Phylogeny of Rosa sections Chinenses and Synstylae (Rosaceae) based on chloroplast and nuclear markers

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Rosa sections Chinenses and Synstylae contain approximately 39 wild species mainly distributed in East Asia and are closely related according to previous studies. But the specific relationships within these two sections were still obscure due to limited sampling, low genetic variation of molecular markers, and complex evolutionary histories. In this study, we used four chloroplast (\textit{ndhC-trnV}, \textit{ndhF-rpl32}, \textit{ndtj-trnF} and \textit{psbJ-petA}) and two nuclear (\textit{ITS} and \textit{GAPDH}) markers with an extensive geographic and taxonomic sampling to explore their evolutionary history. Our phylogenetic analyses suggested that Rosa sections Chinenses and Synstylae defined in traditional taxonomic system are not monophyletic and close to sections Chinenses and Synstylae. Additionally, our results showed incongruence between chloroplast and nuclear markers, and the patterns of incongruence might be due to ancient hybridization (genetic introgression). One putative hybrid species and three samples identified as interspecific hybrids are further discussed in terms of topological incongruence, biological characters and distribution patterns.

1. Introduction

Hybridization has long been recognized as an important driving force in plant evolution (Abbott, 1992; Mallet, 2007; Paun et al., 2008; Rieseberg, 1995, 1997), which can lead to high intraspecific genetic diversity, creation of new species or ecotypes, and reticulate evolution (Anderson, 1948; Linder and Rieseberg, 2004; Whitham et al., 1994). As a result, it is particularly challenging to reconstruct species relationships for groups including hybrids. In the genus Rosa L. (Rosaceae), interspecific hybrids are easily formed, which have contributed substantially to the diversity in this genus (Atienza et al., 2005; Rehder, 1940: Wissemann, 2003). Many hybridization events have been reported in wild roses (Atienza et al., 2005; Joly et al., 2006; Matthews, 1920; Schanzer and Kutlunina, 2010). The whole section Caninae seems to be of hybrid origin (Fougère-Danezan et al., 2015; Ritz et al., 2005; Wissemann, 1999). Some species of Rosa sects. Rosa and Synstylae were also shown to have undergone hybridization during their evolutionary histories (Joly et al., 2006; Ohba et al., 2000). Indeed, hybridization has been recognized as one of the major difficulties in the taxonomy of roses even since the early times (Crépin, 1889; Linnaeus, 1753).

The genus Rosa comprises about 150–200 species widely distributed in the temperate and subtropical regions of the northern hemisphere (Ku and Robertson, 2003; Yü et al., 1985). The widely adopted taxonomic system built by Rehder (1940) divided Rosa species into four subgenera, i.e., Hesperodos Cocke
cell, Hulthemia (Dumort.) Focke, Platyrhodon (Hurst) Rehder and Rosa. The first three subgenera contain only one or two species, while species from the subgenus Rosa are further divided into ten sections, i.e., Banksiae Lindl., Bracteatae Thory, Caninae (DC.) Ser., Carolinea Crép., Cinnamomeae (DC) Ser., Gallicanae (DC.) Ser., Indicas Thory, Laevigatae Thory, Pimpinellifoliae (DC.) Ser., and Synstylae DC. We use R. sect. Chinenses (DC.) Ser. in this study instead of R. sect. Indicas since the former is the oldest valid name while the latter is a synonym (Seringe, 1818; Thory, 1820). Because R. sect. Cinnamomeae includes the type species (\textit{R. cinnamomea} L.) of the genus (Barrie, 2006; Jarvis, 1992), the section name Cinnamomeae is invalid and should be replaced by the autonym (R. sect. Rosa; Art. 22.1, McNeill et al., 2012). So we use R. sect. Rosa instead of R. sect. Cinnamomeae in this study.

Many attempts were made to reconstruct the phylogeny of this genus, most of which suggested that the divisions of most subgenera and sections based on morphology were artificial (Bruneau
And Rosa sects. Chinenses and Synstylae were resolved as closely related. For example, both Matsumoto et al. (1998) and Wu et al. (2000) found that R. sect. Chinenses was nested within R. sect. Synstylae, which is similar to a recent phylogenetic study of 39 Chinese wild roses (Qiu et al., 2012). Both nuclear and chloroplast DNA data from Wissemann and Ritz (2005) and Bruneau et al. (2007) suggested that sections Chinenses and Synstylae formed a clade with other sections, which may have been due to sparse taxon sampling, garden-grown samples that could be introgressed, and low variation in genetic markers. In an analysis of amplified fragment length polymorphism (AFLP) by Koopman et al. (2008), two East Asian species (R. wichurana and R. multiforma Thunb.) from sect. Synstylae formed a clade, and two other species (R. arvensis Huds. and R. moschatula Mill.) from Europe were grouped with sections Rosa and Caninae. However, in these studies, both the sampling and the molecular markers used were limited. Further research with more extensive taxon sampling and molecular data from both nuclear and chloroplast genomes is needed to solve the phylogenetic relationships within sections Chinenses and Synstylae.

