

Mitochondrial phylogeography of a leafminer parasitoid, *Diglyphus isaea* (Hymenoptera: Eulophidae) in China

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Received 14 October 2005; accepted 14 April 2006

Available online 25 April 2006

Abstract

Diglyphus isaea is one of the primary parasitoids of agromyzid leafminers. Mitochondrial cytochrome oxidase subunit I sequences from 10 Chinese localities were used to investigate geographic variation within this widespread and morphologically uniform parasitoid. Phylogeographic analyses and sequence variation indicated that this species was actually a species-complex. We used an amber fossil record of the genus *Entedon* (Eulophidae: Entedoninae) as a reference to estimate the divergence time of major clades within *D. isaea*. With reference to the fossil record of its hosts, our results indicate that associations between parasitoids and leaf-miners could have been possibly established 38.2 million years ago.

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Keywords: Phylogeography; Genetic variation; Cryptic species; *Diglyphus isaea*; COI; Divergence time

1. Introduction

Diglyphus isaea (Walker, 1838) (Hymenoptera: Eulophidae) is an important parasitoid of agromyzid leafminers (Diptera: Agromyzidae), which includes the economically important genus *Liriomyza* (Hendrickson and Barth, 1979; Heinz et al., 1988, 1993; LaSalle and Parella, 1991; Sher et al., 1996). Some *Liriomyza* species, including *L. huidobrensis* and *L. sativae*, have recently been reported from most provinces of mainland China. These have been considered to have been imported via international trade of vegetables and flowers (Jiang, 1997; Kang, 1996).

For agriculture pest management strategies, biological control is among the most important methods. However, without correct identifications of parasitoids and their hosts, it is difficult to use the former to control the latter consis-

tently. *D. isaea* is recorded from nine species of *Liriomyza* (Noyes, 2002) and commercialized to control these leafminers. It is common in all provinces in mainland China (Zhu et al., 2000) as well as in other regions of the earth (Noyes, 2002). Although not studied in detail, the flying capability of *D. isaea* is considered to be weak. Furthermore, it can be difficult to distinguish species using solely morphology because considerable homoplasticity exists in eulophids (Ubaidillah et al., 2003). Historically, 10 scientific names are associated with *D. isaea* (Gonzalez et al., 1979; Zhu et al., 2000). Misidentification obscures our understanding of various aspects of the biology and ecology of this species.

Phylogeographic analysis can be used to delineate haplotype clusters that might correspond to morphologically cryptic species and to identify possible causes of divergence (Avice, 2000). Recent investigations of variation in gene sequences of nuclear ribosomal ITS1 shows extensive cryptic diversity, uncovering five species under the name of *D. isaea* (Sha et al., in press). However, these results are strongly

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dependent on the treatment of insertions/deletion sites (indels). Length variation in RNA genes renders homology determination among sites a problematic, time-consuming procedure (Brower and DeSalle, 1998). The rapid evolution and selective constraints make mitochondrial protein-encoding genes useful molecular markers for separating species (Hebert et al., 2003; Hebert et al., 2004a,b; Wares and Cunningham, 2001). In this paper, the primary objectives are: (1) to investigate the genetic diversity within and among Chinese *Diglyphus*; (2) to evaluate whether or not Chinese *D. isaea* is a species-complex; (3) to estimate the divergence time of major clades, if any.

2. Materials and methods

2.1. Samples

Ten geographical groups of *D. isaea* were sampled mainly from western China. Individuals from related genera were also obtained for outgroup analysis (Zhu et al., 2000). To ensure that only morphological forms of *D. isaea* were included in the study, all specimens were identified by CDZ before DNA extraction (Zhu et al., 2000). All specimens used in this study are listed in Table 1 and all sites of specimen collection are marked in map (Fig. 1). Voucher specimens were deposited in the Institute of Zoology, Chinese Academy of Sciences (IZCAS). Total DNA was extracted with phenol/chloroform and DNA was precipitated with ethanol as described by Sambrook et al. (1989).

2.2. Sequences

Fragments from COI were amplified with standard polymerase chain reaction (PCR) protocols and directly sequenced after purification. Two pairs of primers were used for PCR and sequencing: COISF 5'-TAAGATTTTG ATTATT(AG)CC(TA)CC-3' and COI2191, 5'-CCCGGT AAAATTTAAATATAAACTTC-3', COI2193, 5'-TTTT TTGGTCATCCAGAAGT-3' and COI2613, 5'-ATTGC AAATACTGCACCTAT-3'. The forward primer of the first pair was designed in the laboratory of the Institute of Zoology. Others were taken from Simon et al. (1994) and Chen et al. (2004). PCR was carried out in standard 25 µl reactions of 35 thermal cycles. Each reaction contained 0.125 µl exTaq polymerase (250 U, TaKaRa), 2.5 µl exTaq buffer, 2.0 µl MgCl₂ (25 mM), 1.0 µl dNTPs (25 nmol), 0.5 µl primers (10 pmol), 5.0 µl template DNA and 14.0 µl dH₂O. Each cycle consists of melting temperature at 94 °C for 30 s, annealing at 48 °C for 45 s, and extension at 72 °C for 1 min. PCR products were purified using Minipore protocols (Qiagen) and sequencing reactions were conducted with ABI Big Dye protocols.

2.3. Data analyses

Sequences were first aligned by Clustal W (Thompson et al., 1997) with default parameters and then manually

checked in BioEdit (Hall, 1999). *Diglyphus bimaculatus* was selected as an outgroup since it was considered to be morphologically intermediate between genera *Diglyphus* and *Cirrospilus* (Zhu et al., 2000). Maximum parsimony (MP) was implemented with the heuristic search option in PAUP 4.0b10 (Swofford, 2002). One hundred random stepwise addition replicates via TBR were conducted. All informative characters in MP analyses were weighted equally and unordered. Bootstrapping proportions (BSP) (Felsenstein, 1985) with 1000 replicates were used for branch support.

