi-square test implemented in PAUP* 4.0b10 (Swofford, 2000). The program DAMBE (Xia and Xie, 2001) was used to test the nucleotide substitution saturation in our sequence data partitioned by locus and/or codon positions. The PHT (partition homogeneity test) in PAUP* was employed to test whether the sequences of different loci could be analyzed simultaneously.

2.3. Phylogenetic reconstruction and taxonomic account

Phylogenetic trees were constructed by simultaneously analyzing alignments of concatenated sequences of the three loci using the maximum likelihood function in PAUP* as well as Bayesian Inference (MrBayes 3.1; Ronquist and Huelsenbeck, 2003). Modeltest 3.7 (Posada and Crandall, 1998) was used to select the most representative nucleotide substitution model for analyses of the concatenated sequences of the three loci as well as for partitioned data sets of the concatenated sequences with the following arrangements: (1) three data sets partitioned by gene locus: *ND2*, *COI* and *28S* and (2) three data sets partitioned by codon position and also gene locus, with the two mitochondrial genes treated as a whole: $mtCP_{1+2}$ (*ND2* + *COI*, 1st + 2nd codon positions), $mtCP_3$ (*ND2* + *COI*, 3rd codon position) and 28S.

In the ML analysis, the model was assigned according to the result of model selection for the concatenated sequences [number of substitution types (Nst), substitution rates matrix (Rmat), nucleotide frequencies (Freqs), proportion of invariable sites (*I*) and gamma distribution shape parameter (*a*)]. The optimal tree was searched using the heuristic method, with the initial trees obtained by randomly addition of taxa (1000 replicates, with 10 trees held at each step), and then branches swapped with the TBR (tree-bisection-reconnection) algorithm. Node confidences were assessed by bootstrap analyses (200 replicates).

Table 1
Taxon sampling and GenBank accession numbers of DNA sequences.

Genus	Subgenera	Species	Collection sites	*COI*	*ND2*	*28S*
Amiota	–	*protuberantis* Cao and Chen, 2009	Hainan, China	HQ011958	FJ360720[a]	HQ011929
Apsiphortica	–	*longiciliata* Cao and Chen, 2007	Yunnan, China	HQ011959	HQ011982	HQ011930
Apenthecia	*Apenthecia*	*argentata* Tsacas, 1983	Kakamega, Kenya	HQ011960	HQ011981	HQ011931
Phortica	*Allophortica*	*sexpunctata* (Séguy, 1938)	Kakamega, Kenya	HQ011961	HQ011983	HQ011932
	Alloparadisa subgen. n.	*helva* Chen and Gao, 2008	Yunnan, China	EU500837[b]	EU500840[b]	HQ011933
		sobodo Burla, 1954	Kakamega, Kenya	HQ011962	HQ011984	HQ011934
	Shangrila subgen. n.	*hani* Zhang and Shi, 1997	Sichuan, China	EU431943[b]	EU431926[b]	HQ011935
		pinguiseta Cao and Chen, 2009	Sichuan, China	EU431946[b]	EU431924[b]	HQ011936
	Ashima subgen. n.	*angulata* Prigent and Chen, 2008	Kakamega, Kenya	HQ011963	HQ011985	HQ011937
		machoruka Prigent and Chen, 2008	Kakamega, Kenya	HQ011964	HQ011986	HQ011938
		melanopous Prigent and Chen, 2008	Kakamega, Kenya	HQ011965	HQ011987	HQ011939
		manjano Prigent and Chen, 2008	Kakamega, Kenya	HQ011966	HQ011988	HQ011940
		curvispina Prigent and Chen, 2008	Kakamega, Kenya	HQ011967	HQ011989	HQ011941
		afoliolata Chen and Toda, 2005	Hainan, China	HQ011968	HQ011990	HQ011942
		xishuangbanna Cheng and Chen, 2008	Yunnan, China	HQ011969	HQ011991	HQ011943
		foliiseta Duda, 1923	Guangdong, China	HQ011970	HQ011992	HQ011944
		foliisetoides Chen and Toda, 2005	Hainan, China	HQ011971	HQ011993	HQ011945
		nudiarista Cheng and Chen, 2008	Yunnan, China	HQ011972	HQ011994	HQ011946
		sagittaristula Chen and Wen, 2005	Yunnan, China	HQ011973	HQ011995	HQ011947
		saltiaristula Chen and Wen, 2005	Yunnan, China	HQ011974	HQ011996	HQ011948
		speculum (Máca and Lin, 1993)	Jiangxi, China	EU500838[b]	EU500839[b]	HQ011949
		huiluoi Cheng and Chen, 2008	Yunnan, China	HQ011975	HQ011997	HQ011950
		tanabei Chen and Toda, 2005	Guangdong, China	HQ011976	HQ011998	HQ011951
		spinosa Chen and Gao, 2005	Yunnan, China	HQ011977	HQ011999	HQ011952
		glabra Chen and Toda, 2005	Guangdong, China	HQ011978	HQ01200	HQ011953
		longipenis Chen and Gao, 2005	Yunnan, China	HQ011979	HQ01201	HQ011954
	Phortica	*okadai* (Máca, 1977)	Tokyo, Japan	EU431942[b]	EU431920[b]	HQ011955
		magna (Okada, 1960)	Tokyo, Japan	EU431941[b]	EU431919[b]	HQ011956
		omega (Okada, 1977)	Yunnan, China	HQ011980	HQ01202	HQ011957

[a] Determined in He et al., 2009b.
[b] Determined in He et al., 2009a.