Rosa sects. Chinenses and Synstylae are mainly distributed in East Asia. And species from these two sections include most of the wild ancestors of modern cultivated roses, such as R. multiforma, R. luciae Franch. & Rochebr. in R. sect. Synstylae and R. chinensis var. spontanea (Rehder & E.H. Wilson) T.T. Yu & T.C. Ku, R. odorata var. gigantea (Crep.) Rehder & E.H. Wilson in R. sect. Chinenses (Ku and Robertson, 2003; Rehder, 1940; Yü et al., 1985). Species of R. sect. Synstylae can be distinguished by their exserted and columnated stamens, which is similar to R. chinensis sects. Synstylae. In Flora of China (Yü et al., 1985), R. section Synstylae has been divided into two series (ser. Brunonianae Yu and Ku and Multiflorae Yu and Ku). Species of ser. Multiflorae have peltate or serrate stipules while those in ser. Brunonianae have entire ones. Rosa sect. Chinenses has similarly exerted styles, but the free styles and much bigger hips distinguish this section from sect. Synstylae. R. section Chinenses contains three species that are all endemic to China (Ku and Robertson, 2003). The ploidy of approximately a third wild species of these two sections have been reported in previous studies, and nearly all of them are diploid (Cole and Melton, 1986; Roberts et al., 2009; Ueda and Akimoto, 2001; Yokoya et al., 2000). Thus, unlike R. sects. Caninae and Rosa (Joly et al., 2006; Ritz et al., 2005), polyploidization does not seem to be an important factor in the evolution of R. sects. Chinenses and Synstylae. However, hybridization seems to be at work in these two sections as suggested by previous studies (Ohba et al., 2000; Meng et al., 2011) and numerous intermediate morphological characters from specimens and field observations.

In this study, we used four chloroplast and two nuclear genetic markers to reconstruct the phylogeny of R. sects. Chinenses and Synstylae. Our sampling covered the whole distribution area and most wild species of these two sections. Species belonging to other sections and subgenus were also sampled. The objectives of this study are: (1) to reconstruct a more comprehensive and better-resolved phylogeny of Rosa sections Chinenses and Synstylae, and (2) to test the significance of species hybridization in these two sections.

2. Materials and methods

2.1. Taxon sampling

Samples for this study were obtained from various sources. Most of our samples were collected in the field and the voucher specimens were deposited in the Herbarium of Chengdu Institute of Biology (CDBI) after identified with Flora of China (Ku and Robertson, 2003; Yü et al., 1985) and Flora Europaea (Tutin et al., 1978). Others were obtained from cultivated material or herbarium specimens at the Royal Botanic Gardens Kew and Edinburgh, Wageningen University Botanical Garden, and Botanic Garden Meise in Belgium. In total, we sampled 126 individuals (including three outgroups) of which 85 (representing 34 species) belonging to sect. Synstylae, ten (representing three species) belonging to sect. Chinenses, and three putative hybrids related to these two sections. According to previous studies (Eriksson et al., 2003; Potter et al., 2007, 2002), Potentilla parvifolia Fisch. and Rubus idaeos Focke were chosen as outgroups. All samples with detailed information are listed in Supplementary Table S1.

2.2. Lab protocols

Total genomic DNA was extracted from silica-gel dried or fresh leaves using the Tiangen plant genome DNA extraction kit (Tiangen Biotech., Beijing, China).

<table>
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<tr>
<th>Table 1</th>
<th>Primers used in this study.</th>
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Four chloroplast regions (ndhC-trnV, ndhf-rpl32, ndhj-trnV, and psbJ-petA), nuclear ribosomal internal spacers (nrITS) and a single copy nuclear gene coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used in this study. Primers used in this study are listed in Table 1. Polymerase chain reaction (PCR) amplification were performed in 25 μL volumes containing Taq DNA polymerase buffer (Tiangen, Beijing) with 2 mM MgCl₂, 20 μM of dNTP, 0.2 μM of each primer, 5 U of Taq DNA polymerase and 0.5 μL of genomic DNA (20–100 ng). Conditions for amplification consisted of an initial denaturation at 95 °C for 4 min, followed by 34 cycles at 95 °C for 45 s, then at 53 °C (49 °C for GAPDH) for 45 s and at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Amplifications were conducted using a PTC-0200 thermocycler (Bio-rad, Beijing).