The best-fit model of DNA evolution was selected by comparing log-likelihood scores of 56 models of base substitution using the program Modeltest (Posada and Crandall, 1998). Maximum likelihood analyses were based on the selected model using the heuristic search algorithm. A Neighbor-joining phenogram was constructed basing on uncorrected 'p' distances model in MEGA3 (Kumar et al., 2004). Nodal support was evaluated by 100 bootstrap replicates.

Bayesian analyses were performed with MrBayes 2.0 (Huelsenbeck and Ronquist, 2001). All Bayesian analyses were initiated with random starting trees. The Markov Chain Monte Carlo analyses were run for 2×10^6 generations to allow for adequate time of convergence. Trees were sampled every 100 generations. After approximately 300,000 generations, the log-likelihood values of each sampled tree stabilized. We used the last 17,000 sample trees to estimate the 50% majority rule consensus tree and the Bayesian posterior probabilities (BPP). Uncorrected pairwise distance was computed in MEGA3 (Kumar et al., 2004).

2.4. Estimation of the most recent common ancestors

We included samples of the eulophid genus *Entedon Dalman, 1820*, because congeneric species (identified by S.G.A. Compton and CDZ in University of Leeds, UK) were available in Dominican Amber dating from around 20–25 million years ago (Lambert et al., 1985). Other extant entedonine genera were also recognizable from this period of amber (Gumovsky, 2001). Two species of *Eulophus* were reported from the Tertiary Epoch (1.8–65 million years ago) (Statz, 1938), which covers too long a time span to be useful for calibrations. Thus, we used *Entedon* as the calibrating point (22.5 million years ago). *Euderus* was also chosen as our initial outgroup taxon.

The Markov Chain Monte Carlo analysis (MCMCAS) was carried out using Bayesian Evolutionary Analysis Sampling Trees (BEAST, Ver. 1.1.2; Drummond and Rambaut, 2004). A randomly generated cladogram assuming the coalescent process was used as the initial tree. The chain length was set to be 2 million generations. MCMCAS was used to estimate the mutation rates of the fragment of COI and the most recent common ancestors (MRCAs) of different clades found by the MP analysis.

3. Results

Twenty-nine COI sequences of *D. isaea* were collected from different localities. Fifteen COI sequences of the related species were also obtained (Table 1). All sequences were deposited in GenBank (Table 1). Aligned sequences used in the final analyses are available upon request.

3.1. Sequence composition

COI sequences were easily aligned with few insertions or deletions. The alignment yielded a 745 bp fragment, which corresponds to bases 1818–2568 of the mitochondrial genome of *Drosophila yakuba* (Clary and Wolstenholme, 1985). Once aligned, no indels were detected. However, with the reference to the mitochondrial genome of *D. yakuba*, six

