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Molecular phylogenetic evidence for paraphyly of *Ceratovacuna* and *Pseudoregma* (Hemiptera, Hormaphidinae) reveals late Tertiary radiation

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Abstract

Ceratovacuna and *Pseudoregma* are important groups in Cerataphidini (Hemiptera, Hormaphidinae) that not only produce soldier aphids in galls on the primary hosts but also produce horned soldiers on the herbaceous secondary hosts. However, due to sampling bias in previous studies, the phylogenetic relationships of these two genera remain inconclusive. In this study, based on more extensive sampling and examination of both mitochondrial (cytochrome *c* oxidase subunit I (COI); cytochrome *b* (Cytb)) and nuclear (elongation factor-1α (EF-1α); long-wavelength opsin (LWO)) genes, we reconstructed the phylogenetic relationships of *Ceratovacuna* and *Pseudoregma*. Phylogenetic analyses, along with morphological evidence, suggested that these two genera belong to the paraphyletic groups with species clustered into three main groups. The monophyly of *Ceratovacuna* and *Pseudoregma* as a whole was generally supported by all analyses. Monophyly of *Pseudoregma* was also supported. The estimated divergence times demonstrated that diversification of *Ceratovacuna* and *Pseudoregma* occurred approximately at 10 mya. The relatively low resolution for the basal relationships of the three main clades may indicate that these two genera have experienced a rapid radiation along with speciation burst of their secondary hosts during the late Tertiary.

Keywords: Divergence time, diversification, geographical isolation, phylogeny

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Introduction

Ceratovacuna Zehntner (1897) and *Pseudoregma* Doncaster (1966) are closely related genera that belong to Cerataphidini in Hormaphidinae (Hemiptera, Aphididae). Most cerataphidini aphids alternate between their primary hosts, *Styrax* (Styracaceae), where they produce galls, and their secondary hosts (usually Asteraceae and bamboos) (Ghosh, 1988; Stern *et al*., 1997*a*). Almost all species of Cerataphidini produce soldier aphids in galls on their primary hosts, but many *Pseudoregma* and *Ceratovacuna* species also produce horned soldiers on their secondary hosts. Behavioral, morphological, and phylogenetic evidence suggest that primary and secondary host soldiers are not homologous and that horned soldiers evolved after the primary host soldiers (Aoki & Kurosu, 1989, 2010; Fukatsu *et al*., 1994). The unusual

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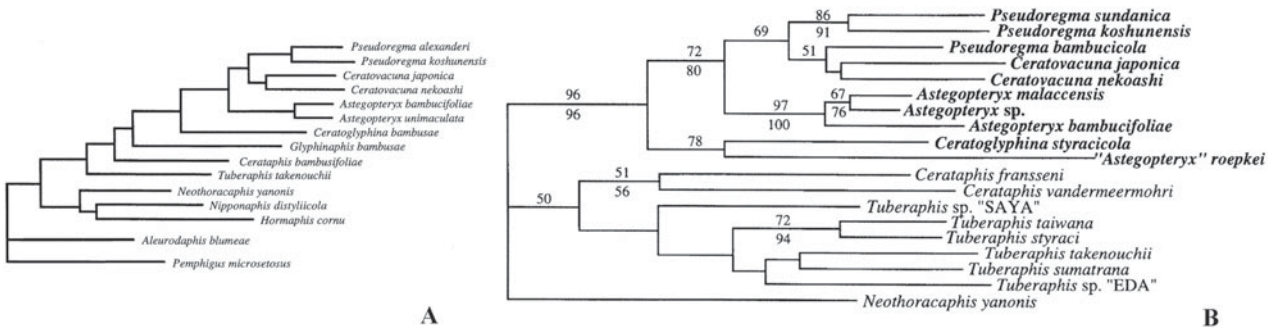

Fig 1. Phylogeny of Cerataphidini based on mitochondrial COI and COII (Stern, 1994) (a) and Stern (1995) (b).

biology of the horned soldiers suggests that they are interesting groups for investigating social behavior (Aoki, 1987; Dixon, 1985, 1998; Stern, 1994, 1995, 1998; Stern *et al*., 1997*a*, *b*; Costa, 2006). However, to better understand the origin and history of sociality in these species, their evolutionary history must be clarified first.

The genus *Ceratovacuna* contains 21 known species (Qiao *et al*., unpublished data), while *Pseudoregma* includes 12 species (Qiao *et al*., unpublished data). Using mitochondrial genes, Stern (1994) performed the first molecular phylogenetic study of Cerataphidini and found that *Ceratovacuna* and *Pseudoregma* each formed a monophyletic group (fig. 1a). However, using the same mitochondrial sequences but replacing *P. alexanderi* with *P. bambusicola* and *P. sundanica*, the monophyly of *Ceratovacuna* and *Pseudoregma* was not supported (fig. 1b) (Stern, 1995). The phylogenetic relationships of *Ceratovacuna* and *Pseudoregma* have been discussed in several works later (Stern *et al*., 1997*a*; Stern, 1998; Fukatsu *et al*., 2001). However, these studies have mainly focused on the life history and evolution of soldier aphids. While the monophyly of *Pseudoregma* was supported in most of these studies, the status of *Ceratovacuna* remained inconclusive. In addition, these studies were based on mitochondrial genes, and sampling bias existed due to specific study aims (e.g. evolution of life history and soldier aphids). Therefore, a detailed investigation of the phylogenetic relationships of these two genera is needed.

In addition to their uncertain phylogenetic history, the species of both *Ceratovacuna* and *Pseudoregma* are widely distributed across complex habitats in South-East Asia. Their distribution ranges include Java in the south, Japan and Korea in the north, eastern India in the west, and Taiwan and Japan in the east (Stern *et al*., 1997*a*). Considering their similar distributions, the two genera provide good models for investigating the structure of inter- and intraspecific genealogical patterns and examining how aphid species diversification correlates with historical events that have shaped the speciation of co-distributed taxa.

To test the monophyly of *Ceratovacuna* and *Pseudoregma*, we used a more extensive sampling and both mitochondrial (cytochrome *c* oxidase subunit I (COI); cytochrome *b* (Cytb)) and nuclear (elongation factor-1α (EF-1α); long-wavelength opsin (LWO)) genes to reconstruct the phylogenetic relationships of these two related genera. We also estimated divergence times and tested whether geographical barriers and host evolution have been the possible driving forces for species differentiation in both genera.

Materials and methods

Taxon sampling

We sampled 12 species of *Ceratovacuna* and five species of *Pseudoregma*. Specimens for slide-mounting were stored in 75% ethanol. *Ceratovacuna* sp1., *Ceratovacuna* sp2., *Ceratovacuna* sp3., *Ceratovacuna* sp4., and *Ceratovacuna* sp5. have recently been recognized as new species based on morphological and molecular data, and this information will be published separately. Samples for molecular experiments were stored in 95% ethanol. All samples were preserved at −30°C. On the basis of current knowledge of the phylogenetic relationships within Hormaphidinae and Cerataphidini (Stern, 1998; Ortiz-Rivas *et al*., 2010; Huang *et al*., 2012), outgroups were chosen from *Aleurodaphis* van der Goot (1917), *Astegopteryx* Karsch (1890), *Cerataphis* Lichtenstein (1882), *Ceratoglyphina* van der Goot (1917), *Tuberaphis* Takahashi (1933) in Cerataphidini; *Hamamelistes* Shimer (1867) and *Hormaphis* Osten-Sacken (1861) in Hormaphidini; *Neohormaphis* Noordam (1991) and *Nipponaphis* Pergande (1906) in Nipponaphidini, respectively. Multiple outgroups are indispensable to reliably test the monophyly of the two genera. All voucher specimens were deposited in the National Zoological Museum of China (NZMC) at Institute of Zoology, Chinese Academy of Sciences (Beijing) (voucher numbers see table 1). Collection information for all samples, including collection localities, host plants, and collection dates are listed in table 1.