Table 2
Primers used for PCR and sequencing in this study.

Primer name	Primer sequence (5′–3′)	Utility	References
COI-1	ATCGCCTAAACTTCAGCCAC	PCR/sequencing	Wang et al. (2006)
COI-2	TCCATTGCACTAATCTGCCA	PCR/sequencing	Wang et al. (2006)
COI-F1	CGCCTAAACTTCAGCCACTT	PCR/sequencing	He et al. (2009b)
COI-R1	CCTAAATTAGCTCATGTAGAC	PCR/sequencing	He et al. (2009b)
ND2-H1	AAGCTACTGGGTTCATACC	PCR/sequencing	Park (1999)
ND2-T1	ATATTTACAGCTTTGAAGG	PCR/sequencing	Park (1999)
ND2-T2	GCTTTGAAGGCTATTAGTT	PCR/sequencing	He et al. (2009b)
ND2-T3	AGGCGATAGATTGTAAATC	PCR/sequencing	He et al. (2009b)
28S-F1	GACTACCCCCTGAATTTAAGCAT	PCR/sequencing	Kim et al. (2000)
28S-F2	CAACCTCAACTCATATGGGAC	PCR/sequencing	Present study
28S-R1	CCATCTTTCGGGTCACAGCAT	PCR/sequencing	Present study
28S-R2	GTTCACCATCTTTCGGGTCA	PCR/sequencing	Present study

Bayesian inference was conducted with either a unitary model for the concatenated sequences, or site-specific models for data sets partitioned by the two above-mentioned schemes. Inference criteria (Nst, substitution rates, invariable sites) were selected according to the results of model, and parameter estimates (Rmat, Freqs, *I* and *a*) did not assume linkage. In addition, the sampling frequency were set as every 100 generations, and numbers of chains = 4. The two parallel runs were stopped after the average deviation of split frequencies fell well below 0.01. Therefore, in all analyses, full runs of 2,000,000 generations were performed. In each analysis, 5000 early-phase samples were discarded as burn-in for each run yielding a total of 30,002 trees per run to construct a 50% majority consensus tree with nodes characterized by posterior probability.

2.4. Biogeographic analysis

Based on the phylogenetic tree constructed in the present study (Fig. 2), we used the program DIVA (dispersal–vicariance analysis) version 1.2 (Ronquist, 1996) to reconstruct the ancestral distributions in the genus *Phortica*. To avoid the basal trichotomy (*Amiota*, *Apsiphortica*, (*Apenthecia*, *Phortica*)) in the tree (Fig. 2), the genus *Amiota* was removed. In DIVA optimization, the current distribution of the genus *Phortica*, which covers the likely ancestral distributions of this genus, was divided into four unit areas: P (Palearctic region), sC (southern China), seA (Southeast Asia) and Af (Afrotropical region). In addition, based on the distribution of most of the extant *Phortica* species, we restricted the maximum number of unit areas allowed in ancestral distributions as four (i.e., the total number of unit areas inhabited by the terminals).

2.5. Estimation of divergence times

Based on the Bayesian tree inferred without data partitioning, we estimated the divergence time at each node using r8s1.71 (Sanderson, 2003). Given the absence of internal calibration point in the subfamily Steganinae of family Drosophilidae, we used a "outgroup" molecular clock of *ND2 + COI* sequences of species from the genus *Drosophila*, subfamily Drosophilinae. This clock was "calibrated" using the divergence times among these species estimated

