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The Phylogenetic Relationships Between Mytilarioideae and the Other Subfamilies of Hamamelidaceae Inferred from the ITS Sequences of Nuclear Ribosomal DNA*

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Abstract: The phylogenetic relationships between Mytilarioideae and the other subfamilies of Hamamelidaceae based on internal transcribed spacers (ITS) and 5.8S coding region of nuclear ribosomal DNA were shown in this study. The monophyly of Mytilarioideae including *Mytilaria* and *Chunia* was strongly supported, which suggests that Mytilarioideae is an independent subfamily. The ITS data indicated that there was a sister relationship between Exbucklandioideae and Rhodoleioideae (with 95% bootstrap value supporting), and Hamamelidioideae and Disanthoideae (with 58% bootstrap value supporting) respectively. All taxa of Hamamelidaceae sampled in this study formed a monophletic group and *Liquidambar formosana* was cladistically basal within the family, which supported the result of our last study in 1997.

Keywords: phylogenetic relationship, Mytilarioideae, ITS sequence
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The comprehensive classification of the family Hamamelidaceae was proposed by Harms[1]. He recognized the five subfamilies, i. e., Disanthoideae, Hamamelioideae, Rhodoleioideae, Bucklandioideae (= Exbucklandioideae) and Liquidambaroideae (= Altingioideae). This classification was adopted by several authors (with minor changes), such as Vink[2] and Bogle[3]. However, Chang[4] separated the subfamily Exbucklandioideae into two subfamilies: Mytilarioideae for *Mytilaria* and *Chunia*, *the latter was* the new genus described by Chang[5] in 1948, and Exbucklandioideae for only *Exbucklandia*. Endress[6] provided an emended classification of the family with four subfamilies, which combined Exbucklandioideae, Mytilarioideae, and Disanthoideae into the subfamily Exbucklandioideae. The objectives of this study were to reconstruct the phylogenetic relationships between Mytilarioideae and the other subfamilies of Hamamelidaceae based on the divergences data of ITS sequences, and to discuss if the subfamily Mytilarioideae is an independent subfamily.

1 Materials and methods

1.1 DNA isolation and PCR amplification

Eight taxa representing four subfamilies and an outgroup were sampled in this study (Tab. 1). The sequences of ITS regions of *Hamamelis mollis* and *Liquidambar formosana*[7], which representing

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the subfamilies Hamamelioideae and Altingioideae respectively, were used in this study. Total DNAs were extracted with the CTAB method of Doyle and Doyle[8] followed by purification with DPS (DNA purification system) kit (made by our laboratory). DNA amplification was performed in 2×60 μL reactions following Wen and Zimmer[9]. The PCR products of ITS were purified with the centrifugal filter units (UFC3LTKNB, Millipore).

Tab. 1　Accessions of Hamamelidaceae and an outgroup *Platanus* sampled for the ITS studies[1)]

Species	Subfamily	Voucher	Geographical origin
Chunia bucklandioides	Mytilarioideae	Zhou/974301	Jianfengling, Hainan, China
Disanthus cercidifolius var. *longipes*	Disanthoideae	Hao/974001	Cultivated in Zhongshan Botanical Garden, China
Exbucklandia populnea	Exbucklandioideae	Chen/9703056	Cultivated in South China Botanical Garden, China
Exbucklandia tonkinensis	Exbucklandioideae	Chen/9703057	Cultivated in South China Botanical Garden, China
Mytilaria laosensis	Mytilarioideae	Chen/9703054	Cultivated in South China Botanical Garden, China
Mytilaria laosensis	Mytilarioideae	Shi/9704118	Heishiding, Guangdong, China
Platanus acerifolia	Platanaceae	Shi/974338	Cultivated in Wuhan Botanical Garden, China
Rhodoleia championii	Rhodolioideae	Xing/6742	Dawushan, Hong Kong, China

1) The classification system was based on Chang H T (1979)

1.2　Sequencing of the PCR products

The ITS regions were sequenced following the dideoxy chain termination method of Sanger[10] using the Sequenase Version 2.0 DNA sequencing kit (US70770, Amersham) and alpha 35S-dATP as a radioactive tracer. C5.8S, ITS4, N5.8S and N18L18[9] were used as sequencing primers to obtain the entire ITS and 5.8S regions from both directions. Products of the sequencing reactions were separated on a 6% polyacrylamide gel at 50 W. Gels were transferred to 3 mm Whatman paper, dried in an oven at 45 ℃ for 3 hours, and exposed to an X-ray film (Kodak XAR) for 3～4 days at room temperature. About 320 nucleotides were obtained per sequencing run.

