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Lineage diversification and hybridization in the *Cayratia japonica–Cayratia tenuifolia* species complex

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ABSTRACT

The *Cayratia japonica–Cayratia tenuifolia* species complex (Vitaceae) is distributed from temperate to tropical East Asia, Southeast Asia, India, and Australia. The spatiotemporal diversification history of this complex was assessed through phylogenetic and biogeographic analyses. Maximum parsimony, neighbor-joining, and maximum likelihood methods were used to analyze sequences of one nuclear (*AS1*) and two plastid regions (*trnL-F* and *trnC-petN*). Bayesian dating analysis was conducted to estimate the divergence times of clades. The likelihood method LAGRANGE was used to infer ancestral areas. The Asian *C. japonica* and *C. tenuifolia* should be treated as an unresolved complex, and Australian *C. japonica* is distinct from the Asian *C. japonica–C. tenuifolia* species complex and should be treated as separate taxa. The Asian *C. japonica–C. tenuifolia* species complex was estimated to have diverged from its closest relatives during the Late Eocene (35.1 million years ago [Ma], 95% highest posterior densities [HPD] = 23.3–47.3 Ma) and most likely first diverged in mid-continental Asia. This complex was first divided into a northern clade and a southern clade during the middle Oligocene (27.3 Ma; 95% HPD = 17.4–38.1 Ma), which is consistent with a large southeastward extrusion of the Indochina region relative to South China along the Red River. Each of the northern and southern clades then further diverged into multiple subclades through a series of dispersal and divergence events following significant geological and climatic changes in East and Southeast Asia during the Miocene. Multiple inter-lineage hybridizations among four lineages were inferred to have occurred following this diversification process, which caused some Asian lineages to be morphologically cryptic.

1. Introduction

The Sino-Japanese floristic region in temperate Asia has remarkably rich biodiversity compared to other temperate floristic regions worldwide. The high species richness of the region is explained by its climatic diversity, complex topography, complex geological history, and the absence of major extinctions during Quaternary glaciations (Qian and Ricklefs, 2000; Harrison et al., 2001; Milne and Abbott, 2002; Milne, 2006; Qiu et al., 2011). Adjacent to temperate Asia, tropical Asia (Indo-Burma and Sundaland) is also known for high biodiversity (Myers et al., 2000). In addition to the high rates of endemics in these floristic regions, some species are widely distributed across the regions. Those species have previously received little attention; however, recent advances in molecular analyses have made it possible to reveal hidden lineages within a species (e.g., Qiu et al., 2011). Phylogeographic analyses of such species may reveal how they spread across floristic regions and help to clarify the geological histories of temperate and tropical Asia.

Cayratia Jussieu (Vitaceae) comprises over 60 species, occurring mainly in tropical and subtropical Asia, Africa, Australasia, and the Pacific islands (Wen, 2007; Lu et al., 2013). *Cayratia japonica* (Thunb.) Gagnep. is distributed from the temperate to tropical regions of East Asia, Southeast Asia, India, and Australia (Jackes, 1987; Ohba, 1999; Hui and Wen, 2007). Two intraspecific varieties are recognized in China: *C. japonica* var. *pseudotrifolia* (W.T. Wang)

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C.L. Li and *C. japonica* var. *mollis* (Wallich ex M.A. Lawson) Momiyama (Hui and Wen, 2007). *Cayratia tenuifolia* (Wight & Arn.) Gagnep. occurs in the Japanese islands of Kyushu and the Ryukyus, as well as in Taiwan, the Malay Peninsula, and Borneo. It was morphologically distinguished from *C. japonica* by differences in dentate leaflet margins (Hatusima and Amano, 1967; Hatusima, 1971) and was treated as a synonym of *C. japonica* var. *dentata* (Makino) Honda (Makino, 1909; Ohba, 1999). Okada et al. (2007) suggested that *C. tenuifolia* might be distinguished from *C. japonica* by differences in the color of the floral disc at anthesis: specifically, yellow in *C. tenuifolia* and orange in *C. japonica*.

The chromosome number of *C. japonica* has been reported as $2n = 40$ in China (Huang et al., 1988). Both *C. japonica* and *C. tenuifolia* contain diploids ($2n = 2x = 40$) and triploids ($2n = 3x = 60$) in Japan (Okada et al., 2003, 2005, 2007; Tsukaya et al., 2012). Consistent with triploids in other species, the triploids of *C. japonica* and *C. tenuifolia* have low fertilities and rarely bear fruit. Moreover, the diploid *C. japonica* of Honshu, Japan, has variable pollen fertility (31–97%; Okada et al., 2003) and is often unable to produce seeds. Both *C. japonica* and *C. tenuifolia* readily propagate vegetatively, and thus some individuals of the species were assumed to spread through vegetative clones in Japan (Okada et al., 2003).

In a previous study, we explored the origins of triploids of *C. japonica* and *C. tenuifolia* in Japan using the single-copy nuclear gene *ASYMMETRIC LEAVES 1* (*AS1*) (Tsukaya et al., 2012). According to the phylogenetic investigation, alleles were divided into three distinct lineages, the majority of which were shared by *C. japonica* and *C. tenuifolia*. These results suggested that the two species were not phylogeneticaly distinct from each other and may be members of a species complex (Tsukaya et al., 2012). In addition, the majority of diploids and all triploids were heterozygous for the *AS1* genotypes and consisted of two alleles with distinct lineages, suggesting that lineage diversifications were followed by lineage admixtures through hybridizations. Furthermore, triploids of each species originated from independent hybridizations.