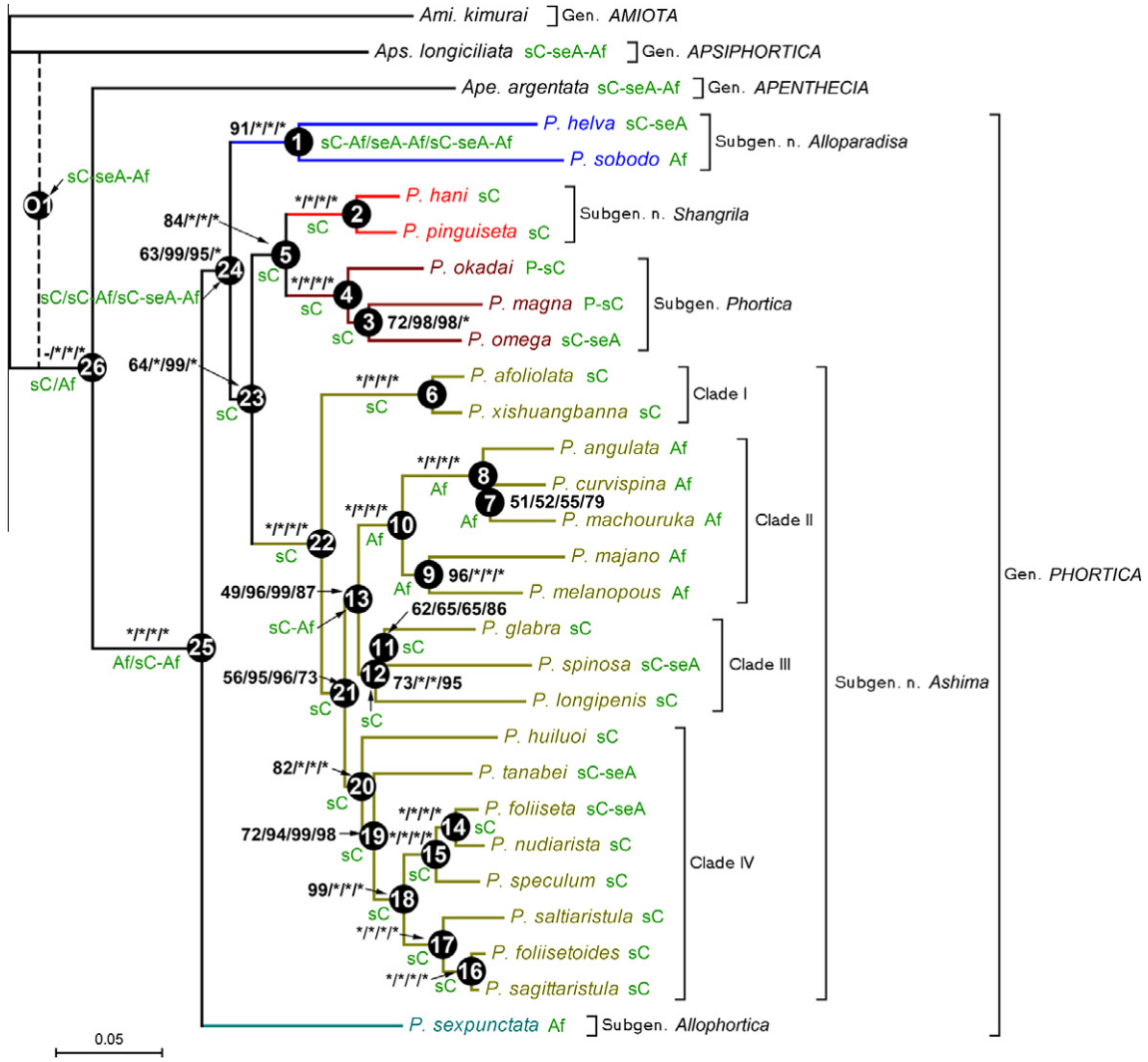

Fig. 2. Bayesian tree constructed with a unitary model for the concatenated sequences of the three genes *ND2*, *COI* and *28S*. The numbers (substituted with asterisks when equal to 100) above branches indicate the node confidences in different analyses, separated by slashes and given in the order of (1) bootstrap percentages of the ML analysis; (2) posterior probabilities (PP) of the Bayesian analysis with unitary model; (3) PP of the Bayesian analysis with data partitioned by gene locus (*ND2*, *COI* and *28S*); (4) PP of Bayesian analysis with data partitioned by codon position and also gene locus ($mtCP_{1+2}$, $mtCP_3$ and *28S*). Letters below branches indicating the estimated ancestral area(s) on the corresponding ancestral node; letters to the right of the species names represent respective current distribution; those to the right of the generic names of *Apenthecia* and *Apsiphortica* represent the respective likely ancestral distribution. Abbreviations: P (Palearctic region), sC (southern China), seA (Southeast Asia) and Af (Africa).

by Tamura et al. (2004) [i.e., 62.9 Mya (Million years ago) between the subgenera *Drosophila* and *Sophophora*, 62.2 Mya between the *D. melanogaster* and *D. willistoni* species groups, 54.9 Mya between the *D. melanogaster* and *D. obscura* species groups, 17.7 Mya between *D. pseudoobscura* and *D. subobscura*, 12.6 Mya between *D. melanogaster–D. simulans* and *D. erecta*, and 5.4 Mya between *D. melanogaster* and *D. simulans*]. We calculated the genetic distances for each of these taxon-pairs using the corresponding *ND2* + *COI* sequences [available from the GenBank entries BK006335 (Drosophila 12 Genomes Consortium, 2007), NC_001709 (Lewis et al., 1994), FJ899745 (Torres et al., 2009), NC_005781 (Ballard, unpublished data), BK006338 (*Drosophila* 12 Genomes Consortium) and NC_001322 (Clary and Wolstenholme, 1985)]. Then, a regression line of $T = 2452.4D^{2.1048}$ ($R^2 = 0.87$) was calculated from a plot of the above-calculated genetic distances (with the Tamura–Nei model, and gamma-distributed rates among sites) on the corresponding divergence times (i.e., *T*). The time for the ancestor of the genus *Phortica* was figured out based on this regression line, and this time was used as calibration point in the time estimation using the software r8s and the r8s bootstrap kit (Eriksson, 2007), following the procedure as in Gao et al. (2011). In our r8s analyses, one of the outgroup taxa, *Amiota protuberantis*, was pruned from the tree to avoid the basal trichotomy. The mean divergence times, their standard deviation and the 95% confidence intervals were therefore estimated.