1.3　Sequence analysis and phylogenetic reconstruction

The DNA sequences were assembled and the boundaries between the coding and spacer regions were determined by comparison with the sequences of carrot (Daucus carota[11]). The assembled sequences were aligned using CLUSTAL X[12].

Phylogenetic analyses were performed using PAUP 3.1.1[13]. The amount of support for monophyletic groups revealed in the most parsimonious tree (MPT) was examined with 100 bootstrap replicates with the random addition and the heuristic search options. The phylogenetic tree was rooted using *Corylus chinensis* (Betulaceae) and *Platanus acerifolia* (Platanaceae) as the complex outgroup.

2　Results and discussion

The total length of the ITS1, 5.8S and ITS2 regions of *Mytilaria*, *Chunia* and *Exbucklandia* are 672, 684 and 680～687 bp respectively. The Kimura two-parameter distances are presented in Tab. 2. The maximum parsimony analysis generated two MPTs with a total length of 1 173 steps, a consistency index (CI) of 0.743, a homoplasy index (HI) of 0.257, a retention index (RI) of 0.542 and a

rescaled consistency index (RC) of 0.403, treating gaps as missing data. The strict consensus tree from the two MPTs is shown in Fig. 1.

Tab. 2 Kimura two-parameter sequence divergence values of taxa in Mytilarioideae and the other subfamilies of Hamamelidaceae. Below the diagnoal are the absolute distances; and above the diagonal are the Kimura two-parameter distances. Gaps were eliminated from the comparison

Species	1	2	3	4	5	6	7	8	9	10	11
P. acerifolia 213	—	0.425	0.396	0.399	0.380	0.380	0.372	0.377	0.379	0.396	0.423
Cor. chinensis	321	—	0.393	0.383	0.364	0.370	0.371	0.375	0.376	0.381	0.407
H. mollis 58	299	297	—	0.240	0.250	0.262	0.257	0.249	0.250	0.262	0.327
D. cercidifolius 153	301	289	181	—	0.208	0.228	0.229	0.238	0.238	0.244	0.322
E. populnea 124	287	275	189	157	—	0.032	0.164	0.215	0.216	0.232	0.310
E. tonkinensis 125	287	279	198	172	24	—	0.172	0.226	0.228	0.240	0.315
R. championii 110	281	280	194	173	124	130	—	0.233	0.234	0.242	0.314
M. laosensis 123	285	283	188	180	162	171	176	—	0.001	0.131	0.298
M. laosensis 140	286	284	189	180	163	172	177	1	—	0.132	0.299
C. bucklandioides 157	299	288	198	184	175	181	183	99	100	—	0.319
L. fomosana 63	319	307	247	243	234	238	237	225	226	241	—

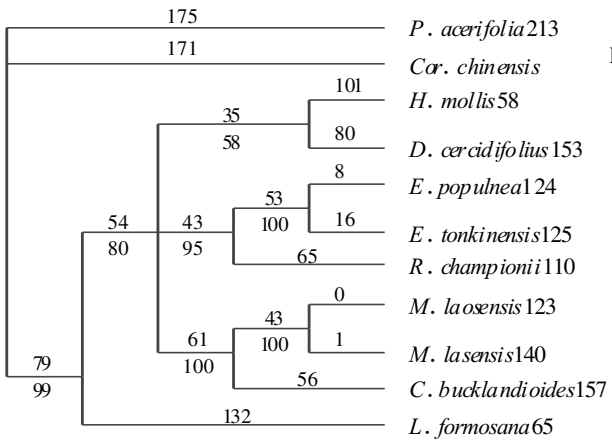

Fig 1 The strict consensus tree of the two most parsimonious trees of Mytilarioideae and the other subfamilies of Hamamelidaceae based on the ITS sequences of nuclear ribosomal DNA (1 173 steps, CI = 0.743, RI = 0.542, RC = 0.403). The tree was rooted using *Corylus chinensis* and *Platanus acerifolia* as the complex outgroup. Bootstrap confidence values (100 replicates) are given below the branches whereas the branch lengths are indicated above the branches.

2.1 The phylogenetic relationships between Mytilarioideae and the other subfamilies

In the strict consensus tree (Fig. 1), the monophyletic group of Mytilarioideae that includes *Mytilaria* and *Chunia* was shown (the bootstrap value is 100%). A sister group, i.e., Hamamelidioideae and Disanthoideae, was suggested, but not very strong (58%) bootstrap analysis supported. However they had a long branch length respectively (101 in Hamamelidioideae and 80 in Disanthoideae, see Fig. 1). This result agreed to the morphological results of Huang[14] and Pan et al[15], which suggested that Disanthoideae was more closely related to Hamamelidioideae than the other subfamilies based on the characters of paracytic stomatal apparatuses and pentamerous flowers.