DNA extraction, PCR and sequencing

The CTAB DNA isolation technique (Vavre *et al*., 1999) was used to extract genomic DNA. A 700 bp fragment of COI was amplified using primers LepF (5′-ATTCAACCAATCATAAAGATATTGG-3′) and LepR (5′-TAAACTTCTGGATGTCCAAAAAATCA-3′) from Foottit *et al*. (2008). About 800 bp of Cytb was amplified using primers Cp1 (5′-GATGATGAAATTTTGGATC-3′) and Cp2 (5′- CTAATGCAATAACTCCTCC-3′) from Harry *et al*. (1998). The primers for amplification of EF-1α were EF3 (5′-GAACGTGAACGTGGTATCAC-3′) and EF2 (5′- ATGTGAGCAGTGTGGCAATCCAA-3′) (Palumbi, 1996). LWO sequences were amplified using primers OPSETF1 (5′-GGYRTYACNATTTTYTTCTTRGG-3′) and OPSETR1 (5′-GANCCCCADATYGTNAATAAYGG-3′) (Benjamin Ortiz-Rivas, private communication). PCR for Cytb, EF-1α, and LWO was performed in 30 µl reaction volumes: 3 µl 10 × PCR buffer, 2.4 µl dNTPs, 20 µl dd

Table 1. Related information of aphid samples examined in this study.

Genera and species	Voucher No.	Locality	Host plant	GenBank Accession Nos: COI/Cytb/EF-1α/LWO
Ceratovacuna Zehntner				
Ceratovacuna sp1.	14734	Fujian: Wuyishan Mt.	Bamboo (Poaceae)	JX282701
Ceratovacuna sp2.	14535	Fujian: Wuyishan Mt.	Bamboo (Poaceae)	JX282738/JX282646
Ceratovacuna sp2.	14774	Fujian: Wuyishan Mt.	*Indocalamus tessellates* (Poaceae)	JX282706/JX282652/JX282819/JX282869
Ceratovacuna sp2.	14786	Fujian: Wuyishan Mt.	*I. tessellates* (Poaceae)	JX282707/JX282653/JX282820/JX282870
Ceratovacuna sp2.	14791	Fujian: Wuyishan Mt.	*I. tessellates* (Poaceae)	JX282709/JX282654/JX282821/JX282871
Ceratovacuna sp2.	14792	Fujian: Wuyishan Mt.	*Rhus chinensis* (Anacardiaceae)	JX282710/JX282656
Ceratovacuna sp2.	14804	Fujian: Wuyishan Mt.	*I. tessellates* (Poaceae)	JX282711/JX282657
Ceratovacuna sp2.	26876	Fujian: Wuyishan Mt.	*I. tessellates* (Poaceae)	JX282708/JX282658/JX282841/JX282900
Ceratovacuna sp2.	25626	Guizhou: Suiyang County	Bamboo (Poaceae)	JX282712/JX282655/JX282822
Ceratovacuna sp3.	15367	Tibet: Motuo County	*Imperata arundinacea* (Poaceae)	JX282713/JX282659/JX282823/JX282874
Ceratovacuna sp4.	26749	Zhejiang: Anji County	Bamboo (Poaceae)	JX282760/JX282660/JX282839/JX282898
Ceratovacuna sp4.	26819	Zhejiang: Suichang County	*Phyllostachys heterocycla* (Poaceae)	JX282761/JX282661/JX282840/JX282899
Ceratovacuna sp5.	26095	Guangxi: Luocheng County	Poaceae	JX282694/JX282683/JX282833/JX282895
C. graminum (van der Goot, 1917)	14503	Fujian: Wuyishan Mt.	*I. arundinacea* (Poaceae)	JQ916425/JX282640/JX282824/JX282853
C. graminum (van der Goot, 1917)	27035	Zhejiang: Tianmu Mt.	*Oplismenus* (Poaceae)	JX282763/JX282643/JX282842/JX282901
C. graminum (van der Goot, 1917)	27085	Zhejiang: Linan City	Poaceae	JX282764/JX282644/JX282843
C. graminum (van der Goot, 1917)	15655	Guizhou: Daozhen County	Poaceae	JX282641/JX282641/JX282825/JX282876
C. graminum (van der Goot, 1917)	25601	Guizhou: Suiyang County	Poaceae	JQ916441/JX282642
C. hoffmanni (Takahashi, 1936)	14568	Fujian: Wuyishan Mt.	Bamboo (Poaceae)	JX282695/JX282662
C. lanigera Zehntner, 1897	14599	Guangxi: Jiuwandashan Mt.	*I. arundinacea* (Poaceae)	JX282715/JX282612/JX282795/JX282859
C. lanigera Zehntner, 1897	17383	Zhejiang: Taishun County	Poaceae	JX282726/JX282805/JX282880
C. lanigera Zehntner, 1897	18052	Fujian: Wuyishan Mt.	*Miscanthus floridulus* (Poaceae)	JX282737/JX282626/JX282807/JX282883
C. lanigera Zehntner, 1897	17322	Zhejiang: Taishun County	Poaceae	JQ916434/JX282624/JX282801/JX282878
C. lanigera Zehntner, 1897	14600	Guangxi: Jiuwandashan Mt.	*I. arundinacea* (Poaceae)	JX282721/JX282610/JX282797/JX282860
C. lanigera Zehntner, 1897	14655	Guangxi: Jiuwandashan Mt.	*I. arundinacea* (Poaceae)	JX282722/JX282621/JX282800/JX282866
C. lanigera Zehntner, 1897	14871	Fujian: Longyan County	*M. floridulus* (Poaceae)	JX282725/JX282623/JX282794/JX282872
C. lanigera Zehntner, 1897	26919	Fujian: Sha County	*Miscanthus* (Poaceae)	JX282729/JX282631/JX282812/
C. lanigera Zehntner, 1897	17357	Zhejiang: Taishun County	Poaceae	JQ916435/JX282622/JX282810/
C. lanigera Zehntner, 1897	18054	Fujian: Wuyishan Mt.	*I. arundinacea* (Poaceae)	JX282740/JX282627/JX282809/JX282884
C. lanigera Zehntner, 1897	17370	Zhejiang: Taishun County	Poaceae	JX282724/JX282625/JX282806/JX282879
C. lanigera Zehntner, 1897	26378	Taiwan: Shouka Village	Phragmites australis (Poaceae)	JX282728/JX282630/JX282814/JX282897
C. lanigera Zehntner, 1897	14645	Guangxi: Luocheng County	*I. arundinacea* (Poaceae)	JX282716/JX282615/JX282793/JX282862
C. lanigera Zehntner, 1897	19504	Hainan: Changjiang County	Phragmites australis (Poaceae)	JX282731/JX282629
C. lanigera Zehntner, 1897	15670	Guizhou: Daozhen County.	*I. arundinacea* (Poaceae)	JX282714/JX282618/JX282803/JX282877

Table 1. (*Cont*.)