Table 1
Detailed information about sampled specimens

| Species name | Specimen number | Locality and altitude | Sex | GenBank number |
|--------------------------------------|-----------------|--------------------------------------|--------|----------------|
| <i>Aulogmnus</i> | | | | |
| <i>A. sp GXjxsts 2 1095</i> | Aulo2 | Jinxiushengtangshan; GUANGXI; 1095 m | Female | DQ149154 |
| <i>Cirrospilus</i> | | | | |
| <i>C. diallus QHgemglmd 103 2880</i> | Cirr103 | Geermuguolemude; QINGHAI; 2880 m | Female | DQ149155 |
| <i>C. pictus BJmtg 101 1300</i> | Cirr101 | Mentougou; BEIJING; 1300 m | Female | DQ149156 |
| <i>C. variagatus SXhyhs 4 1475</i> | Cirr4 | Hengyuanhengshan; SHANXI; 1475 m | Male | DQ149157 |
| <i>C. vittatus QHgem 104 2880</i> | Cirr104 | Geermuguolemude; QINGHAI; 2880 | Female | DQ149158 |
| <i>Diglyphus</i> | | | | |
| <i>D. begini TBls 187 3650</i> | Dig187 | Lasa; XIZANG; 3650 m | Male | DQ149159 |
| <i>D. bimaculatus TBjz 128 4700</i> | Dig128 | Jiangzirelong; XIZANG; 4700 m | Female | DQ149160 |
| <i>D. bimaculatus TBls 130 3650</i> | Dig130 | Lasa; XIZANG; 3650 m | Female | DQ149161 |
| <i>D. crassinervis QH 119 2900</i> | Dig119 | Delinghabangexiang; QINGHAI; 2900 m | Female | DQ149162 |
| <i>D. isaea BJnky 113 50</i> | Dig113 | Weigongcun; BEIJING; 50 m | Female | DQ149163 |
| <i>D. isaea BJnky 190 50</i> | Dig190 | Weigongcun; BEIJING; 50 m | Male | DQ149164 |
| <i>D. isaea GSmxhlc 118 2700</i> | Dig118 | Mingxianhonglingcun; GANSU; 2700 m | Female | DQ149165 |
| <i>D. isaea GSzq 141 2350</i> | Dig141 | Zhouqushatan; GANSU; 2350 m | Female | DQ149166 |
| <i>D. isaea GXdx 114 5 200</i> | Dig114 | Daxin; GUANGXI; 200 m | Male | DQ149167 |
| <i>D. isaea GZfjs 110 2200</i> | Dig110 | Fanjingshan; GUIZHOU; 2200 m | Male | DQ149168 |
| <i>D. isaea GZfjs 111 2200</i> | Dig111 | Fanjingshan; GUIZHOU; 2200 m | Female | DQ149169 |
| <i>D. isaea LNxy 145 200</i> | Dig145 | Xiuyanshi; LIAONING; 200 m | Male | DQ149170 |
| <i>D. isaea QHdlhbg 122 2900</i> | Dig122 | Delinghabangexiang; QINGHAI; 2900 m | Male | DQ149171 |
| <i>D. isaea QHdlhbg 123 2900</i> | Dig123 | Delinghabangexiang; QINGHAI; 2900 m | Female | DQ149172 |
| <i>D. isaea QHgem 103 2880</i> | Dig103 | Geermu; QINGHAI; 2880 m | Female | DQ149173 |
| <i>D. isaea QHq1 108 2790</i> | Dig108 | Qilianshan; QINGHAI; 2790 m | Male | DQ149174 |
| <i>D. isaea QHq1 11 2790</i> | Dig11 | Qilianshan; QINGHAI; 2790 m | Female | DQ149175 |
| <i>D. isaea QHq1 126 2790</i> | Dig126 | Qilianshan; QINGHAI; 2790 m | Female | DQ149176 |
| <i>D. isaea SCbxhpg 107 2200</i> | Dig107 | Baoxinghepinggou; SICHUAN; 2200 m | Female | DQ149177 |
| <i>D. isaea SCbxhpg 152 2200</i> | Dig152 | Baoxinghepinggou; SICHUAN; 2200 m | Female | DQ149178 |
| <i>D. isaea SCbxhpg 153 2200</i> | Dig153 | Baoxinghepinggou; SICHUAN; 2200 m | Male | DQ149179 |
| <i>D. isaea SCbxhpg 175 2200</i> | Dig175 | Baoxinghepinggou; SICHUAN; 2200 m | Female | DQ149180 |
| <i>D. isaea SCbxhpg 176 2200</i> | Dig176 | Baoxinghepinggou; SICHUAN; 2200 m | Female | DQ149181 |
| <i>D. isaea SCbxhpg 6 2200</i> | Dig6 | Baoxinghepinggou; SICHUAN; 2200 m | Female | DQ149182 |
| <i>D. isaea SCgzkd 124 2400</i> | Dig124 | Ganzikangding; SICHUAN; 2400 m | Female | DQ149183 |
| <i>D. isaea SCgzkd 174 2400</i> | Dig174 | Ganzikangding; SICHUAN; 2400 m | Female | DQ149184 |
| <i>D. isaea SXhyhs 14 3 1475</i> | Dig14 | Henyuanhengshan; SHANXI; 1475 m | Male | DQ149185 |
| <i>D. isaea SXzzhzz 115 1350</i> | Dig115 | Zhouzhihouzhenzi; SHANXI; 1350 m | Female | DQ149186 |
| <i>D. isaea SXzzhzz 168 1350</i> | Dig168 | Zhouzhihouzhenzi; SHANXI; 1350 m | Female | DQ149187 |
| <i>D. isaea SXztsbs 180 2750</i> | Dig180 | Zhouzhitanbaishan; SHANXI; 2750 m | Male | DQ149188 |
| <i>D. isaea SXztsbs 181 2750</i> | Dig181 | Zhouzhitanbaishan; SHANXI; 2750 m | Female | DQ149189 |
| <i>D. isaea TBbsbd 136 4390</i> | Dig136 | Bangsubangda; XIZANG; 4390 m | Female | DQ149190 |
| <i>D. isaea TBls 138 3650</i> | Dig138 | Lasa; XIZANG; 3650 m | Female | DQ149191 |
| <i>D. minoerus TBjzrl 101 1 4700</i> | Dig101 | Jiangzirelong; XIZANG; 4700 m | Male | DQ149192 |
| <i>D. pachyneurus QHdl 121 2900</i> | Dig121 | Delinghabangexiang; QINGHAI; 2900 m | Male | DQ149193 |
| <i>Entedon</i> | | | | |
| <i>Entedon sp2 BJmtg 2 1280</i> | Ent2 | Mengtougou; BEIJIAN; 1280 | Female | DQ149194 |
| <i>Entedon sp3 SCbx 3 2200</i> | Ent3 | Baoxinghepinggou; SICHUAN; 2200 m | Female | DQ149195 |
| <i>Euderus</i> | | | | |
| <i>Euderus sp1 BJzhwy 101 100</i> | Eud101 | Zhiwuyuan; BEIJING; 100 m | Female | DQ149196 |
| <i>Eulophus</i> | | | | |
| <i>Eulophus sp2 TBcd 102 3400</i> | Eulo102 | Changdu; XIZANG; 3400 m | Female | DQ149197 |



Fig. 1. Map showing sampling sites for *Diglyphus isaea* (also see Table 1). Five main clades numbers (correspond to Fig. 2) were marked on every site.

deletions were detected between sites 1996–2001 from all of our samples. In total, 249 sites were variable (33.4%) and 200 of them were potentially informative for parsimonious analyses (26.8%). Average frequencies were 29.3% for A, 12.4% for C, 15.6% for G and 42.7% for T. The COI sequences showed a strong adenine and thymine (AT) bias, 72%, as typical for insect mitochondrial genomes (Crozier and Crozier, 1992, 1993; Simon et al., 1994; Arias and Sheppard, 1996). Compared to the first and second positions, the third position had as expected a stronger AT bias of 90.4%. All sequences were translated into amino acids using the mtDNA genetic code of *D. yakuba*.