It should be indicated here because no any samples of *Exbucklandia* was compared in our former publication①, the wrong *Exbucklandia* and Hamamelioideae formed a rather weak clade (50% bootstrap value supported) in our former publication[7]. After the data of the two species of the *Exbucklan-*

① The species *Exbucklandia tonkinensis* in our last publication[7] was sampled by mistake. It should be the species *Mytilaria laosensis* according to our present experimental tests.

dia were analyzed in this study, the strict consensus tree showed that a sister group relationship between the Exbucklandioideae and the Rhodoleioideae was strongly supported (95%) by the bootstrap analysis (Fig. 1). This result agreed with Pan et al[15], who indicated that Exbucklandioideae and Rhodoleioideae had the same cyclocytic stomatal apparatuses. The ITS data of this study also suggested that all taxa of Hamamelidaceae sampled here formed a monophyletic group and the genus *Liquidambar* was cladistically basal within the family(99%), which is the same as our former study[7]. There are the sister relationships among the clades of Hamamelidioideae and Disanthuoideae, Mytilarioideae, and Exbucklandioideae and Rhodoleoideae supported by the bootstrap value of 80%.

2.2 Mytilarioideae is an independent subfamily

Controversies concerning the classification of *Mytilaria* and its phylogenetic position have been existing for a long time. Harms[1] recognized the five subfamilies. However, the position of the *Mytilaria* was insufficiently known at Harms time. Chang[5] segregated *Mytilaria* and *Chunia* in the new (and six) subfamily Mytilarioideae, leaving only *Exbucklandia* in Exbucklandioideae. Huang and Lee[16] supported the segregation on the basis of wood anatomy. Takhtajan[17], on the other hand, listed subfamily Chunioideae (rather than Mytilarioideae) as one of the six subfamilies in Hamamelidaceae, but did not indicated which of the three genera of Exbucklandioideae he would assign to it or his reasons for removing them from the Exbucklandioideae. Pan et al[15] and Pan and Yang[18] supported the treatment of *Mytilaria* as an independent subfamily on the features of stephanocytic stomatal apparatuses and the basic chromosome number($\chi=13$). In this study the phylogenetic analysis based on the ITS data supports strongly that Mytilarioideae is considered as an independent subfamily.

Endress[7] recognized that the genera of *Mytilaria*, *Chunia*, *Exbucklandia* and *Disanthus* seemed to form a good natural group by many morphological charactersistics. So he combined those four genera (three subfamilies) into the subfamily Exbucklanioideae. Bogle[3] indicated that the fact of *Chunia* shared multilacunar nodal anatomy with *Mytilaria* provided evidence to support the close relationship between the two genera implied by Chang. However, on the basis of other morphological evidence, he would prefer to see *Chunia* and *Mytilaria* maintained within the subfamily Exbucklandioideae as a tribe Mytilareae rather than segregated as a subfamily Mytilarioideae. The phylogenetic analyses based on ITS sequence data in this study shows that there are the high level divergences among the *Disanthus*, *Exbucklandia*, and *Mytilaria* and *Chunia* (see the branch lengths in Fig. 1 and the Kimura two-parameter distances in Tab. 2) and they are in the different clades respectively with strong bootstrap values supporting (Fig. 1). Therefore the data of ITS sequences in this study do not support the treatment of combining those four genera into the subfamily Exbucklandioideae.

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壳菜果亚科(金缕梅科)与相关亚科的核糖体 DNA ITS 区序列分析及系统发育关系

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摘 要: 测定和分析了金缕梅科(Hamamelidaceae)壳菜果亚科等各亚科代表属的核糖体 DNA ITS 区和 5.8S 编码区序列. 应用最大简约法构建的分子系统树表明: 壳菜果亚科 (Mytilarioideae)植物形成 1 个单系类群[含壳菜果属(*Mytilaria*)和山桐材属(*Chunia*)], 支持壳菜果亚科作为 1 个独立的亚科. 本研究所得到的 ITS 数据表明马蹄荷亚科(Exbucklandioideae)与红花荷亚科(Rhodoleioideae), 金缕梅亚科(Hamamelidioideae)与双花木亚科(Disanthoideae)分别为姐妹群关系(其自展数据支持率各为 95%和 58%). 分子系统树还表明, 测得的所有金缕梅科植物形成一单系类群(其自展数据支持率为 99%), 枫香亚科(Liquidambaroideae)为该科最基础的 1 个分支. 这结果与 1997 年所得到的结论相一致.

关键词: 系统进化关系, 壳菜果亚科, ITS 区
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