Genera and species	Voucher No.	Locality	Host plant	GenBank Accession Nos: COI/Cytb/EF-1α/LWO
C. lanigera Zehntner, 1897	19528	Hainan: Jianfeng Mt.	*I. arundinacea* (Poaceae)	JX282727/JX282611/JX282808/JX282886
C. lanigera Zehntner, 1897	14651	Guangxi: Jiuwandashan Mt.	Poaceae	JX282718/JX282617/JX282802/JX282865
C. lanigera Zehntner, 1897	26055	Guangxi: Lingui County	Poaceae	JX282813/JX282813/JX282813/JX282894
C. lanigera Zehntner, 1897	14647	Guangxi: Jiuwandashan Mt.	*I. arundinacea* (Poaceae)	JX282717/JX282616/JX282811/JX282863
C. lanigera Zehntner, 1897	14654	Guangxi: Jiuwandashan Mt.	*I. arundinacea* (Poaceae)	JQ916431/JX282619
C. lanigera Zehntner, 1897	15366	Tibet: Motuo County	*I. arundinacea* (Poaceae)	JX282723/JX282620/JX282799/JX282873
C. lanigera Zehntner, 1897	17348	Zhejiang: Taishun County	Poaceae	JX282720/JX282613
C. lanigera Zehntner, 1897	14674	Guangxi: Jiuwandashan Mt.	Poaceae	JQ916432/JX282632/JX282798/JX282867
C. lanigera Zehntner, 1897	19576	Hainan: Ledong County	Poaceae	JX282730
C. lanigera Zehntner, 1897	17398	Zhejiang: Taishun County	Poaceae	JQ916436/JX282804/JX282881
C. lanigera Zehntner, 1897	22277	Hainan: Ledong County	Poaceae	JX282741
C. longifila (Takahashi, 1910)	20982	Hainan: Wuzhi Mt.	Bamboo (Poaceae)	JX282693/JX282638/JX282826/JX282888
C. nekoashi (Sasaki, 1910)	24046	Yunnan: Kunming City	Poaceae	JX282748/JX282637/JX282827/JX282890
C. nekoashi (Sasaki, 1910)	18420	Hainan: Wuzhi Mt.	Poaceae	JX282750
C. nekoashi (Sasaki, 1910)	20988	Hainan: Yuanmen County	Bamboo (Poaceae)	JX282749
C. panici (van der Goot, 1917)	14598	Guangxi: Jiuwandashan Mt.	Poaceae	JX282704/JX282634/JX282815/JX282858
C. panici (van der Goot, 1917)	14648	Guangxi: Jiuwandashan Mt.	Bamboo (Poaceae)	JX282702/JX282636/JX282816/JX282864
C. panici (van der Goot, 1917)	14678	Guangxi: Jiuwandashan Mt.	Bamboo (Poaceae)	JX282705/JX282633/JX282818/JX282868
C. panici (van der Goot, 1917)	27015	Zhejiang: Tianmu Mt.	*Oplismenus* (Poaceae)	JX282703/JX282635/JX282817
C. silvestrii (Takahashi, 1927)	12553	Guizhou: Chishui County	unknown	JQ916424/JX282645/
C. silvestrii (Takahashi, 1927)	24537	Guizhou: Kuankuoshui District	Bamboo (Poaceae)	JX282752/JX282647/JX282829/JX282891
C. silvestrii (Takahashi, 1927)	23896	Yunnan: Xishuangbanna District	*Dendrocalamus membramaceus* (Poaceae)	JX282754/JX282648/JX282828
C. silvestrii (Takahashi, 1927)	24844	Yunnan: Binchuan County	Bamboo (Poaceae)	JX282751/JX282650/JX282830/JX282892
C. silvestrii (Takahashi, 1927)	24884	Yunnan: Kunming City	Bamboo (Poaceae)	JQ916440/JX282651/JX282832
C. silvestrii (Takahashi, 1927)	24877	Yunnan: Kunming City	Bamboo (Poaceae)	JX282753/JX282649/JX282831/
Pseudoregma Doncaster				
P. alexanderi (Takahashi, 1924)	16032	Yunnan: Longling County	Bamboo (Poaceae)	JX282773
P. alexanderi (Takahashi, 1924)	20936	Hainan: Ledong County	Bamboo (Poaceae)	JQ916437
P. alexanderi (Takahashi, 1924)	21353	Hainan: Yuanmen County	Bamboo (Poaceae)	JQ916438/JX282774
P. alexanderi (Takahashi, 1924)	21399	Hainan: Ledong County	Bamboo (Poaceae)	JX282732
P. bambucicola (Takahashi, 1921)	17406	Zhejiang: Taishun County	Unknown	JX282734/JX282668/JX282782/JX282882
P. bambucicola (Takahashi, 1921)	22006	Guangdong: Chebaling District	Bamboo (Poaceae)	JQ916439/JX282669/JX282784/JX282889
P. bambucicola (Takahashi, 1921)	22530	Sichuan: Chengdu City	Bamboo (Poaceae)	JX282736/JX282671/JX282786
P. bambucicola (Takahashi, 1921)	14204	Taiwan: Taibei City	Bamboo (Poaceae)	JX282733
P. bambucicola (Takahashi, 1921)	26014	Guangxi: Guilin City	Bamboo (Poaceae)	JX282746/JX282787/JX282893

Table 1. (*Cont.*)