3. Results

3.1. Sequence characterization

The alignment of the concatenated sequences is of 3512 nucleotide positions (1026, 1539 and 947 for *ND2*, *COI* and *28S*, respectively) in length, of which 1224 (512, 540 and 172, respectively) are variable, and 841 (347, 408 and 86, respectively) are parsimony-informative. The Chi-square test revealed no significant base composition heterogeneity among the taxa employed, even at the 3rd codon positions of the *ND2* or *COI* sequences ($p = 0.5499$ and 0.1856, respectively). None of the DAMBE test yielded observed index of substitution saturation (I_{ss}) greater than the critical one

($I_{ss.c}$), with the exceptions that, only for an extreme asymmetrical tree, I_{ss} is greater than $I_{ss.c}$ at the 3rd codon position of the *ND2* and *COI* sequences ($P = 0.0191$ and $P = 0.000$, respectively). Therefore, there is little substitution saturation in our sequence data.

In the PHT test, no significant phylogenetic incongruence was found among the three data partitions ($p = 0.136$ for *ND2* vs. *COI*, 0.25 for *ND2* vs. *28S*, and 0.102 for *COI* vs. *28S*), indicating that the three data partitions could be analyzed simultaneously in the phylogenetic reconstruction.

3.2. Model selection and phylogenetic reconstruction

The result of model selection using Modeltest 3.7 is shown in Table 3. The majority consensus trees of the Bayesian analyses under different data partitioning schemes yielded same tree topology, therefore, only the one deduced with a unitary substitution model is shown (Fig. 2). The node confidences in the ML analysis (bootstrap percentages, or BPs), as well the posterior probabilities (PPs) obtained by Bayesian analyses with site-specific models, are also shown in Fig. 2.

The results in Fig. 2 and Table 4 indicate monophyly of the genus *Phortica* (PP = 1.00 in all the three analyses; BP = 100). In addition, the results suggest that the genus *Phortica* is closer related to the genus *Apenthecia* than to *Amiota* or *Apsiphortica* (PP = 1.00). Five major lineages were recovered within the genus *Phortica*: (1) the new subgenus *Alloparadisa* to be established in the present study (PP = 1.00; BP = 91); (2) the new subgenus *Shangrila* to be established in the present study (PP = 1.00; BP = 100); (3) the subgenus *Phortica* s.s. (PP = 1.00; BP = 100); (4) the new subgenus *Ashima* to be established in the present study (PP = 1.00; BP = 100); and (5) the subgenus *Allophortica* represented by only one species. The subgenus *Allophortica* appears to be first branching branches among the five subgenera, with the remaining ones (*Alloparadisa*, *Ashima*, *Phortica* s.s. and *Shangrila*) collectively forming a large monophyletic cluster (PP = 0.95–1.00; BP = 63). Within this large cluster, the new subgenus *Alloparadisa* can be considered the most basal one, with the remaining three subgenera clustered together (PP = 0.99–1.00; BP = 64). The genera *Shangrila* and *Phortica* cluster as sister taxa (PP = 1.00; BP = 84).

Within the new subgenus *Ashima*, four groups, henceforth referred to as Clades I–IV, were recovered. Among them, the Clade II exclusively consisting of African species was placed as the sister taxon of the Clade III consisting of Chinese and Southeast Asian species. However, the Bayesian and ML support for this cladistic relationship are relatively low (PP = 0.87–0.99; BP = 49). The Clades II and III collectively were suggested as closer to the Clade IV than to Clade I (PP = 0.73–0.96; BP = 56).

Table 4
Divergence times estimated for the nodes of the Bayesian tree. Branch/node codes correspond to those shown in Fig. 2.

Node	Clade	Divergence time (Mya)	
		Mean	95% CI*
O1	Tree root	39.7	31.9–49.5
26	Gen. *Apenthecia* + gen. *Phortica*	32.6	27.5–38.2
25	Gen. *Phortica*	22.7**	–
24	Subgen. *Alloparadisa* + *Shangrila* + *Phortica* + *Ashima*	20.7	18.4–22.3
23	Subgen. *Shangrila* + *Phortica* + *Ashima*	19.2	17.0–21.5
22	Subgen. *Ashima*	14.0	11.9–16.4
21	Clade II + clade III + clade IV	12.4	10.4–14.9
20	Clade IV	11.1	8.7–13.8
19		10.1	7.8–12.7
18		7.7	5.6–9.7
17		4.5	3.1–6.0
16		1.2	0.5–2.9
15		4.6	3.2–6.4
14		2.7	1.7–5.4
13	Clade II + clade III	11.5	9.9–14.4
12	Clade III	10.4	8.8–13.0
11		9.8	8.4–12.3
10	Clade II	8.9	7.3–10.8
9		7.3	5.5–9.6
8		4.1	3.1–5.4
7		3.7	2.8–4.9
6	Clade I	3.0	1.9–4.4
5	Subgen. *Shangrila* + *Phortica*	16.0	13.1–19.5
4	Subgen. *Phortica*	10.0	7.1–12.9
3		8.4	6.2–10.9
2	Subgen. *Shangrila*	6.1	4.4–9.0
1	Subgen. *Alloparadisa*	16.4	13.2–19.2

* CI = confidence interval.
** The calibration point.