3.2. Phylogenetic analyses

Maximum-parsimony analyses produced 45 equally most parsimonious trees, each with 582 steps, a consistency index (CI) of 0.581, and a retention index (RI) of 0.787. The strict consensus showed five major clades or groups in Chinese *D. isaea* (Fig. 2). For Groups I–II, bootstrap analysis revealed high branch support. Other groups were weakly supported, with BSPs under 60. Because of the low support, Groups III, IV and V were treated as one clade.

The likelihood ratio test chose the model TVM+I+G. The model allowed for equal transition rates and nucleotide substitution with invariable sites plus a G distribution of variable-rate sites. Maximum likelihood analysis produced the same results as those from MP analysis. The 50% majority rule consensus tree and NJ trees were completely compatible with the MP tree (Fig. 3).

3.3. Sequence divergence

Uncorrected *p* distances of interspecies in six genera are shown in Table 2. In the Eulophidae, the interspecies

sequence divergences between the Euderinae and Eulophinae ranged from 11.4–17.4%, while that between the Entedoninae and Eulophinae ranged from 12.6–19.7%. In the Eulophinae, the interspecies divergence among genera *Aulogymnus*, *Cirrospilus*, *Diglyphus* and *Eulophus* were around 10.6%. In *Diglyphus*, the variation of two samples of *D. bimaculatus* was 2.3%. The interspecies divergences were about 9.5% in this genus. Because this paper focused on variation within *D. isaea*, the value of 9.5% divergence implies the presence of multiple species within *Diglyphus*.

The uncorrected pairwise distance among all samples of *D. isaea* ranged from 0–17.4%. With reference to Group I, divergence between Groups II–V exceeded 9.5% (Table 3), which indicated the presence of multiple cryptic species. The result was similar to those from phylogenetic analysis.

Variation among members of Group II, ranging from 1.1–3.8%, was much lower than the delimiting value of 9.5%. Similarly, in Group I the uncorrected pairwise nucleotide divergence ranged from 0–6.3%. Except for sample 114, the divergence between both samples 118 and 122 and the others ranged from 10.2–17.9%; these divergences indicated the presence of cryptic species.

3.4. Divergence time

Mitochondrial DNA sequences diverge at 1–1.5% per million years for invertebrates (Palumbi and Wilson, 1990). In our study, after calibration with amber wasps, the evolution rates were 3.225×10^{-3} , 8.742×10^{-4} and 1.511×10^{-2} for the 1st, 2nd and 3rd codons, respectively. Five MRCAs of major branches I–V were identified to be as follows (Fig. 4): 29.9, 23.0, 36.5, 34.0 and 34.4 Ma, respectively. The MRCA of Groups I–V of *D. isaea* was estimated at 38.2 Ma.