Genera and species	Voucher No.	Locality	Host plant	GenBank Accession Nos: COI/Cytb/EF-1α/LWO
P. bambucicola (Takahashi, 1921)	26107	Guangxi: Baotan County	Bamboo (Poaceae)	JQ916443/JX282783/JX282896
P. bambucicola (Takahashi, 1921)	Y8937	Taiwan: Taibei City	Bamboo (Poaceae)	JX282735
P. bambucicola (Takahashi, 1921)	Y8955	Hunan: Jishou City	*Bambusa ventricosa* (Poaceae)	JX282747/JX282670
P. koshunensis (Takahashi, 1924)	14586	Guangxi: Jiuwandashan Mt.	*Fargesia semicoriacea* (Poaceae)	JQ916429/JX282676
P. koshunensis (Takahashi, 1924)	14602	Guangxi: Jiuwandashan Mt.	Bamboo (Poaceae)	JQ916430/JX282678/JX282780/JX282861
P. koshunensis (Takahashi, 1924)	14606	Guangxi: Jiuwandashan Mt.	Bamboo (Poaceae)	JX282700/JX282679/JX282781
P. koshunensis (Takahashi, 1924)	15651	Guizhou: Daozhen County	Bamboo (Poaceae)	JQ916433/JX282677/JX282779/JX282875
P. panicola (Takahashi, 1921)	13489	Yunnan: Xishuangbanna District	Poaceae	JX282697/JX282672/JX282772
P. panicola (Takahashi, 1921)	14550	Fujian: Wuyishan Mt.	*I. tessellates* (Poaceae)	JQ916427/JX282673/JX282775/JX282854
P. panicola (Takahashi, 1921)	14554	Fujian: Wuyishan Mt.	*Arthraxon* sp. (Poaceae)	JQ916428/JX282675/JX282776
P. panicola (Takahashi, 1921)	14556	Fujian: Wuyishan Mt.	*Phyllostachys nigra* (Poaceae)	JX282699/JX282674/JX282777/JX282855
P. sundanica (van der Goot, 1917)	19559	Hainan: Ledong County	*Musa paradisiaca* (Musaceae)	JX282742/JX282680/JX282791/JX282887
P. sundanica (van der Goot, 1917)	19654	Hainan: Changjiang County	Zingiberaceae	JX282744/JX282681/JX282788
P. sundanica (van der Goot, 1917)	24286	Hainan: Limu Mt.	*Alpinia zerumbet* (Zingiberaceae)	JX282745/JX282682/JX282790
P. sundanica (van der Goot, 1917)	23942	Yunnan: Xishuangbanna District	Zingiberaceae	JX282743/JX282789
Cerataphidini				
Aleurodaphis blumeae van der Goot, 1917	15597	Guizhou: Leigongshan Mt.	unknown	JX282771/JX282690/JX282844/JX282902
Aleurodaphis mikaniae Takahashi, 1925	15017	Sichuan: Baoxing County	*Parasenecio* sp. (Asteraceae)	JX282765/JX282687
Aleurodaphis mikaniae Takahashi, 1925	15638	Guizhou: Daozhen County	unknown	JX282766/JX282685/JX282851
Astegopteryx rhapidis (van der Goot, 1917)	Y8927	Hainan: Wenchang County	*Cocos nucifera* (Arecaceae)	JX282769/JX282848/JX282904
Astegopteryx bambusae (Buckton, 1893)	14592	Guangxi: Shiwandashan Mt.	Bamboo (Poaceae)	JX282768/JX282692/JX282849/JX282905
Cerataphis bambusifoliae Takahashi, 1925	14553	Fujian: Wuyishan Mt.	Bamboo (Poaceae)	JN032723 DQ493861/JX282906
Ceratoglyphina bambusae van der Goot, 1917	14466	Fujian: Wuyishan Mt.	Bamboo (Poaceae)	JN032718/JX282691/DQ493856
Ceratoglyphina phragmitidisucta Qiao *et* Zhang, 1999	14811	Fujian: Wuyishan Mt.	Bamboo (Poaceae)	JX282850
Chaitoregma tattakana (Takahashi, 1925)	13308	Guizhou: Fanjing Mt.	Bamboo (Poaceae)	JN032707
Tuberaphis xinglongensis (Zhang & Zhong, 1982)	Y8928	Hainan: Wenchang County	*C. nucifera* (Arecaceae)	JX282767/JX282684/JX282847/JX282903
Hormaphidini				
Hormaphis betulae Mordvilko, 1901	14708	Jilin: Changbai Mt.	*Betula ermanii* (Betulaceae)	JX282846
Hormaphis betulae Mordvilko, 1901	14994	Jilin: Changbai Mt.	*Betula* sp. (Betulaceae)	JX282770/JX282688/JX282845/JX282907
Hormaphis betulae Mordvilko, 1901	15214	Jilin: Changbai Mt.	*Betula* sp. (Betulaceae)	JN032726/JX282686/DQ493864
Hormaphis cornu (Shimer, 1867)		USA: Georgia, Athens	*Hamamelis virginiana*	EU701682
Nipponaphidini				
Euthoracaphis oligostricha Chen, Fang & Qiao, 2009	15299	Yunnan: Kunming City	*Machilus yunnanensis* (Lauraceae)	JN032727/DQ493865
Neohormaphis wuyiensis Qiao, Jiang & Guo, 2008	14525	Fujian: Wuyishan Mt.	*Quercus* sp. (Fagaceae)	JN032721/
Thoracaphis quercifoliae Ghosh, 1988	14526	Fujian: Wuyishan Mt.	*Quercus* sp. (Fagaceae)	JN032722/JX282689/DQ493859/JX307714

H_2O, 0.4 unit Taq DNA polymerase (all from TransGen Biotech, Beijing, China) and 0.6 µl 10 µM forward and reverse primers (synthesized by Invitrogen Biotech, Shanghai, China). The PCR volumes for COI were the same as those for Cytb, except for 19.4 µl dd H_2O and 0.8 µM primers. All PCR reactions included a 95°C hot start for 5 min and a 72°C extension for 10 min at the end. The cycling conditions for COI included 34 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min. The cycling conditions for Cytb included 35 cycles of denaturation at 92°C for 1 min, 48°C for 1.5 min, and 72°C for 1 min. The PCR thermal regime for EF-1α and LWO included 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1.5 min. Sequencing reactions were performed using the same primers that were used for PCRs using ABI 3730 automated sequencer (ABI, USA).

Sequence alignment and phylogenetic analyses

Sequences were assembled using Seqman II (DNASTAR) and checked manually. Multiple alignments were performed with ClustalX 1.81 (Thompson *et al*., 1997). Nucleotide composition, conserved sites, variable sites, and parsimony informative sites were calculated using MEGA 4 (Tamura *et al*., 2007). We used the coding regions of EF-1α and LWO in phylogenetic analyses. Introns of EF-1α and LWO were removed according to the GT-AG rule and comparison with the cDNA sequences of *Hormaphis similibetulae* [GenBank accession number: DQ493866] and *Cerataphis* sp. D15 [GenBank accession number: AJ539465], respectively. A partition homogeneity test was implemented with PAUP* 4.10b (Swofford, 2002) and used to evaluate the appropriateness of combining different genes into one dataset. The results of the partition-homogeneity test indicated that all genes can be grouped to four combined datasets (COI + Cytb, EF-1α + LWO, COI + Cytb + EF-1α and COI + Cytb + EF-1α + LWO) ($P < 0.01$). For Bayesian analysis, partitions were made for each gene separately using their own parameters derived from Modeltest 3.7 (Posada & Crandall, 1998).

Phylogenetic analyses were conducted in PHYML (Guindon & Gascuel, 2003) and MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2005). Modeltest was used to determine the best-fit nucleotide substitution model for maximum likelihood (ML) analysis. The combined data were partitioned into mitochondrial versus nuclear data, and the best model chosen under the Akaike Information Criterion (AIC) (Posada & Buckley, 2004) for COI, COI + Cytb, EF-1α, EF-1α + LWO, and COI + Cytb + EF-1α + LWO was GTR + I + G. ML analysis was performed using PHYML, and nodal support was evaluated by bootstrap analysis with 1000 replicates. The values of model parameters were treated as unknown variables with uniform prior probabilities, and were estimated during the analyses. Four chains were run, starting from a random tree and proceeding for 15 million Markov chain Monte Carlo generations, with tree sampling every 100 generations. Four independent runs were conducted to verify the results. The first 2500 trees (25% of the total) were discarded as burn-in. For the remaining trees, 50% majority-rule consensus trees were generated. An examination of the log-likelihood scores, the average standard deviation of split frequencies (< 0.01), and potential scale reduction factor (PSRF) for branch lengths suggested that the burn-in periods were long enough for chains to become stationary.