Amplification products were purified using the Universal DNA Purification Kit (Tiangen, Beijing, China) after gel-separation. Purified products were directly sequenced at Invitrogen Biotech. (Shanghai, China).

Heterozygous (double) peaks were observed in chromatograms of most nuclear (ITS and GAPDH) sequences, which might be caused by different alleles. A strategy similar to Joly et al. (2006) was used to sample all the alleles. Sequences with reliable chromatograms were used directly. When heterozygous peak occurred at only one site, cloning was not considered necessary since the alleles could be easily distinguished. If sequences had more than one heterozygous peak or could not be directly sequenced, cloning was performed. Amplification and purification were performed as previously described except that the PCR volume was 50 μL. The purified PCR products were cloned with pEASY-T1 Cloning Kit (Transgen, Beijing, China) following the manual provided by the manufacturer. Finally, five to twenty positive clones were sequenced.

2.3. Sequence treatment, alignment and gap coding

Sequences were edited and assembled using Sequencher v4.1.4 (GeneCodes Inc., Ann Arbor, MI, USA). For nuclear sequences, the samples that had only one heterozygous peak were separated into two sequences labeled as S1 and S2. Cloned sequences were treated following the strategy of Yang et al. (2013). First, PCR errors were corrected by comparing the cloned sequences with the original sequences from direct sequencing. Second, if more than two cloned sequences of one sample shared identical bases, they were treated as one consensus sequence. Third, PCR-mediated recombination and pseudogene sequences were excluded.

Sequences were aligned using ClustalW (Thompson et al., 1994) in BioEdit v7.0.9.0 (Hall, 1999) and manually adjusted when necessary. Single base repeats as well as ambiguously aligned indels were excluded. Other substitutions and indels were considered as variations. Indels in the regions of unambiguous alignment were coded following the modified complex indel coding (Müller, 2006; Simmons and Ochoterena, 2000) for maximum parsimony (MP) analyses and simple coding (Simmons and Ochoterena, 2000) for Bayesian Inference (BI) using SeqState v1.4.1 (Müller, 2005).

2.4. Phylogenetic analysis

The chloroplast genome are maternally inherited, and our preliminary analyses did not show obvious incongruence among the four chloroplast DNA (cpDNA) regions, so we combined them to get better resolution of the phylogeny. All the concatenated chloroplast dataset and the nuclear datasets were analyzed using MP, maximum likelihood (ML) and BI. MP analyses were conducted with PAUP* v4.0 b10 (Swofford, 2002). For each dataset we performed a heuristic tree search using a set of 1000 random addition sequence. MP bootstrap support was summarized from 1000 bootstrap trees.

Model selection was performed in jModelTest v2.1.1 (Darriba et al., 2012) with the default setting to select the best-fit DNA substitution model for each dataset. The best-fit models and parameter values determined by Akaike Information Criterion (AIC) (Akaike, 1974) are presented in Supplementary Table S2.

ML analyses were performed using RAxML-HPC2 on XSEDE v8.0.24 (Stamatakis, 2014) in the Cipres Science Gateway (Miller et al., 2010). All parameters were set according to the best-fit models or the most similar ones available in Cipres. ML bootstrap support was summarized from 5000 bootstrap trees.

BI analyses were conducted using MrBayes v3.2.1 (Ronquist et al., 2012). All parameters were set according to the best-fit or the most similar model available in MrBayes v3.2.1. The Markov chain Monte Carlo (MCMC) algorithm was run for 30,000,000 (50,000,000 for GAPDH) generations with one cold and three heated chains, starting with a random tree and sampling one tree every 1000 generations. The first 7,500,000 (12,500,000 for GAPDH) generations were treated as burn-in. An adequate burn-in value for each analysis was assessed using the software Tracer 1.5 (Ramrunt and Drummond, 2009) and a 50% majority-rule consensus tree was then computed.

Phylogenetic networks were constructed for the nuclear datasets using SplitsTree v4.13.1 (Huson and Bryant, 2006). Phylogenetic network analyses were performed using the NeighborNet algorithm with Kimura 2-parameter (K2P) distances and Ordinary Least Square Method.