3.3. Reconstruction of ancestral areas in the genus Phortica

So far four species has been described and assigned in the subgenus *Allophortica*: *P. fenestrate*, *P. sexpunctata* and *P. vumbae* from Africa, *P. oldenbergi* from Germany (Máca, 2003; Prigent and Chen, 2008). *P. oldenbergi* has not been rediscovered since it was described by Duda (1924). Bächli et al. (2004) and Máca (2003) considered it as an introduced species from Africa to Europe. Accordingly, in our DIVA optimization, we assumed the ancestral

Table 3
Results of model selection using Modeltest 3.7.

Model	Data set					
	ND2 GTR + I + G	*COI* GTR + I + G	$mtCP_{1+2}$ TIM + I + G	$mtCP_3$ K81uf + I + G	*28S* GTR + I + G	Concatenated sequence GTR + I + G
Freqs						
A	0.3825	0.3212	0.2593	0.4567	0.3634	0.3334
C	0.0654	0.0752	0.1669	0.0438	0.1304	0.0972
G	0.0331	0.1513	0.1682	0.0190	0.1671	0.1297
T	0.5190	0.4523	0.4056	0.4805	0.3390	0.4397
Rmat						
[A–C]	0.2904	23.1598	1.0000	1.0000	0.3236	1.5144
[A–G]	10.1319	79.6073	5.2227	112.8812	4.4621	11.6839
[A–T]	0.2880	84.8577	2.9442	0.2159	1.7201	7.3021
[C–G]	5.4028	40.8399	2.9442	0.2159	0.4669	3.4373
[C–T]	6.0444	1477.1377	14.9027	112.8812	2.5917	41.6187
[G–T]	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
I	0.3448	0.5015	0.5801	0.0690	0.5810	0.5038
a	0.5102	0.4321	0.4735	0.3998	0.7196	0.6195

distribution of *Allophortica* as Africa, which is the same as the current distribution of *P. sexpunctata*.

The results of the DIVA analysis are shown on the tree in Fig. 2. There are nine alternative, equally optimal reconstructions, each of which requiring 13 dispersals. The inferred ancestral distribution for the most recent common ancestor (MRCA) of the genera *Apenthecia + Apsiphortica + Phortica* (node O1) was sC-seA-Af (i.e., southern China + Southeast Asia + Africa), while that for the MRCA of the genera *Apenthecia + Phortica* was sC or Af, and that for the MRCA of *Phortica* was Af or sC-Af. The ancestral distribution for the new subgenus *Alloparadisa* was ambiguous, and that for the subgenera *Ashima + Phortica + Shangrila* was sC. Each of the subgenera *Ashima*, *Phortica* and *Shangrila* was suggested to have retained the ancestral distribution (sC), with the exception (in the subgenus *Ashima*) that the MRCA of the Clades II and III may have dispersed from southern China to Africa followed by a vicariance between these two areas, probably led to an allopatric diversification of the Clades II and III in Africa and southern China, respectively. In addition, as suggested by the distribution of some species (e.g., *P. foliiseta*, *P. spinosa* and *P. tanabei*), dispersals, presumably from southern China to Southeast Asia, may have occurred after the speciation of these species.

3.4. Divergence times in the genus Phortica

Based on some divergence times estimated in Tamura et al. (2004), the divergence time between the subgenus *Allophortica* and the rest of the genus *Phortica* (node 25, Fig. 2) was calculated as 22.7 Mya (the early-Miocene sub-epoch). The estimated times for the other nodes based on this calibration point are shown in Table 4. The divergence time between the genera *Apenthecia* and the genus *Phortica* was 32.6 (95% confidence interval, i.e., 95% CI: 27.5–38.2) Mya in the Oligocene epoch. Within the genus *Phortica*, following the splitting of the subgenus *Allophortica*, the subgenus *Alloparadisa* diverged from the lineage of the subgenera *Ashima + Phortica + Shangrila* at 20.7 (95% CI: 18.4–22.3) Mya in early-Miocene. The subgenus *Ashima* diverged from the lineage of the subgenera *Shangrila + Phortica* at 19.2 (95% CI: 17.0–21.5) Mya, also in early-Miocene, and the latter two subgenera (*Phortica* and *Shangrila*) diverged from each other at 16.0 (95% CI: 13.1–19.5) Mya at the end of early-Miocene. Within the subgenus *Apsiphortica*, the divergence between the Asian and African lineages occurred at 16.4 (95% CI: 13.2–19.2) Mya at the end of early-Miocene. The four major clades in the subgenus *Ashima* arose largely during mid-Miocene, with the splitting between the Clade II of Africa and its Asian counterpart (i.e., Clade III) falling close to the beginning of late-Miocene.