Divergence time estimation

The new species *Unicohormaphis sorini* Wegierek & Zyla (Wegierek & Zyla, 2011), which belongs to Cerataphidini, was found in Baltic amber and dates back to the Eocene. Since fossils generally indicate the minimum age of a species, we assigned a conservative age of 57 mya (with normal prior distribution) as a calibration point to represent the minimum age for the most recent common ancestor (MRCA) of Cerataphidini in our study. Based on a mitochondrial tRNA/COII dataset and calibrated nucleotide substitution rate, von Dohlen *et al*. (2002) calculated the mean of divergence estimates between East-Asian and North American *Hormaphis* as 25.5 mya. We also used this age as a calibration point to verify the results of fossil calibration. Based on mitochondrial and nuclear data, the divergence times among species were estimated using BEAST (v1.6.1, Drummond & Suchard, 2010) under a relaxed molecular clock and uncorrelated lognormal model, respectively. A GTR model of nucleotide substitution with gamma rate heterogeneity among sites and a proportion of invariant sites and Yule speciation process were used.

After determining optimal parameters over several preliminary runs, multiple analyses were run for 10 million generations, and 25% of the samples were removed as burn-in. The results were visualized in Tracer v1.5 (Rambaut & Drummond, 2008), where the stationarity was assessed by ensuring that the effective sample size (ESS) values were high for most parameters. The results proved repeatable over multiple independent runs. Thus, a chronogram was reconstructed based on results from a representative single run and visualized using the program FigTree v1.3.1 (Rambaut, 2009).

Results

Sequence characters and phylogenetic analyses

COI sequences were obtained from 85 samples. The sequence length varied from 608 to 703 bp, and the final alignment was of 574 bp. Seventy-eight Cytb sequences were obtained with length from 666 to 814 bp. Their final alignment was 665 bp. The length of the EF-1α sequences obtained from 67 samples varied between 990 and 1168 bp, with a final alignment of 806 bp. Forty-eight LWO sequences ranging from 948 to 1198 bp were obtained, and their 636 bp alignment was used for phylogenetic analyses. All sequences were submitted to GenBank (see table 1 for accession numbers). The intraspecific pairwise distances were 0–4.5% for *C. lanigera* and much lower in the other species (0–1.6%) (Suppl. 1).

Phylogenetic analyses

The Bayesian and ML analyses based on COI did not support the monophyly of *Ceratovacuna* and *Pseudoregma*, when all species studied here were included; however, the monophyly of the two genera as a whole was suggested (ML/BI: 78/1.00) in the phylogeny including most related species from other genera (fig. 2). Except *C. silvestrii* and *P. alexanderi*, the taxonomic status of most species in these two genera was supported. Although with low resolution, all sampled species tended to cluster into three main groups: I, *C. silvestrii* + *C. hoffmanni* + *Ceratovacuna* sp1. + *Ceratovacuna* sp2.; II, *C. nekoashi* + *Ceratovacuna* sp3. + *C. graminum* + *Ceratovacuna* sp5. + *C. panici* + *C. lanigera*; and III,

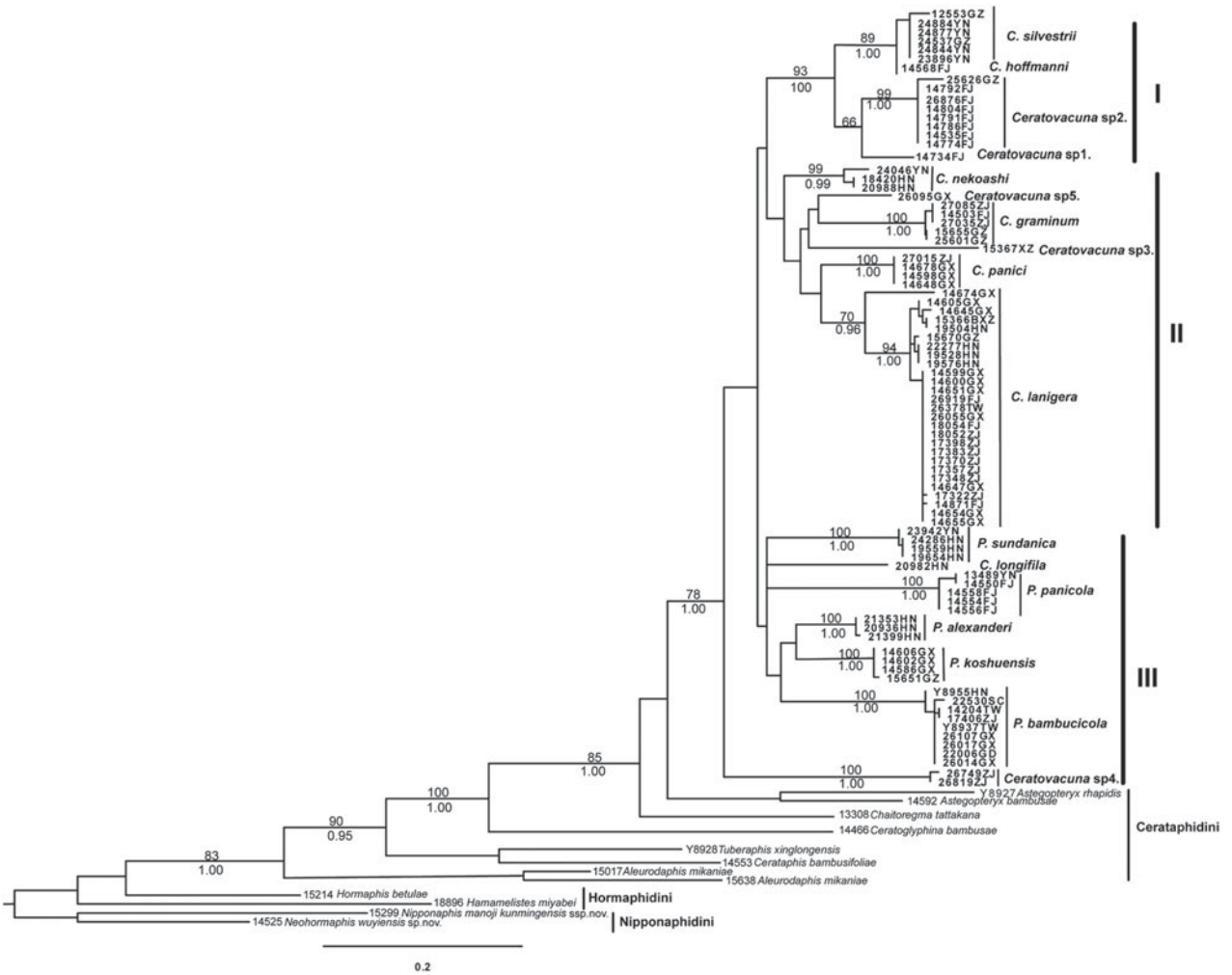

Fig 2. Phylogenetic relationships between genus *Ceratovacuna* and *Pseudoregma* based on maximum likelihood analysis of mitochondrial gene COI. Bootstrap percentages (≥50%) from ML are shown above the branch, posterior probabilities (≥50%) of Bayesian analysis are shown below the branch.

P. sundanica+*C. longifila*+*P. panicola*+*P. alexanderi*+*P. koshunensis*+*P. bambucicola*. *Ceratovacuna* sp4., was located as a sister clade of all other species. The monophyly of group I was supported (ML/BI: 93/1.00). One sample (No. 14674) of *C. lanigera* formed a sister group with all other samples of this species (ML/BI: 70/0.96).

Phylogenetic analysis based on EF-1α (fig. 3) and the combined datasets of COI+Cytb (Suppl. fig. 1), EF-1α+LWO (Suppl. fig. 2), and COI+Cytb+EF-1α+LWO (fig. 4) yielded trees with similar topology to the COI tree. The monophyly of *Ceratovacuna* and *Pseudoregma* as a whole was supported in all analyses. In the EF-1α tree, monophyly of group II (ML/BI: 59/1.00) and *Pseudoregma* (ML/BI: 69/1.00) was supported, respectively. However, the monophyly of *C. silvestrii* in group I was not resolved.