2.5. Molecular dating analysis

Since topologies obtained from the nuclear dataset were weakly supported, we selected the concatenated chloroplast dataset to perform molecular clock analyses in BEAST v 1.7.5 (Drummond et al., 2012) to estimate divergence times. We used the GTR+I+G model that is similar to the best-fit model to execute the analysis with an uncorrelated lognormal relaxed clock model and a Yule process of speciation. Two independent Markov chain Monte Carlo (MCMC) runs were set to 80,000,000 generations with parameters sampled every 1000 generations and 25% burn-in. Samples were combined and convergence of chains was checked in Tracer v 1.5 (Rambaut and Drummond, 2009). Although many fossils of Rosa were found, most of them were hard to assign to an extant species. Thus, we chose the oldest fossil R. germerensis (55.8–48.6 Ma; Idaho, USA) (Edelman, 1975) as the only calibration point. We used this age to constrain the root node (separation of Rosa from outgroups) with a prior normal distribution centered on 50.0 Ma and a 97.5% confidence interval between 51.0 and 49.0 Ma.

3. Results

3.1. Phylogeny of concatenated chloroplast DNA regions

After removing ambiguous sites (e.g. single base repeats), our aligned chloroplast ndhC-trnV, ndhf-rpl32, ndhj-trnF, and psbJ-petA DNA regions contained 763 base pairs (bp), 1275 bp, 1319 bp, and 1051 bp, respectively. The final concatenated cpDNA matrix was composed of 126 taxa and 4408 bp, of which 755 (17.1%) were variable and 396 (9.0%) were potentially parsimony informative.

The results of the MP, ML and BI analyses were congruent on major lineages. MP bootstrap supports (MPBS), ML bootstrap supports (MLBS) and BI posterior probabilities (PP) were shown on the 50% majority-rule consensus tree from BI analysis (Fig. 1). The monophyly of Rosa was well supported by all analyses (100% MPBS, 100% MLBS, 1.00 PP). In addition, two well-supported clades

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Fig. 1. Phylogram resulting from Bayesian Inference of concatenated chloroplast dataset. Double slashes on branches indicate branch lengths not in proportion. Numbers above branches are posterior probability values; numbers below branches are bootstrap values from MP/ML analyses. Clades, subclades or lineages are indicated by labels on branches and gradual colors of boxes covered on taxa names.
("Rosa" Clade, and Synstylae and allies Clade) were recovered within Rosa.

"Rosa" Clade included sections Caroliniae, Rosa, and one species from section Pimpinellifoliae, as well as two species from section Synstylae (Fig. 1). All accessions of Rosa glomerata Rehder & E. H. Wilson and Rosa abyssinica R. Br. (Sect. Synstylae) were nested in "Rosa" Clade. The monophyly of R. abyssinica is strongly supported by all analyses and sister to R. glomerata 2 from Ebian (Sichuan, China). The other two samples of R. glomerata from Luding (R. glomerata 1) and Yingjing (R. glomerata 3) counties (Sichuan, China) formed another subclade with high support values.

Synstylae and allies Clade included most samples from section Synstylae, all samples from section Chinenses, as well as samples from subgenus Platyrhodon and sections Banksiaceae, Caninae, Gallicanae, Laevigatae and Pimpinellifoliae (Fig. 1). Both sections Chinenses and Synstylae were not recovered as monophyletic. Asian species of sections Chinenses and Synstylae formed a well-supported subclade (Asian) in which seven well-supported lineages (A-G) were resolved. The only Synstylae species (R. setigera Michx.) distributed in North America formed another subclade (North American). European Synstylae species clustered with Caninae species and formed the subclade European II while the remaining Caninae species clustered in a subclade we named European I. These four subclades formed a well-supported (83% MPBS, 90% MLBS, 1.00 PP) clade named Core Synstylae in this study.

3.2. Phylogeny of ITS region

The final ITS dataset contained 224 accessions and 622 aligned nucleotides, of which 168 (27.0%) were variable and 103 (16.6%) were parsimony informative. The phylogenetic analyses of the ITS dataset using MP, ML, and BI methods showed similar topologies. The backbone of the phylogenetic tree had weak support at many nodes (Fig. 2). The genus Rosa was resolved as monophyletic and well supported. But neither section Synstylae nor section Chinenses was resolved as monophyletic (Fig. 2). Unlike cpDNA results, R. cymosa from sect. Banksiaceae was sister to the remaining Rosa species. Additionally, none of the cladates identified by cpDNA was supported here except R. setigera species, the only Synstylae species endemic to North America. Most accessions of "Rosa" Clade in the chloroplast tree formed a clade. Moreover, R. glomerata and R. abyssinica showed a closer relationship to species from Core Synstylae clade.