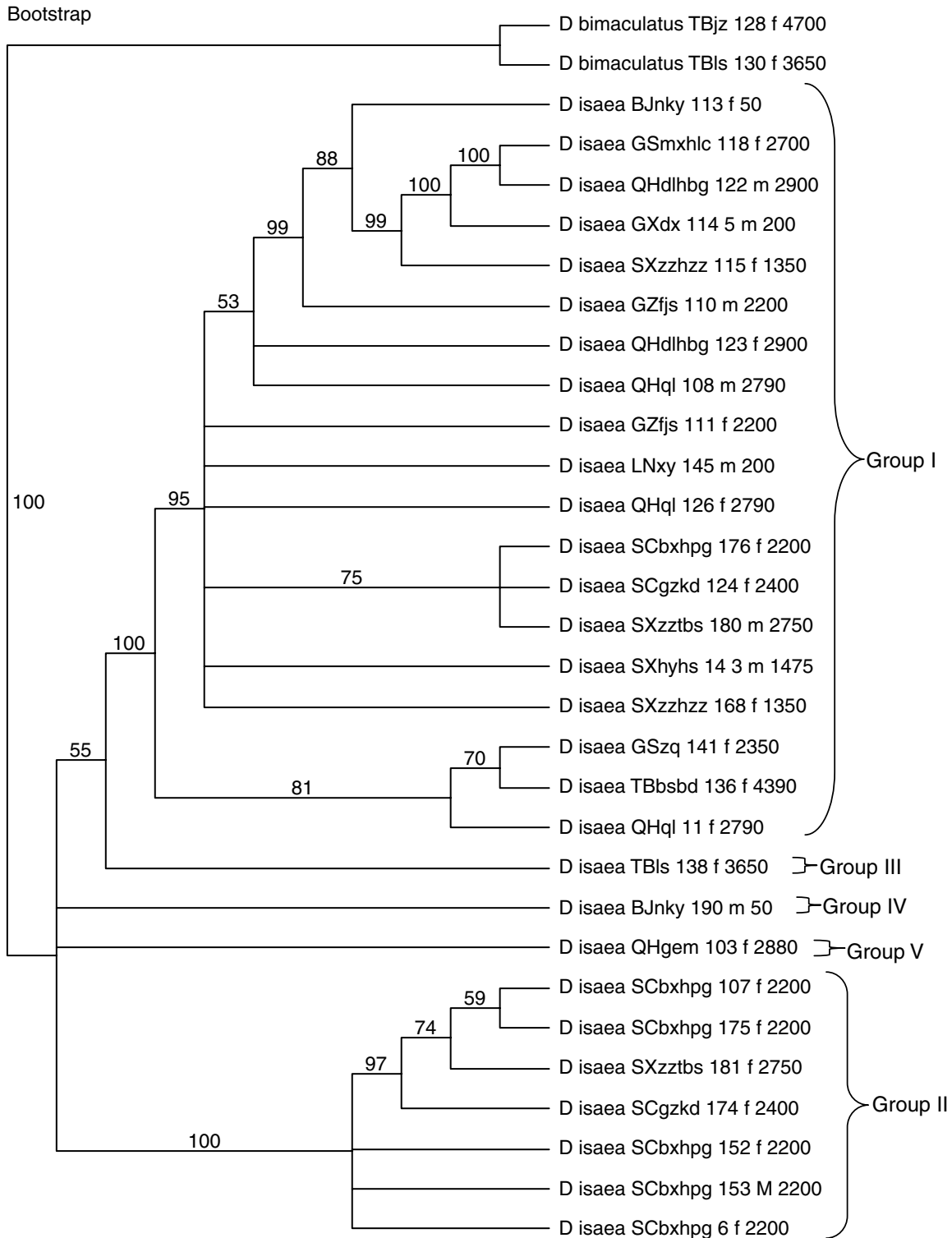


Fig. 2. Parsimonious phylograms inferred from mitochondrial protein-coding gene, cytochrome *c* oxidase subunit I (COI) partial sequences (Tree number = 45; Bootstrap values shown above branches; Tree length = 561, CI = 0.581, RI = 0.7873).

4. Discussion

Cryptic species can be defined as morphologically indistinct lineages that are genetically, and thus reproductively isolated. A detailed morphological examination of a large series of *D. isaea* from China reveals that the species exhibit

little morphological variation (Zhu et al., 2000). Current phylogeographic analysis and sequence divergence results show five main clades in *D. isaea*, all of which likely represent species. Cryptic species or species-complexes also may be common in the Eulophidae because homoplasticity is common (Ubaidillah et al., 2003). New biodiversity has

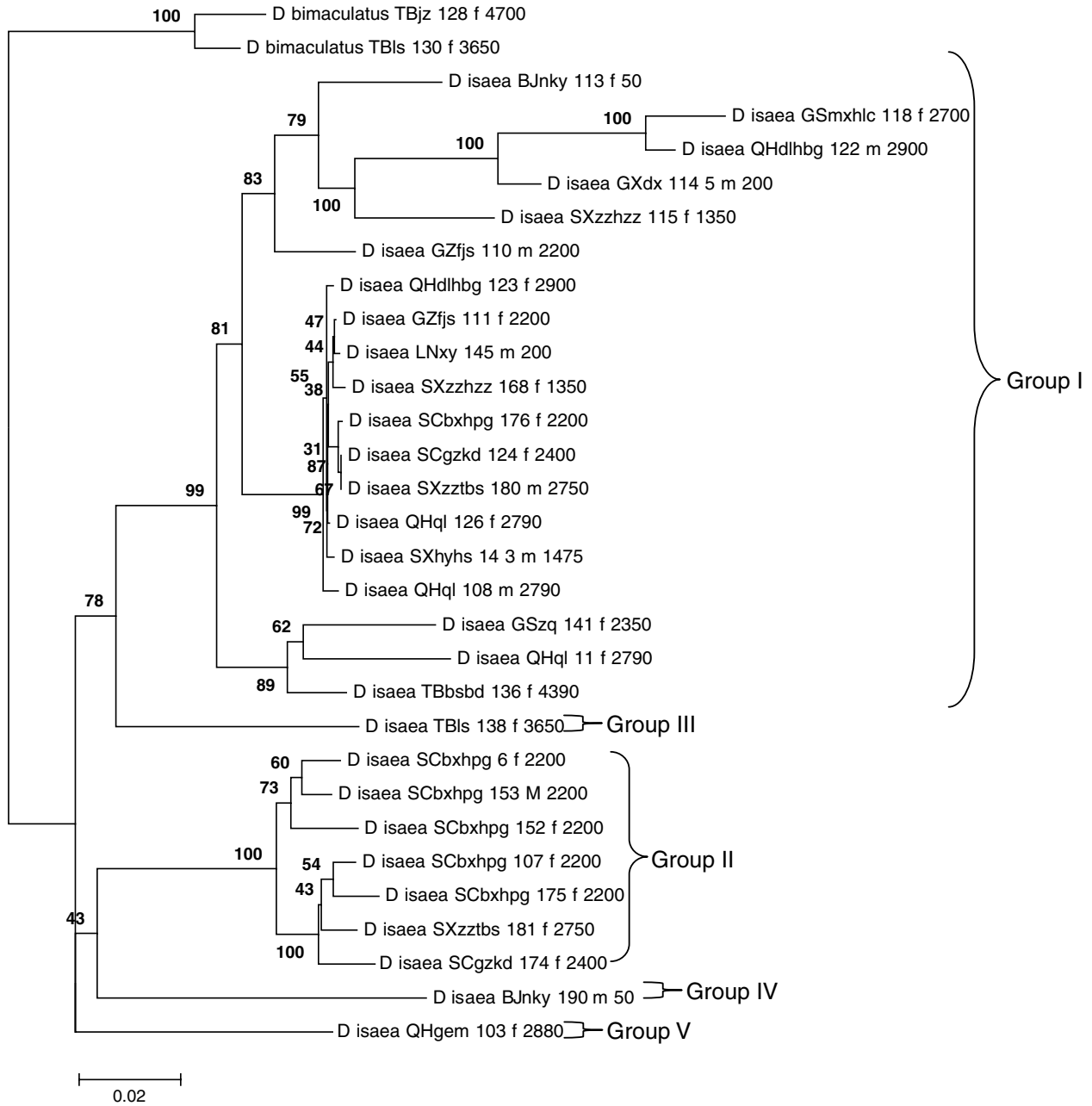


Fig. 3. Neighbour-joining phylogram inferred from COI part sequence using an *p*-distance model. Substitution to include = transitions + transversions. Rates among sites = uniform rates.

been also documented in other families of chalcidoids, such as within the Encyrtidae (Hoy et al., 2000), Eurytomidae (Claridge and Askew, 1960), Aphelinidae (Hafez and Douth, 1954) and Agaonidae (Molbo et al., 2003). Indeed, numerous species complexes occur in parasitoid Hymenoptera (Gordh, 1982). Often one widespread species is found to consist of sympatric sibling species or allopatric forms that are reproductively isolated (Gordh, 1982).