The results of combined datasets improved the resolution of species relationships. The sister relationship of *C. silvestrii*, *C. hoffmanni* with *Ceratovacuna* sp2. (ML/BI: 91/1.00), and *C. lanigera*, *C. nekoashi* with *C. panici* (ML/BI:70/1.00) were supported based on the combined data from COI+Cytb (Suppl. fig. 1). Similarly, *P. panicola* with *P. sundanica* (ML/BI: 85/0.99), *C. silvestrii* with *Ceratovacuna* sp2. (ML/BI: 52/1.00), and *Ceratovacuna* sp3. with *Ceratovacuna* sp5. (ML/BI: 97/1.00) were all clustered into sister clades in the analysis based on a combined dataset of EF-1α+LWO (Suppl. fig. 2). Bayesian analysis of the combined dataset COI+Cytb+EF-1α+LWO strongly supported the monophyly of three main groups (fig. 4).

Divergence time

The divergence times were estimated separately for mitochondrial and nuclear data, and the 95% confidence intervals of most nodes were largely congruent (fig. 5; table 2). The analysis based on mitochondrial data included more taxa and was used to evaluate possible factors that influenced both species divergence and the formation of distribution patterns. The MRCA of *Ceratovacuna* and *Pseudoregma* were approximately 48.9 mya (95% highest posterior density, HPD: 35.6–54.9 mya). Most species divergences in the two genera

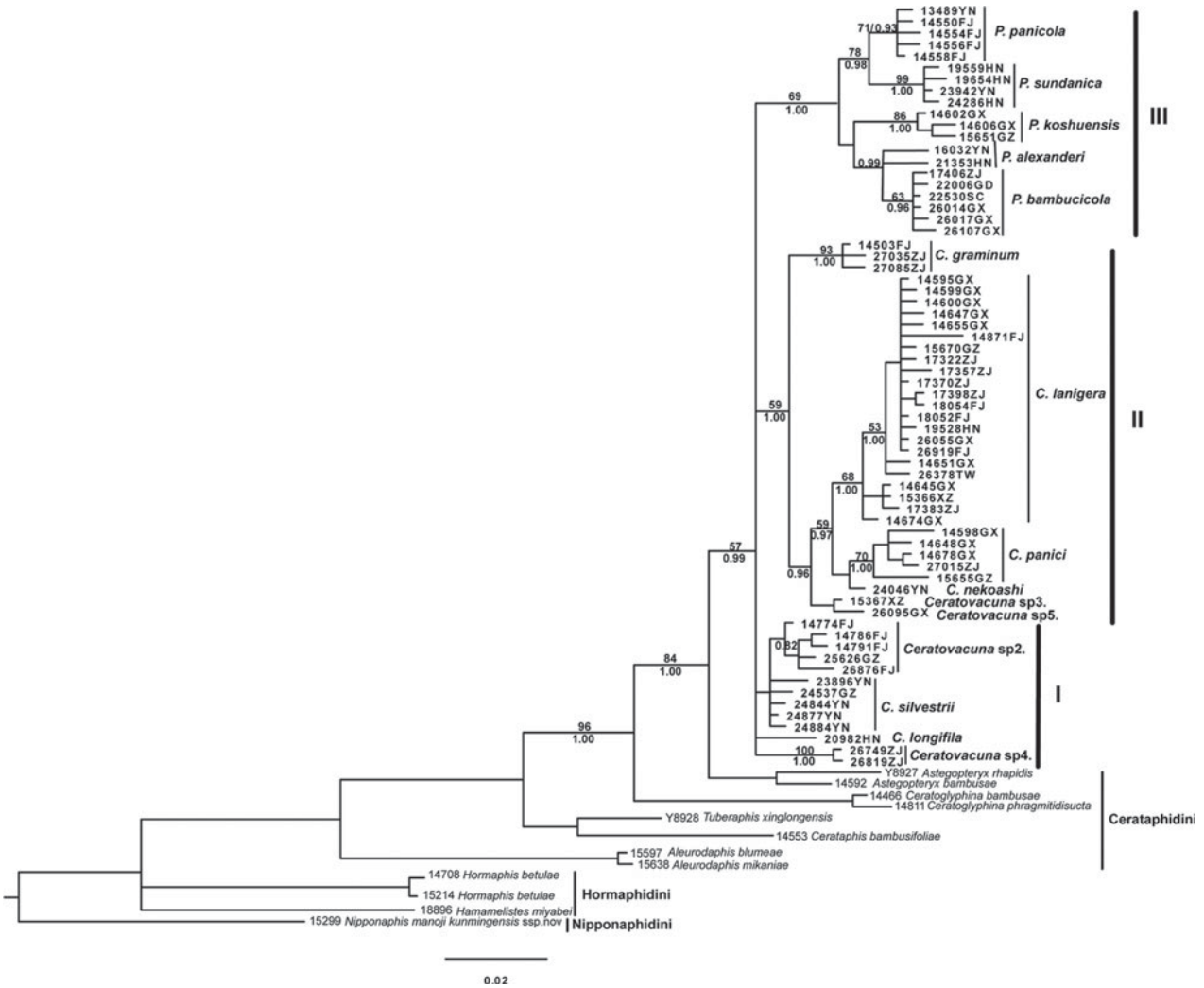

Fig 3. Phylogenetic relationships between genus *Ceratovacuna* and *Pseudoregma* based on Bayesian analysis of nuclear gene EF-1α. Bootstrap percentages (≥50%) from ML are shown above the branch, posterior probabilities (≥50%) of Bayesian analysis are shown below the branch.

occurred in the late Tertiary. The earliest speciation time was 24.3 mya (HPD: 9.5–37.9 mya) for *C. lanigera*, and the divergence of *Ceratovacuna* sp4. occurred at 8.8 mya (HPD: 4.9–13.1 mya).

Discussion

Phylogenetic relationships of Ceratovacuna *and* Pseudoregma

Phylogenetic analyses based on both mitochondrial and nuclear genes have not reconstructed the monophyly of *Ceratovacuna* and *Pseudoregma*. Some previous studies suggested the monophyly of *Pseudoregma* (Stern, 1994, 1998; Stern *et al*., 1997*b*; Fukatsu *et al*., 2001). In the present study based on more sampling and both mitochondrial and nuclear data, monophyly of *Pseudoregma* was supported by phylogenetic analyses based on nuclear genes and combined datasets (figs 3 and 4), while the monophyly of *Ceratovacuna* was unresolved in all analyses. The monophyly of the two genera as a whole was strongly supported in all analyses. These results are also supported by morphological evidences. The pleural grooves on the pronotum of apterous viviparous females were an important morphological characteristic in distinguishing *Ceratovacuna* and *Pseudoregma* (Ghosh, 1988). The species of *Pseudoregma* have two pleural grooves on their pronotum that are separated by a median ridge, while *Ceratovacuna* species have no obvious pleural grooves. As more species are identified and described, distinguishing these two genera based on the occurrence/absence of pleural grooves on pronotum may be problematic, because this morphological characteristic varies within and among species. Molecular phylogenetic analyses and morphological characters, such as the arrangement and number of wax plates as well as pleural grooves on the pronotum of apterous viviparous females, provide evidence that *Ceratovacuna* and *Pseudoregma* should be redefined. In the future, more data are needed to test this assumption.