3.3. Phylogeny of GAPDH region

PCR amplification of GAPDH region failed for five samples, and one sequence (DQ091174, R. setigera) was obtained from GenBank (Joly et al., 2006). Finally, the GAPDH dataset included 272 accessions and 915 aligned nucleotides that contained 418 (45.7%) variable sites and 270 (29.5%) potential parsimony informative sites. Phylogenetic analyses of GAPDH dataset using three methods (MP, ML, and BI) did not show obvious incongruence except support values (Fig. 3). The monophyly of the genus Rosa was well-supported, while the phylogenetic relationships within Rosa were very different from the chloroplast and ITS trees. Two early-diverging clades (One included species from subgenus Platyrhodon, sections Banksiaceae and Laevigatae, and another included species from section Pimpinellifoliae.) were sister to the remaining species forming a polytomy. The positions for species of section Synstylae from Europe and North America, and species from sections Caninae, Caroliniae, Rosa, and Gallicanae were unresolved. Other species (mainly Asian) from sections Chinenses and Synstylae showed a little more resolution. R. glomerata formed a clade sister to species of Rosa soulieana group. Seven accessions of R. abyssinica formed a clade and another one clustered with species of R. soulieana group.

3.4. Phylogenetic networks

Phylogenetic networks of nuclear ITS and GAPDH regions (Figs. 4 and 5) were similar to the corresponding phylogenetic trees (Figs. 2 and 3) and showed clearer backbones of genus Rosa. Species from sections Chinenses, Gallicanae, and some sequences from section Caninae were nested in Synstylae. Section Chinenses was split into different groups in both networks.

3.5. Divergence times

The maximum clade credibility (MCC) tree (Supplementary Fig. S1) generated from divergence time analyses was congruent with the cpDNA phylogenetic tree (Fig. 1) in major clades. The age of the first divergence event in Rosa, which is the divergence time between "Rosa" Clade and Synstylae and allies Clade, was estimated to be 40.9 Ma (95% highest posterior density (HPD): 30.0–50.2 Ma). The crown age of Core Synstylae was estimated to be 24.2 Ma (95% HPD: 15.4–33.7 Ma), and the crown age of subclade Asian was 18.4 Ma (95% HPD: 11.2–26.4 Ma).

4. Discussion

4.1. Low resolution and phylogenetic incongruence

Our results provided some significant information for the evolutionary history of genus Rosa, while resolution was limited between closely related species. Low resolution in phylogeny can be due to causes such as insufficient data, noisy sequences, rapid diversification, polyploidization, and reticulate evolution (Baurain et al., 2007; Calvino et al., 2008; Campbell et al., 2007; Lo et al., 2009; Xu et al., 2012). In this study, we sampled most species of sections Chinenses and Synstylae covering the whole distribution area. The concatenated chloroplast dataset had much lower parsimony informative (PI) rate than nuclear markers (Table 2), while a better resolved phylogeny was obtained. It seems that insufficient variation is not the key reason in this case. Many reticulate evolution events were indicated by the nuclear networks (Figs. 4 and 5). Hybridization is easy for Rosa species because of overlapping distribution areas and flowering times, common generalist pollinators, and fertile crossbreeds (Jesse et al., 2006; Kevan et al., 1990; Matthews, 1920). Many studies have reported that hybridization has occurred in this genus not only between closely related species but also between species from different sections (Matthews, 1920; Mercure and Bruneau, 2008; Ritz et al., 2005; Schanzer and Kutulina, 2010; Uggla and Carlson-Nilsson, 2005; Wissemann and Hellwig, 1997). Most species from sections Chinenses and Synstylae are diploid, but polyploidy is very common in other sections especially Caninae and Rosa. A lot of studies have documented polyploidization events and evaluated its significance for the evolution of the genus Rosa (Erlanson, 1938; Joly et al., 2006; Wissemann, 2002). Therefore, we attribute the low resolution of nuclear phylogenies to the complex evolutionary history of this genus rather than insufficient data. Although the resolution was low in nuclear phylogenies, some moderately to strongly supported clades were recovered. Numerous inconsistencies (R. glomerata, R. abyssinica and R. rubus group) were revealed between the chloroplast and nuclear phylogenies (Figs. 1–3). Either abiotic (stochastic errors and systematic errors) or biological factors (convergent evolution, genetic introgression following hybridization, incomplete lineage sorting,
Fig. 2. Phylogram resulting from Bayesian Inference of nrITS dataset. Numbers above branches are posterior probability values; numbers below branches are bootstrap values from MP/ML analyses. Taxa colors corresponding to Fig. 1 indicate their position in chloroplast phylogeny.
Fig. 3. Phylogram resulting from Bayesian Inference of GAPDH dataset. Double slashes on branches indicate branch lengths not in proportion. Numbers above branches are posterior probability values; numbers below branches are bootstrap values from MP/ML analyses. Taxa colors corresponding to Fig. 1 indicate their position in chloroplast phylogeny.
Therefore, we rather attribute the discordance to incomplete lineage sorting (Knowles and Carstens, 2007). In our study, the divergence time often corresponds to recent divergence events with insufficient reasonable explanation in these cases. Incomplete lineage sorting does not seem to have undergone hybridization events. Incongruent phylogenetic relationships among our gene trees could be caused by introgression/hybridization. Incomplete lineage sorting often corresponds to recent divergence events with insufficient time to fully sort gene polymorphism from the ancestors (Knowles and Carstens, 2007). In our study, the divergence time of those species (Fig. S1) seemed to be long enough for a full lineage sorting. Therefore, we rather attribute the discordance to genetic introgression than incomplete lineage sorting in this case.