Only a few studies have examined this species complex in other regions. In Australia, *C. japonica* is found along the east coast of Queensland (Jackes, 1987). Although phylogenetic relationships between Australasian *C. japonica* and other Australian species of Vitaceae were investigated (Rossetto et al., 2001, 2007), the analysis did not include the Asian *C. japonica*. The phylogenetic relationship between *C. japonica* in Australia and Asia has not yet been examined.

In this study, we used expanded taxon sampling and additional molecular markers to determine the phylogenetic relationships among the *C. japonica–C. tenuifolia* species complex across most of its distribution. Details of divergences and hybridizations among major lineages were assessed. Divergence times of the main lineages and the ancestral area of the species complex were estimated, and the divergence history of this clade was also investigated. The species complex is widely distributed across temperate to tropical regions from East Asia to Australia and provides an appropriate model system for determining the origins of intraspecies complex lineages.

2. Materials and methods

2.1. Materials and determinations of ploidy levels

Cayratia japonica and *C. tenuifolia* were treated as members of the *C. japonica–C. tenuifolia* species complex in the present study, as suggested by Tsukaya et al. (2012). We collected 116 accessions of the *C. japonica–C. tenuifolia* species complex from native habitats in Australia, China, Japan, Korea, Taiwan, Indonesia, Malaysia, and Myanmar (details of accessions are shown in Supplementary Table 1). Both *C. japonica* var. *pseudotrifolia* and *C. japonica* var. *mollis* were included (Supplementary Table 1; *C. japonica* var. *pseudotrifolia*: ID No. 66, *C. japonica* var. *mollis*: ID No. 82, 83). Nine individuals representing eight species (including an unidentified specimens), *C. maritima* Jackes, *C. yoshimurae* (Makino) Honda, *Tetrastigma planicaule* Gagnep., *T. obtectum* (Wall. ex M.A. Lawson) Planch. ex Franch., *T. lanceolarium* Planch., *Cyphostemma juttae* (Dinter & Gilg) Desc., and *Cyphostemma mappia* (Lam.) Galet, were also included as outgroups (Supplementary Table 2).

The ploidy level of each individual was determined by direct observation, flow cytometry, and/or morphological analyses. Triploids can be distinguished from diploids based on their leaf morphology and fruit development (Okada et al., 2003): diploids have thin leaves consisting of 3–5 leaflets, while triploids have thick leaves consisting of 5–7 leaflets (Supplementary Table 1). In addition, diploids have fruits, while triploids do not.

2.2. DNA extraction, PCR amplifications, and sequencing

Genomic DNA was extracted from silica gel-dried leaves using an ISOPLANT II Kit (Nippon Gene, Tokyo, Japan) following the manufacturer's protocol. One nuclear (the *AS1*) and two plastid (*trnL-F* and *trnC-petN*) markers were used. The two-chloroplast regions *trnL-F* and *trnC-petN* were amplified and sequenced using trnL-c and trnF-f (Taberlet et al., 1991), and trnC and petN2R primers (Lee and Wen, 2004), respectively.

The *AS1* was originally isolated from *Arabidopsis thaliana* (L.) Heynh., and the *AS1* encodes the MYB transcription factor required for polarity-dependent growth of lateral organs and plant-immune response (Byrne et al., 2000; Nurmberg et al., 2007). The *AS1* orthologs are well conserved in many angiosperms such as *Antirrhinum majus* (*PHANTASTICA*, Waites et al., 1998) and *Zea mays* (*rough sheath2*, Phelps-Durr et al., 2005). To the best of our knowledge, two examples of phylogenetic analyses applying the *AS1* orthologs have been reported (Tsukaya et al., 2012; Hinsinger et al., 2013).

For the *AS1*, the primers AS1-F1 (5′-ATAGGGATGCCAAATCTTGC-3′) and AS1-R1 (5′-ACCTTTGATTCTATCTCTTC-3′) were used for amplification and sequencing. Amplification was carried out under the following conditions: incubation at 94 °C for 2 min followed by 20 cycles of touchdown PCR (denaturation at 94 °C for 15 s, annealing at 68 °C for 30 s, reducing the temperature by 0.9 °C per cycle, followed by extension at 68 °C for 1 min), and then 20 cycles of non-touchdown PCR (denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s, and extension at 68 °C for 1 min), with a final extension for 7 min at 68 °C. PCR reactions were performed with high-fidelity KOD-plus DNA polymerase (Toyobo, Osaka, Japan). PCR products were cleaned using the QIAquick PCR purification kit (Qiagen, Tokyo, Japan) or ExoSAP-IT (USB Corp., Cleveland, OH, USA). The BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) was used for sequencing reactions.

When sequences of alleles could not be determined because of two or more heterozygous sites in the *AS1*, TA-cloning was conducted to obtain the allele sequences. After purified PCR products were cloned, following Ishikawa et al. (2009), at least eight white colonies were used for PCR amplification and sequencing.

2.3. Phylogenetic analyses

Sequences were initially aligned with Clustal X (Thompson et al., 1997) and then modified manually to minimize the number of insertions and deletions (indels). Phylogenetic relationships were analyzed with the maximum parsimony (MP), neighbor-joining (NJ; Saitou and Nei, 1987), and maximum likelihood (ML) methods. PAUP* 4.0_b10 software (Swofford, 2003) was used for MP and NJ analyses. All gaps were coded as missing. All characters were weighted equally for the MP analysis. The analysis was conducted through a heuristic search with the TBR branch-swapping option. In total, 100 rounds of random sequence