3.5. Systematic account of the genus Phortica

3.5.1. Subgenus Allophortica Máca, 2003

Type species: *Phortica fenestrata* Duda, 1939.

3.5.1.1. Diagnosis. Surstylus with dense, longer prensisetae; median rod of aedeagus strongly expanded; aedeagal apodeme very strong (modified from Máca, 2003).

3.5.2. Subgenus Phortica

3.5.2.1. Diagnosis. Additional plate present between cerci and tenth sternite; aedeagal median rod developed, basally with bridges articulated with anterodorsal corners of gonopod (after Chen et al., 2007).

3.5.3. Subgenus Sinophthalmus Coquillett 1904

Type species: Sinophthalmus picta (Coquillett, 1904).

3.5.3.1. Diagnosis. Arista without branches, but with some minute pubescence (modified from Máca, 2003).

3.5.4. Alloparadisa Chen, subgen. n.

Type species: *Phortica varipes* Duda, 1926.

3.5.4.1. Diagnosis. Abdominal sixth tergite very large; epandrium small, tapering laterally; cercus narrowed and elongated, with numerous, slender setae ventrally; surstylus elongated, stick-like; 10th sternite simple; hypandrium strongly protruding anterolaterally; aedeagus basally fused to gonopods (modified from Máca, 2003 for the *varipes* group).

3.5.4.2. Etymology. A combination of the Greek words: all + paradis, meaning like *Paradisea* birds.

3.5.5. Ashima Chen, subgen. n.

Type species: *Phortica xyleboriphaga* Senior-White, 1921.

3.5.5.1. Diagnosis. Fifth tarsomere of fore leg mostly with one long seta in male (Fig. 1D); abdominal third to fifth tergites nearly brownish black, thin yellow along anterior margins, sometimes with very thin, yellow stripes medially; epandrium broad and with more than 30 setae; paramere short, rod-shaped, basally mostly projected and with a few sensilla, apically always knobbed (after Chen et al., 2005 for the *foliiseta* complex).

3.5.5.2. Etymology. Patronym, the name of a beautiful girl in a legend of the Yi nationality in Yunnan, Southwest China.

3.5.6. Shangrila Chen, subgen. n.

Type species: *Amiota* (*Phortica*) *hani* (Zhang and Shi, 1997).

3.5.6.1. Diagnosis. Aristal dorsal branches short, lacking ventral branches; wing R_{4+5} and M_1 veins nearly parallel; fifth sternite notched posteromedially; cercus with 2 or 3 long, strong setae ventrally; paramere with spike-like processes basolaterally (after He et al., 2009a for the *hani* complex).

3.5.6.2. Etymology. From the Tibetan lection, meaning an enchanting place.

4. Discussion

4.1. Phylogenetic relationships in the genus Phortica

The present study attempted the reconstruction of phylogenetic relationships in the genus *Phortica* using DNA sequences of three genes from 26 species covering most of the major lineages in this genus. A monophyletic status of the grouping of the subgenera *Allophortica*, *Alloparadisa*, *Ashima*, *Phortica* and *Shangrila* was strongly supported. The subgenus *Sinophthalmus* was not investigated in the present study due to unavailability of specimens for DNA sequencing. Nevertheless, species of this subgenus exhibit the diagnostic characters of the genus *Phortica* referred to in the Introduction. Máca (2003) assumed a close relationship of *Sinophthalmus* to *P. foliiseta* group and allies considering its morphology including the structure of male terminalia. Particular affinity between the subgenera *Sinophthalmus* and *Allophortica* was suggested by their morphological commonness in palpus lacking a hollow sense organ and facial carina distinct. These morphological data suggest that a genus *Phortica* including *Sinophthalmus* is very likely to be a natural group, with each of the six subgenera being morphologically well diagnosed, as depicted in the foregoing section "Systematic account of the genus *Phortica*".

It was suggested that the subgenus *Allophortica* assumes a relatively basal position in the genus *Phortica* irrespective of the phylogenetic position of the subgenus *Sinophthalmus*. This relationship was supported by the morphological data, including some plesiomorphic characters in *Allophortica* such as aedeagal apodeme shallowly concave, with paramere and aedeagus holders only slightly developed (Máca, 2003). Meanwhile, the monophyly of *Allophortica* was clearly suggested by the synapomorphic characters shared among its three component species: palpus without hollow sense organ; surstylus with one row of numerous long prensisetae (not clear in *P. oldenbergi* Duda from Europe), paramere lacking sensilla and the aedeagal apodeme thick (Tsacas, 1990; Prigent and Chen, 2008).