The sampled species of these two genera tend to cluster into three main groups. Groups I and II consist of *Ceratovacuna* species, except for *P. sundanica*, which clusters into group I in

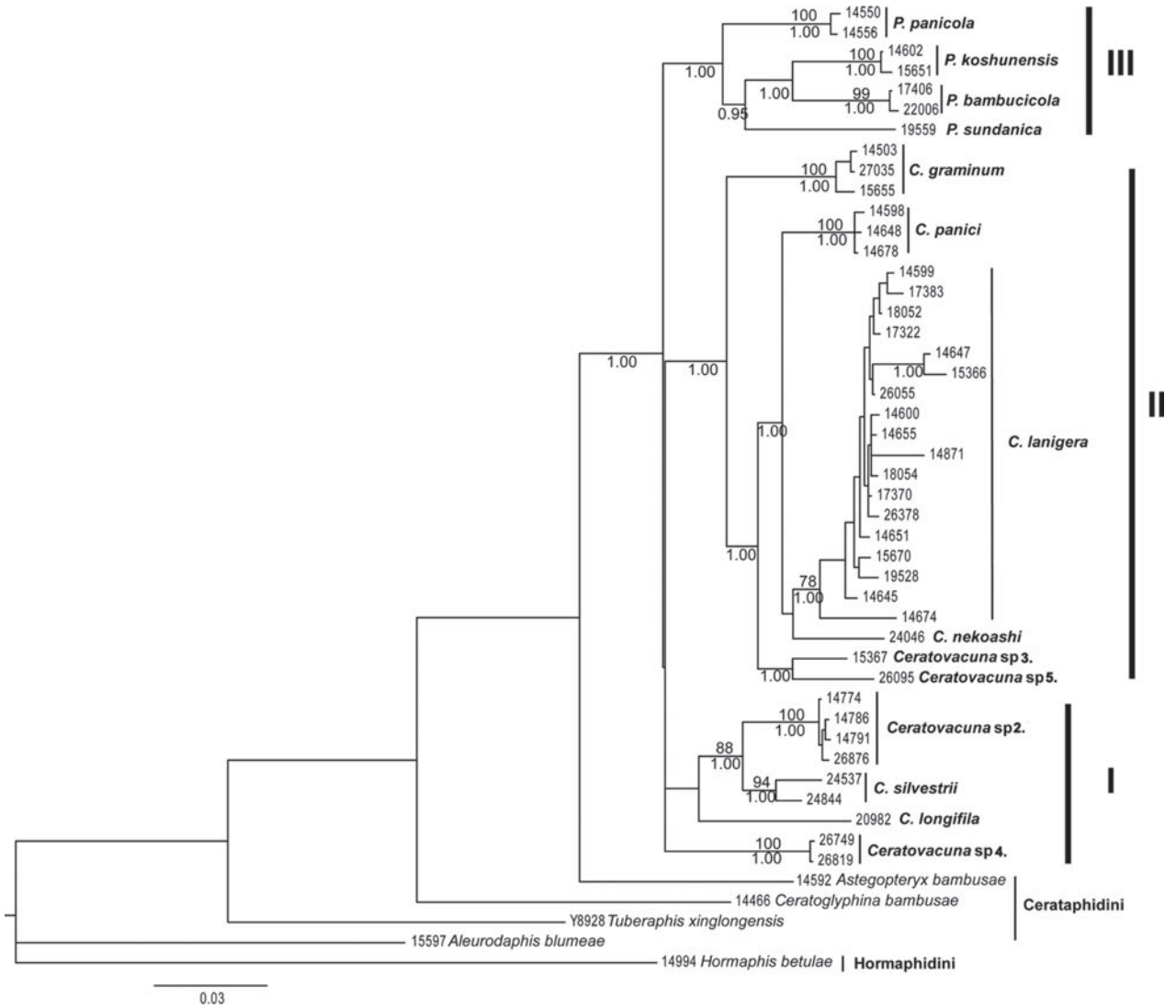

Fig 4. Phylogenetic relationships between genus *Ceratovacuna* and *Pseudoregma* based on Bayesian analysis of the combined datasets of COI, Cytb, EF-1α, and LWO. Bootstrap percentages (≥50%) from ML are shown above the branch, posterior probabilities (≥50%) of Bayesian analysis are shown below the branch.

the analyses based on COI+Cytb (Suppl. fig. 1). All *Pseudoregma* species cluster into group III. Although the individual mitochondrial genes yield low resolution in resolving the topology of deeper nodes, the analyses based on nuclear genes and combined dataset of COI+Cytb+EF-1α+LWO (fig. 4) robustly supported the monophyly of the three main groups. Morphological evidence also supported molecular phylogenetic relationships between species. For example, the relationships of *C. silvestrii* with *Ceratovacuna* sp2., and the species of group II are further supported by their wax plate characteristics. The abdominal tergites of all species in group II have only marginal wax plates, while *C. silvestrii* and *Ceratovacuna* sp2. in group I, along with all species of *Pseudoregma* in group III, all exhibit spinal wax plates and some have pleural wax plates. In addition, wax plates on the head dorsum of *C. silvestrii* and *Ceratovacuna* sp2. are composed of 8–16 wax cells, and both of them are rich in small round or polygonal wax plates on their abdominal tergites. On the other hand, marginal wax plates on the abdominal tergites of all species in group II are composed of fewer than 12 wax cells. Compared to *Pseudoregma*, the wax plates of *Ceratovacuna* are more strongly developed. In contrast to species of *Ceratovacuna*, the posterior margin of the pronotum of *Pseudoregma* species, where some wax plates are distributed, is swollen; while this part of other species of *Ceratovacuna* is flat. Thus, morphological traits supported these three main groups of *Ceratovacuna* and *Pseudoregma*.

In the phylogenetic analyses based on EF-1α (fig. 3), the monophyly of *C. silvestrii* and *P. alexanderi* samples was not supported. The monophyly of *C. silvestrii* was not in the EF-1α+LWO tree. However, these two species could be distinguished in the trees using COI as well as the combined datasets of COI+Cytb and COI+Cytb+EF-1α+LWO. This may be explained by the differential phylogenetic information

Table 2. Estimated divergence times for selected nodes.

Node no.	Node explain	COI		EF-1α	
		Time (mya)	95% HPD range	Time (mya)	95% HPD range
1	*C. graminum* van der Goot	11.4	1.2–20.9	7.8	1.7–15.8
2	*C. panici* van der Goot	10.5	1.5–18.8	7.6	1.3–18.6
3	*C. lanigera* Zehntner	24.3	9.5–37.9	18.5	11.2–28.0
4	*C. nekoashi* Sasaki	12.1	4.2–25.0	16.2	9.9–24.4
5	*Ceratovacuna* sp4.	8.8	4.9–13.1	4.3	0.0–12.9
6	*P. alexanderi* Takahashi	10.0	1.1–16.5	13.4	6.6–17.7
7	*P. koshunensis* Takahashi	11.0	0.6–21.3	8.9	4.7–14.3
8	*P. bambucicola* Noordam	17.7	5.1–25.5	8.8	2.8–18.1
9	*P. panicola* Noordam	15.3	3.4–27.3	9.1	2.7–18.4
10	*P. sundanica* van der Goot	11.8	0.5–24.4	7.2	2.4–14.4
11	*C. silvestrii* Takahashi	11.1	1.5–18.5	12.0	4.8–21.3
12	*Ceratovacuna* sp2.	15.8	4.2–26.8	10.4	3.4–18.4

Node numbers are corresponding to that of fig. 5.