Diglyphus isaea has long been treated as a single species with a wide distributional pattern (Zhu et al., 2000; Noyes, 2002). Our results reveal cryptic diversity in *D. isaea*, which illustrates the value of DNA sequences in discovering hidden diversity. Although our results from COI reveal high

divergence of samples TBLS 138 (Group III) and QHGEM 103 (Group V), sequences from ITS1 suggest both are not distinct species (Sha et al., in press). Because mitochondrial genes evolve faster than nuclear ones, COI may be more suitable for distinguishing species when speciation is rapid. For Group IV, divergence may reflect a recent mixture of reproductively compatible but previously disjunct populations. In 1998, some individuals of *D. isaea* had been intentionally introduced as biological control agents to Beijing from Holland (Chen et al., 2000). Although the experiment was stated to be restricted to the indoors, it is still possible that some of those parasitoids escaped from the laboratory. Such an escape could lead to divergence within and among

Table 2
Uncorrected *p* distance from mitochondrial COI part sequences of different species between different Eulophid genera and between different *Diglyphus* species. Standard error in upper right, distances in lower left

| Species name | Location number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---------------------------------|-----------------|-------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| 1 <i>Autogynmus</i> sp | GXjxsts2 | | [0.013] | [0.013] | [0.013] | [0.013] | [0.013] | [0.013] | [0.013] | [0.013] | [0.012] | [0.012] | [0.012] | [0.012] | [0.012] | [0.012] | [0.013] | [0.013] |
| 2 <i>Cirrospilus diatlius</i> | Qhgem103 | 0.150 | | [0.012] | [0.013] | [0.011] | [0.013] | [0.013] | [0.013] | [0.014] | [0.012] | [0.012] | [0.012] | [0.012] | [0.013] | [0.013] | [0.013] | [0.013] |
| 3 <i>Cirrospilus pictus</i> | BJmtg101 | 0.141 | 0.133 | [0.012] | [0.013] | [0.012] | [0.013] | [0.013] | [0.012] | [0.013] | [0.012] | [0.012] | [0.012] | [0.011] | [0.013] | [0.013] | [0.013] | [0.013] |
| 4 <i>Cirrospilus variagatus</i> | SXhyhs4 | 0.133 | 0.141 | 0.134 | [0.013] | [0.013] | [0.013] | [0.013] | [0.012] | [0.013] | [0.012] | [0.012] | [0.012] | [0.011] | [0.012] | [0.012] | [0.012] | [0.012] |
| 5 <i>Cirrospilus vittatus</i> | QHgem104 | 0.146 | 0.105 | 0.134 | [0.013] | [0.013] | [0.013] | [0.013] | [0.012] | [0.013] | [0.012] | [0.012] | [0.012] | [0.012] | [0.013] | [0.013] | [0.014] | [0.013] |
| 6 <i>Diglyphus begini</i> | TBls187 | 0.137 | 0.146 | 0.137 | 0.128 | 0.145 | 0.152 | [0.013] | [0.012] | [0.013] | [0.012] | [0.011] | [0.011] | [0.011] | [0.013] | [0.013] | [0.013] | [0.013] |
| 7 <i>Diglyphus bimaculatus</i> | TBJzl28 | 0.150 | 0.154 | 0.142 | 0.137 | 0.140 | 0.138 | 0.138 | [0.005] | [0.012] | [0.012] | [0.012] | [0.012] | [0.011] | [0.013] | [0.013] | [0.014] | [0.013] |
| 8 <i>Diglyphus bimaculatus</i> | TBls130 | 0.140 | 0.140 | 0.132 | 0.130 | 0.126 | 0.129 | 0.129 | 0.146 | [0.012] | [0.012] | [0.012] | [0.011] | [0.013] | [0.014] | [0.014] | [0.014] | [0.014] |
| 10 <i>Diglyphus isaea</i> | SCbxbpg152 | 0.134 | 0.132 | 0.125 | 0.111 | 0.133 | 0.113 | 0.119 | 0.107 | 0.132 | [0.005] | [0.014] | [0.014] | [0.011] | [0.012] | [0.012] | [0.013] | [0.012] |
| 11 <i>Diglyphus isaea</i> | SCbxbpg153 | 0.130 | 0.200 | 0.123 | 0.113 | 0.136 | 0.106 | 0.123 | 0.111 | 0.132 | 0.019 | 0.160 | [0.013] | [0.011] | [0.012] | [0.012] | [0.012] | [0.012] |
| 12 <i>Diglyphus minoensis</i> | TBJzrl101 | 0.177 | 0.200 | 0.189 | 0.164 | 0.174 | 0.168 | 0.154 | 0.174 | 0.099 | 0.164 | 0.160 | [0.014] | [0.011] | [0.014] | [0.014] | [0.015] | [0.014] |
| 13 <i>Diglyphus pachyneurus</i> | QHdl121 | 0.128 | 0.119 | 0.110 | 0.119 | 0.119 | 0.106 | 0.109 | 0.095 | 0.137 | 0.098 | 0.101 | 0.153 | [0.013] | [0.012] | [0.012] | [0.013] | [0.013] |
| 14 <i>Eulophus</i> sp2 | TBed102 | 0.129 | 0.138 | 0.140 | 0.106 | 0.153 | 0.141 | 0.142 | 0.130 | 0.165 | 0.130 | 0.122 | 0.180 | [0.012] | [0.012] | [0.012] | [0.012] | [0.012] |
| 15 <i>Entedon</i> sp2 | BJmtg2 | 0.136 | 0.157 | 0.150 | 0.126 | 0.148 | 0.138 | 0.144 | 0.133 | 0.165 | 0.129 | 0.129 | 0.188 | 0.133 | 0.133 | 0.106 | [0.011] | [0.012] |
| 16 <i>Entedon</i> sp3 | SCbx3 | 0.137 | 0.152 | 0.154 | 0.128 | 0.164 | 0.156 | 0.170 | 0.162 | 0.183 | 0.138 | 0.130 | 0.197 | 0.128 | 0.128 | 0.128 | 0.106 | [0.012] |
| 17 <i>Euderus</i> sp1 | BJzhwy101 | 0.142 | 0.149 | 0.138 | 0.114 | 0.142 | 0.161 | 0.157 | 0.154 | 0.164 | 0.134 | 0.130 | 0.174 | 0.140 | 0.121 | 0.126 | 0.115 | |