Máca (2003) suggested a close relationship between *P. hani* and the *P. varipes* group (represented by *P. helva* and *P. varipes* therein), and their closer relationship to *P. sobodo* than to other *Phortica*. In other word, Máca (2003) considered *P. hani* and *P. helva* are closer to each other than either is to *P. sobodo*. Our result differs from that of Máca (2003) by suggesting that, *P. helva* and *P. sobodo* formed a monophyletic group (i.e., the subgenus *Alloparadisa*), while *P. hani* was located in the sister clade of this group (i.e., the assemblage of the subgenera *Ashima* + *Phortica* + *Shangrila*; Fig. 2). The subgenus *Shangrila* including the species in the *P. hani* complex also appear as a monophyletic group, consistent with their habitant distribution [a high elevation zone (ca. 1600–2800 m) range from the Hengduan Mountain in Southwest China to the Shennongjia Mountains in Central China] and some of their synapomorphies proposed recently (He et al., 2009a).

In the present study, the three species *P. magna*, *P. okadai* and *P. omega*, respectively, representing the "*variegata*", "*magna*" and "*omega*" groups (Máca, 1977; Chen and Toda, 1997, 1998), formed a monophyletic group that corresponds to the subgenus *Phortica* s.s. (see the Section 3.4 for detail). There are as many as nearly eighty species have been or are to be classified into the subgenus *Phortica*, and further phylogenetic studies based on dense sampling from each of its component species groups are desirable to elucidate the evolutionary history in this subgenus in the future.

The new subgenus *Ashima* corresponding to the *Phortica foliiseta* species complex was established by Tsacas and Okada (1983) based on the morphological character that arista swollen apically for four species: *P. foliacea* Tsacas and Okada (Taiwan, China), *P. foliiseta* Duda (Taiwan, China), *P. nigrifoliiseta* Takada, Momma and Shima (Malaysia) and *P. phyllochaeta* Tsacas and Okada (Papua New Guinea). Since then, especially during the recent years, more and more new species were classified into this complex, mainly in light of morphology of male genitalia, with not all of these species characterized by apically swollen arista (Chen et al., 2005; Prigent and Chen, 2008; Cheng et al., 2008). Our phylogenetic reconstruction with dense taxon sampling from *Ashima* lends strong support to the monophyly of this newly established subgenus. In addition, the basal position of Clade I in this subgenus is supported by morphological data indicating that species of this subgenus (e.g., *P. afoliolata* and *P. xishuangbanna*) are morphologically distinct from those of the other clades by their typical, apically leaf-shaped arista (Chen et al., 2005).

The Clade II within *Ashima* can be subdivided into two clusters based on morphological criteria: one (including *P. angulata*, *P. curvispina* and *P. machoruka*,) with apically swollen arista and the other one (including *P. manjano* and *P. melanopous*) with apically expanded arista. This morphological relationship was corroborated by the molecular data (Fig. 2). The morphological similarity between *P. sagittaristula* and *P. saltiaristula* in Clade IV is in accordance with the molecular data, which indicate a close relationship between *P. foliisetoides*, *P. sagittaristula* + *P. saltiaristula*. However, there was some conflict between molecular and morphological data in this clade: the molecular data suggested a closer relationship between *P. foliiseta* and *P. nudiarista* than either is related to *P. speculum*, whereas *P. nudiarista* is much more similar to *P. speculum* morphologically, especially regarding male terminalia.

4.2. Historical biogeography of the genus Phortica

Based on the nine alternative, equally optimal DIVA reconstructions of the ancestral distributions, it was indicated that, the ancestral distribution of the genus *Phortica* is Africa or southern China–Africa (Fig. 2: node 25), and two different scenarios could be entertained to explain the change of the distributions in the immediate descendant lineages: (1) the ancestor of the genus *Phortica* had its distribution in Africa. After the sympatric speciation in Africa, the *Allophortica* lineage retained the ancestral distribution, while the lineage of the rest of the genus *Phortica* (Fig. 2: node 24) extended its range to Southern China or even to Southeast Asia; (2) the ancestor of the genus had a wider distribution covering Africa and southern China, vicariance occurred between these two areas, giving rise to *Allophortica* lineage in Africa and the lineage of the rest in Southern China. According to our time estimation, the divergence time between the subgenus *Allophortica* and the rest of the genus *Phortica* falls close to the Oligocene/Miocene boundary (~23 Mya). If this time estimate proves accurate, the former scenario (i.e., the scenario 1 as represented in Figs 3A and B) will be more favored by paleobiogeographic data: by mid-Miocene, a definitive connection between Africa and Eurasia through the Middle East was established, and a precursing filter route, i.e., the Iranian route, had occurred by mid-Eocene (Gheerbrant and Rage, 2006).