### 4.2. Phylogeny of Rosa sections Chinenses and Synstylae

Our analyses all gave strong support to the monophyly of genus *Rosa*. This is consistent with previous molecular phylogenetic studies (Bruneau et al., 2007; Eriksson et al., 2003; Potter et al., 2007; Wissemann and Ritz, 2005). Though this study focused on sections *Chinenses* and *Synstylae*, our extensive sampling did help understanding relationships within the whole genus. As previous studies found (Bruneau et al., 2007; Wissemann and Ritz, 2005; Wu et al., 2000), none of our three phylogenies (Figs. 1–3) supported traditional taxonomy of subgenera and sections (Rehder, 1940; Wissemann, 2003). Our phylogenetic analyses suggested that the sections *Chinenses* and *Synstylae* are not monophyletic since sections *Caninae*, *Chinenses*, *Gallicanae* and *Synstylae* showed very close relationships in our phylogenies. Our nuclear phylogenetic networks (Figs. 4 and 5) also indicated significant interrelations of these four sections in their evolutionary histories. These results were also consistent with a new worldwide study of the whole genus based on several markers (Fougère-Danezan et al., 2015).

Species of sections *Chinenses* and *Synstylae* are mainly distributed in China (Ku and Robertson, 2003). The Chinese species of section *Synstylae* have been divided into two series (ser. *Multiflorae* and *Brunonianae*) in Flora of China (Yu et al., 1983) according to the character of stipules. If the series definition is applied to the whole section, Japanese species (e.g., *R. fujisanensis* Makino and *R. paniculigera* Makino ex Momiy.) should be assigned to ser. *Multiflorae* and others to ser. *Brunonianae*, but these series have not been strictly recovered in our comprehensive molecular phylogenies (Figs. 1–3). The phylogenetic relationships within these two sections were not consistent with traditional taxonomy in many clades either, but a lot of information on their relationships was still provided by our molecular phylogenetic analyses.

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**Fig. 4.** Phylogenetic network from NeighorNet analysis of *nrITS* dataset. Different colors indicate subgenus or sections in traditional taxonomy.
et al., 2008; Scariot et al., 2006), but most studies focused on the section Chinenses. Because this section includes important garden roses or double-petaled varieties. In this study, we sampled all three wild taxa and double-petaled R. odorata (Andrews) Sweet var. odorata. All samples were nested within section Synstylae (Figs. 4 and 5). This relationship was also recovered in previous studies (Bruneau et al., 2007; Fougère-Danezan et al., 2015; Meng et al., 2011; Qiu et al., 2012; Wissemann and Ritz, 2005). Our phylogenies showed that these species together are not monophyletic, and chloroplast phylogeny resolved R. lucidissima as the earliest divergent species in the Asian subclade.