Table 3

Ranges of percentage of uncorrected pairwise distances among distinct groups based on 745 bp of COI for major clades within *D. isaea*

| Clade | Group I | Group II | Group III | Group IV | Group V |
|-----------|-----------|-----------|-----------|----------|---------|
| Group I | 0.1–17.9 | | | | |
| Group II | 10.2–17.4 | 1.1–3.8 | | | |
| Group III | 8.7–16.1 | 10.2–11.7 | 0 | | |
| Group IV | 11.9–17.2 | 11.1–11.9 | 11.5 | 0 | |
| Group V | 10.2–16.4 | 9.4–11.4 | 11.3 | 12.2 | 0 |

local populations. Although it is premature to describe these new taxa in the absence of corroborative evidence such as mating studies, our results suggest that *D. isaea* is a species-complex rather than a single species.

Within *Diglyphus*, the interspecific genetic distance averaged 9.5% or greater. This divergence was higher than that generally reported for mitochondrial genes. For example, in *Halictus*, a genus of bees, the interspecific divergence generally exceeded 5% for the mitochondrial protein coding gene cytochrome oxidase I (COI) and intraspecific sequence divergence did not exceed 4% (Danforth et al., 1998). Arias and Sheppard (1996) found sequence divergence within mitochondrial NADH dehydrogenase subunit 2 to be 2.13% among subspecies of honeybees. In the Agaonidae, Molbo et al. (2003) concluded that interspecific divergence was 4.2–6.62%, also at COI. Our results revealed that the level of divergence was greater in parasitoid *Diglyphus* than between species of agaonids.

Because mtDNA is maternally inherited, implications of gene flow are necessarily somewhat speculative. And because it is possible to have high levels of mtDNA differentiation and yet nuclear gene flow (e.g., Lindell et al., 2005), caution must be exercised in asserting the presence of multiple cryptic species based on mtDNA data alone. Regardless, the best explanation of our data is that *D. isaea* is an assemblage of multiple cryptic species.

Although cryptic species occur in many insects, the mechanisms responsible for their speciation are still poorly understood. Our results provide some preliminary evidence for sympatric speciation. In Group II most samples are from Baoxing and Kangding, Sichuan Province, southwestern China. However one sample, from Baoxin, clusters in Group I, a different species. The same pattern also occurs among samples from Beijing and Geermu. The distribution of five major groups is partially overlapping (Fig. 1), but the genetic distances between groups are highly divergent, reaching the interspecific level.

The potential hosts of *D. isaea*, species of *Liriomyza*, differ in their primary distribution. Some species, like *Liriomyza huidobrensis* and *Liriomyza sativae*, occur in most provinces of mainland China. However, host species are not yet known accurately and alien species already occur in Sichuan Province (Chen, 2003), where sympatric species of *D. isaea* occur. Founder events should occur frequently in parasitoids especially when transmission to new host individuals occurs. This often leads to high rates of speciation. Furthermore, cryptic species exist in a few species in