Assuming the scenario 1, the ancestor of the subgenus *Alloparadisa* (node 1) may have extended its range from southern China to Africa (Fig. 3C), and probably to Southeast Asia as well, and the two daughter lineages diverged as a vicariance between southern China/Southeast Asia and Africa (Fig. 3D). It seemed that most of the diversification of the lineage of the subgenera *Ashima* + *Phortica* + *Shangrila* (node 23) occurred in southern China and the adjacent areas (Fig. 3E–G). There is no indication of geographical isolation between the subgenera *Phortica* and *Shangrila*, even though *Shangrila* may have adapted to habitats of higher elevations (>1600 m), probably along the uplift of the Himalaya–Tibetan Plateau, especially in the western Chinese area in the late Miocene (Harrison et al., 1992). The diversification of the *Ashima* lineage (node 22) initiated and continued to give rise to the Clades I (node 6) and IV in southern China (Fig. 3F and G). Presumably that, the MRCA of the Clades II and III (node 13) of *Ashima* dispersed from southern China to Africa (Fig. 3H), followed by allopatric speciation giving rise to the Clade II in Africa and the Clade III in southern China, respectively (Fig. 3I). According to our time estimation, this dispersal took place between ca. 12.4 and 11.5 Mya in mid-Miocene. Similar hypothesis of "out-of-Asia" has been proposed by Okada (1981) for the genus *Zaprionus* (Drosophilidae), and this hypothesis was supported in Yassin et al.'s (2008, 2010) molecular phylogenetic study. In Yassin et al. (2008), the origin of the genus *Zaprionus* in the Oriental region and its subsequent colonization of Africa were dated back to late-Miocene (>7 Mya).

Taking into account the evolutionary history of the genus *Phortica*, the diversification of each of the subgenera *Shangrila*, *Phortica* and *Ashima* took place largely in southern China since mid-Miocene. One factor facilitating the diversification of these lineages was probably the uplift thrust of the Himalaya–Tibetan Plateau at in the later Miocene, ca. 8 Mya, which resulted in general climate cooling and drying, and the subsequent northern latitude glaciations (http://science.jrank.org/pages/48653/Neogene-climate-Change.html). As mentioned before and as indicated by the current southern China–Southeast Asia distribution of some species of the subgenus *Ashima* (*P. foliiseta*, *P. spinosa* and *P. tanabei*) or the

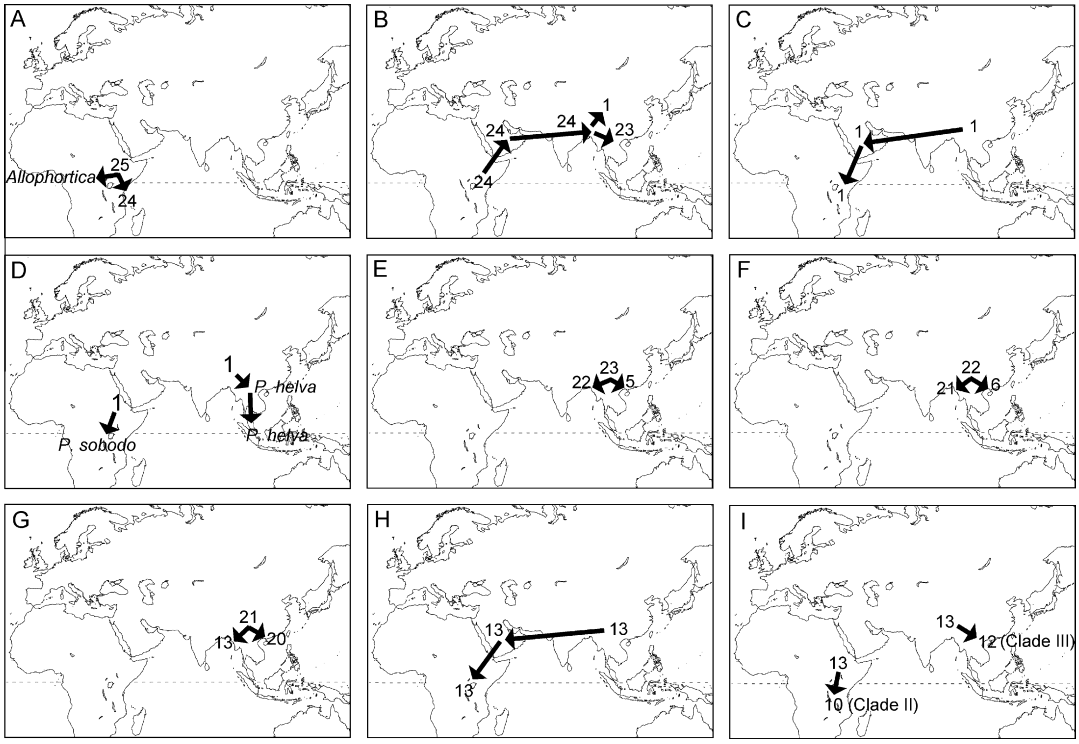

Fig. 3. Some representative scenarios about the hypothetical historical biogeography of the genus *Phortica*. The arrows indicate divergence between sibling groups and/or the direction of dispersals. Each of the numbers in the figure indicates an ancestral species, which was coded by the same number as the corresponding node in Fig. 2.

subgenus *Phortica* (*P. omega*), dispersals from southern China to Southeast Asia may have occurred subsequent to the speciation of these species. Presumably these dispersals were also facilitated by the above-mentioned climate changes associated with the uplift of the Himalaya–Tibetan Plateau.

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