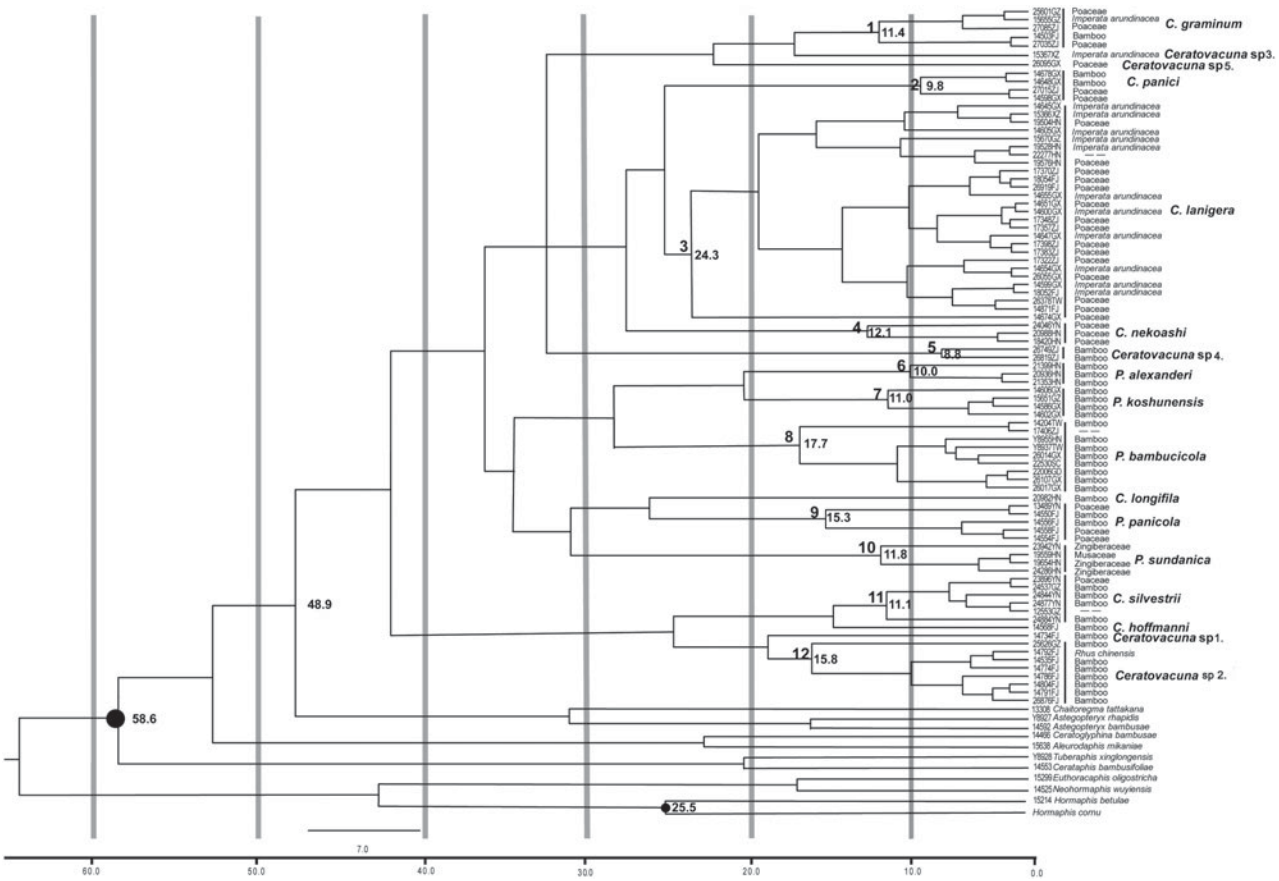

Fig. 5. A chronogram of *Ceratovacuna* and *Pseudoregma* based on the mitochondrial gene COI. Median age estimates are shown behind nodes (mya). Numbers 1–12 corresponding to table 2. Black dot represents two calibration points used in divergence time analyses. Locality abbreviated in capital letters: XZ, Tibet; GZ, Guizhou; YN, Yunnan; HN, Hainan; GX, Guangxi; FJ, Fujian; GD, Guangdong; ZJ, Zhejiang; SC, Sichuan; TW, Taiwan; CQ: Chongqing.

content of these genes. Owing to their slower evolutionary rate, nuclear markers are usually applied to investigate deeper phylogenetic relationships of aphids above genus level, whereas mitochondrial genes are more suitable for resolving relationships at the intra- and interspecific levels (Zhang & Qiao, 2006). *Ceratovacuna* sp2. and *C. silvestrii* are likely to have diverged recently and hence nuclear genes may not yet have sorted completely.

Divergence times and implications for species diversification

The estimated divergence times (fig. 5) indicate that the diversification of most sampled species of *Ceratovacuna* and *Pseudoregma* occurred at approximately 10 mya in the late Tertiary (table 2). Short time intervals are evident among different species. Phylogenetic analyses indicate low resolution of the relationships among the three main groups of the sampled species of *Ceratovacuna* and *Pseudoregma*. Therefore, the divergence times and phylogenetic relationships suggest that *Ceratovacuna* and *Pseudoregma* may have undergone a rapid burst of speciation events in the late Tertiary. The radiation is likely to be related to the evolution of their secondary host plants. The secondary hosts of *Ceratovacuna* and *Pseudoregma* are plants of Poaceae. Owing to climatic cooling, herbaceous angiosperms such as Poaceae and Asteraceae appeared to be dominant in the middle to late Tertiary period (Heie, 1996; von Dohlen & Moran, 2000). In fact, the divergence times in the present study are consistent with the results of Huang *et al*. (2012), which suggest that Cerataphidini most likely acquired its secondary hosts during the middle to late Tertiary period.

Except for *C. lanigera*, intraspecific genetic distances of mitochondrial COI were 0–1.6% in the two genera. However, the distances for *C. lanigera* samples were 0–4.5%. The genetic distances between one sample (No. 14674) from Jiuwan Mountain with other samples varied from 4.0 to 4.5%, which were much larger than the differences between the other species. Phylogenetic analyses based on mitochondrial genes all supported sample No. 14674 forming a separate clade from all other *C. lanigera* samples. Sample No. 14674 has no wax plates on the dorsal side of the head and pronotum, and the wax cells of the marginal wax plates on mesonotum and abdominal tergites are loosely arranged. All other samples of *C. lanigera* exhibit a pair of marginal wax plates on the head dorsum and pronotum, along with a much tighter arrangement of wax cells on the marginal wax plates of the mesonotum and abdominal tergites. Current evidences of genetic distances, phylogenetic topology, and morphological characteristic indicate that there may have been one cryptic species in *C. lanigera*. Geographical isolation of Jiuwan Mountain is likely to be a main factor for intraspecific divergence. However, further investigation based on more samples especially from the Jiuwan Mountain region is needed to test whether this putative cryptic species is valid.

The supplementary material for this article can be found at http://www.journals.cambridge.org/BER

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