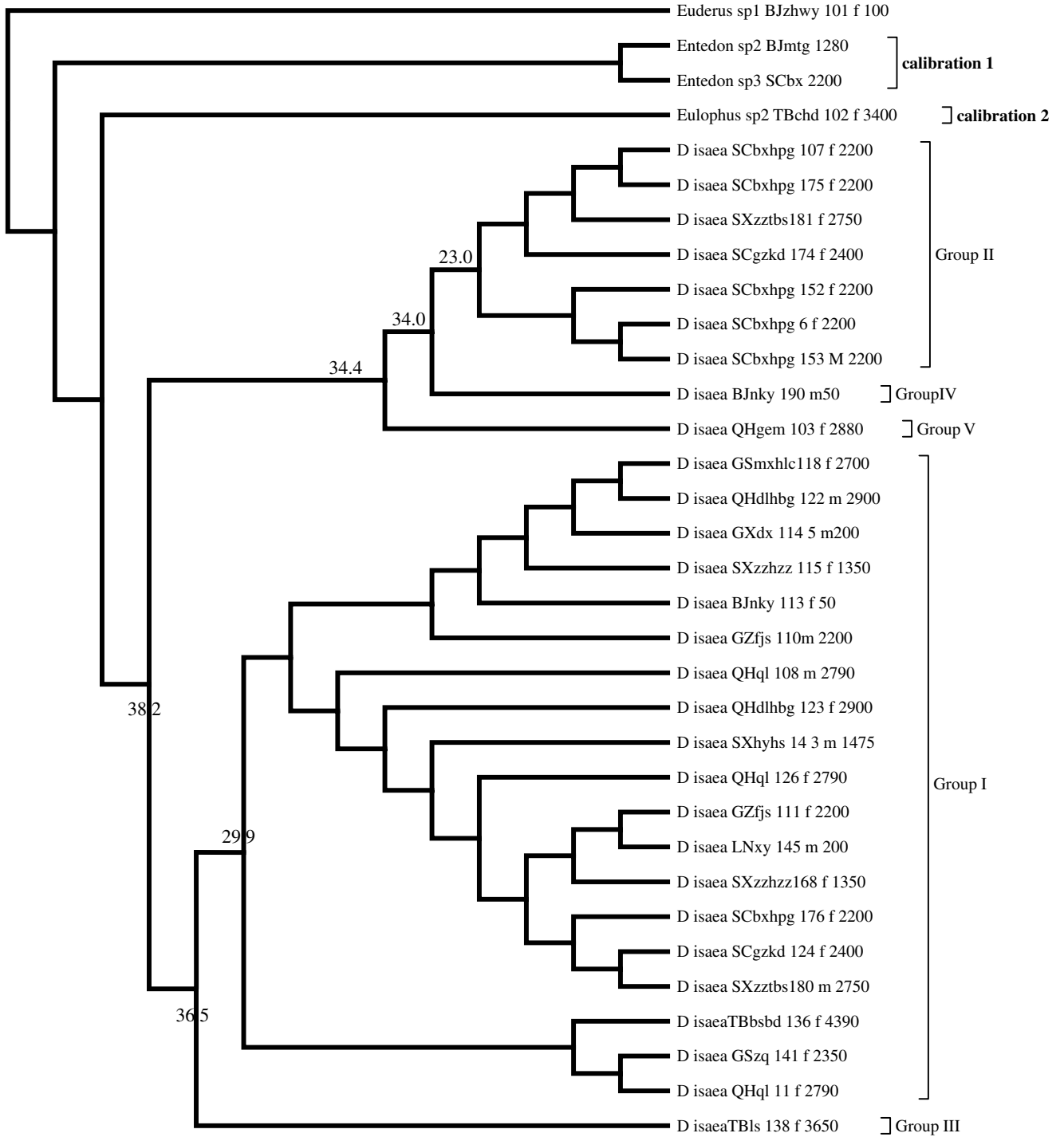


Fig. 4. Appearance time of the common ancestor of the major clades and species (unit = Mya).

Liriomyza (Diptera: Agromyzidae) (Scheffer, 2000; Scheffer and Lewis, 2001, 2005). Different species of *Liriomyza* have different leaf mine morphology, and these shapes significantly influence rates of parasitism (Parkman et al., 1989). Thus, host specificity of these parasitoids could make them prone to speciation because these specialized wasps have narrow ecological tolerances (Futuyma and Moreno, 1988).

Although sympatric speciation is possible, our data cannot refute a hypothesis of allopatric speciation. Baoxing and

Kangding, Sichuan Province, are isolated from other localities by topography and climate. Group II includes samples from both regions. Furthermore, localities of Group III and others are far away between each other; more than 3000 km separate LNxy145 and TBls138. The weak flying abilities of the parasitoids likely hinders migration and gene flow.

Highly divergent mitochondrial polymorphisms can be caused by recent mixture of reproductively compatible but previously disjunct populations. Most samples in this study

were collected from the southwestern China, where isolation and high elevation act against dispersion. Long-term fauna surveys in these regions suggest that migration of parasitoids between these isolated regions is very unlikely to occur.

Most modern insect orders became established in the Middle and especially Late Triassic (Labandeira and Eble, 2000). Among the Hymenoptera, some wasps became parasitoids on the diversity of herbivorous insects, including some leaf-miners. The modern forms of those leaf-miners include the larvae of the four holometabolous orders: Coleoptera, Diptera, Lepidoptera and Hymenoptera. Two typical leaf-mining families, the agromyzids and nepticulids, are attacked by parasitoid wasps from the Chalcidoidea. Characteristic leaf damage types are identified from mid-Cretaceous period (97Ma) (Labandeira, 1994). Our results reveal that the common ancestor of *D. isaea* probably appeared 38.2 million years ago. This implies that the associations between parasitoids and leaf-miners dates back to then. However, as many areas of China are not yet sampled, we cannot be sure how many divergent events actually occurred among Chinese groups.

Further samples from other regions of the world, especially those from United Kingdom, where the holotype of *D. isaea* was collected, can also help to identify which group is of the real *D. isaea*. Collecting from locations within the Groups III, IV, V will further explore the phylogeographic structure in this species. Additional study of molecular variation and biology of this wasp and host will provide more information on species status and speciation.

Acknowledgments

We thank members from the lab in the IZCAS, ROM and Natural History Museum (London) for assistance with sequencing and comments on the manuscript. This work was supported by the National Natural Science Foundation of China (NSFC Grant No. 30330090) and partially by the National Science Fund for Fostering Talents in Basic Research (NSFC-J0030092). CDZ was also supported by a K.C. Wong Fellowship from Royal Society (UK), a scholarship from the Chinese Academy of Sciences and a grant from Natural Environment Research Council (UK) to visit the British Natural History Museum and Imperial College, London (UK) (Feb. 2004–Jan. 2006). The considerate and thoughtful comments of George Heimpel and two anonymous reviewers greatly improved the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.biocontrol.2006.04.008](https://doi.org/10.1016/j.biocontrol.2006.04.008)

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