4.2.2. Rosa abyssinica

*Rosa abyssinica* is the only Synstylae species distributed in East Africa (Ethiopia) and the South Arabian Peninsula (Yemen and Saudi Arabia). There was some discordance regarding the position of this species between chloroplast and nuclear phylogenies. Its exserted and columnated styles provide strong evidence for its inclusion in section Synstylae, while in our chloroplast tree, the four samples formed a well-supported lineage nested in “Rosa” Clade (Fig. 1). We attribute the abnormal position of *R. abyssinica* in chloroplast phylogeny to hybridization/introggression instead of incomplete lineage sorting because the divergence time (40.9 Ma, Fig. S1) seems to be long enough for a full lineage sorting as discussed before. Most previous studies of *Rosa* did not include *R. abyssinica* except the recent analyses of Fougère-Danezan et al.
(2015) in which the results were consistent with ours and suggested that *R. abyssinica* could have a hybrid origin.

### 4.2.3. North American Synstylae

Only one species (*Rosa setigera*) from section *Synstylae* is distributed in North America. It has pink flowers and three leaflets per leaf, which is relatively rare in sect. *Synstylae*. This species is also the only rose that is dioecious (*Focke, 1897; Kevan et al., 1990*). *R. setigera* was recovered as monophyletic with a good support in our phylogenetic trees (Figs. 1–3). And its particular distribution, morphological characters and sexual system match its position in the chloroplast tree as sister to all the other species of Core *Synstylae*.

### 4.2.4. European Synstylae

Eight samples representing three European species (*Rosa arvensis*, *R. sempervirens* L., and *R. phoenicia* Boiss) were investigated in this study. They showed close relationships to the European species from sections *Caninae* and *Gallicanae* rather than other species of sect. *Synstylae*. Previous studies (De Riek et al., 2013; Graham and Primavesi, 1993) have reported frequent hybridization between sections *Synstylae* and *Caninae*, which is an important reason for the complexity of section *Caninae*. In our nuclear results (Figs. 2–5), some copies from these species clustered with *Caninae* species suggesting an interrelation in their evolutionary history. However, those three European species separately formed good monophyletic lineages with obvious divergence in their chloroplast genomes (Fig. 1).

### 4.2.5. Rosa glomerata

*Rosa glomerata* is widely distributed in Southwest China with typical exserted and columnated styles as well as white flowers. But its leathery leaflets with abaxially dense pubescence and prominent veins make it very different from other *Synstylae* species. Nuclear and chloroplast results for this species were inconsistent as mentioned above. *R. glomerata* has an overlapping distribution area with “Rosa” species (*R. moyesii* Hemsl & E.H. Wilson, *R. gracilliflora* Rehder & E.H. Wilson and *R. multibracteata* Hemsley & E.H. Wilson) and showed close relationships in chloroplast tree (Fig. 1). Considering the early divergence (40.9 Ma, Fig. S1) of “Rosa” Clade and *Synstylae* and allies Clade, together with the sympatric distribution with some “Rosa” species we suggest that *R. glomerata* has undergone some ancient hybridization events.

### 4.2.6. Rosa rubus group

This group mainly corresponded to Lineage B in the chloroplast tree and included three species (*R. rubus*, *R. henryi* Boulenger, and *R. sambucina* Koidz.) (Fig. 1). These species are highly similar with long climbing or repent branches, five leaflets per leaf, similar size and shape of leaflets, and white flowers in corymbs. But *R. rubus* is densely pubescent or glandular on the abaxial side of leaflets while leaflets of *R. henryi* and *R. sambucina* are glabrous on both sides. *R. rubus* and *R. henryi* share a large distribution area in central and south China while *R. sambucina* is distributed in Japan and Taiwan (China). In our phylogenetic analyses, the boundaries between *R. rubus*, *R. henryi*, and *R. sambucina* were very obscure. Additionally, in the chloroplast tree one sample of *R. rubus* and three of *R. henryi* were recovered as closely related to sympatric species of *Rosa multiflora* group in Lineage G (Fig. 1). Rechecking of specimens excluded misidentifying of these species. The non-monophyletic positions could be due to incomplete lineage sorting or genetic introgression as mentioned above. Here, the incongruences are more likely the result of genetic introgression/hybridization than incomplete lineage sorting, because lineage B and G diverged about 15 Mya (Fig. S1) which is long enough for a full lineage sorting.

### 4.2.7. Rosa soulieana group

The *Rosa soulieana* group is distributed in Hengduan Mountains (H-D Mountains) and eastern edge of Qinghai-Tibet Plateau (Q-T Plateau) with about five species and varieties. *Rosa soulieana* Crép. is the first described species of this group and occupies the whole distribution area. The other four species and varieties (*R. degenensis* T.C. Ku, *R. derongensis* T.C. Ku, *R. duplicata* T.T. Yu & T.C. Ku, and *R. soulieana* var. *sungpanensis* Rehder) are morphologically similar, narrowly distributed, and sympatric with *R. soulieana*. They were documented according to their different characters of leaflets or flowers (Yu, 1990; Rehder, 1930; Yü et al., 1980). All samples of this group clustered together in our chloroplast (Lineage C; Fig. 1) and ITS analyses (Fig. 2). Our molecular phylogenies did not show a deep divergence in contrast with the morphological diversity exhibited by those species, and despite the fact that H-D Mountains and Q-T Plateau have been suggested as the divergence center of many plant taxa because of their rich topography and complex history (Gao et al., 2013; Sun et al., 2014; Wang et al., 2009).

### 4.2.8. Rosa helenae group

The *Rosa helenae* group included five species (*R. brunonii* Lindl., *R. filipes* Rehder & E. H. Wilson, *R. helenae* Rehder & E. H. Wilson, *R. shangchengensis* T. C. Ku, and *R. weisensis* T. T. Yu & T. C. Ku). They generally have seven (*R. weisensis* has five to seven) leaflets per leaf and corymb or compound corymb inflorescences. In the chloroplast tree (Fig. 1) these species clustered as a subclade of Lineage D that was sister to *R. odorata* and *R. odorata* var. *gigantea* of sect. *Chinenses* (not recovered in nuclear phylogenies). Either introgression or incomplete lineage sorting, or both of them could be the cause for this relationship.

### 4.2.9. Rosa multiflora group

The *Rosa multiflora* group corresponds to the series *Multiflorae* including about 12 species and varieties in our study. *Rosa multiflora* is naturally distributed in China and Japan but now considered as an invasive species in other parts of the world (e.g. Australia, Pakistan and USA). Other species and varieties occur at coastal regions of China and Japan. They formed a clade that further divided into three highly supported lineages (Lineage E, Lineage F and Lineage G) in the chloroplast tree (Fig. 1). Morphological characters are not consistent with our molecular results. For example, the white flowered *R. multiflora* and its pink flowered variety *R. multiflora* var. *cathayensis* did not segregate in two separate clades. *Rosa anemoniflora* has light pink flowers and three leaflets per leaf which is rare in sect. *Synstylae*. However, despite this peculiarity, this species was not monophyletic in our molecular phylogenies.

### 4.2.10. Rosa longicuspis group

This group included two species (*R. longicuspis* and *R. lasiosepalum*) and a variety (*R. longicuspis* var. *sinowilsonii* (Hemsley) T.T. Yu & T.C. Ku) distributed in South China. *Rosa longicuspis* and *R. longicuspis* var. *sinowilsonii* are distributed in Yunnan, Guizhou and Sichuan provinces. *Rosa lasiosepalum* is endemic to Guanxi and Yunnan provinces. They have shiny and coriaceous leaves with prominent veins that are particular in sect. *Synstylae*. Despite these species have different morphological characteristics, they did not exhibit obvious genetic divergences in our analyses.
4.3. Putative hybrids in Rosa sections Chinenses and Synstylae

As mentioned above, hybridization occurred frequently among wild roses and driven the diversification of this genus (Joly et al., 2006; Matthews, 1920; Schanzer and Vagina, 2007; Uggla and Carlson-Nilsson, 2005). Indeed, it generated a lot novel characters (e.g. intermediate characters) and promoted speciation in this genus.

*Rosa lichiangensis* T.T. Yu & T.C. Ku was first documented in 1981 (Tsue-Chih, 1981). It was supposed to be hybrid-origin from our field and specimen observations. And its hybrid origin was confirmed by both molecular and morphological evidences in another study (Zhu and Gao, unpublished). The results of cpDNA and GAPDH in this study also displayed the hybrid scenario of *R. lichiangensis* (Figs. 1 and 3). When documenting hybridization events in plant using ITS sequences, it is necessary to consider concerted evolution. Some studies suggested that homogenization of ITS copies can eliminate one of the parents copies in few generations (Hillis et al., 1991; Wendel et al., 1995; Aguilar et al., 1999). Our ITS results support the same scenario in *R. lichiangensis* (Fig. 2). The homogenization of parental alleles in the ITS sequences resulted in the display of maternal (*R. soulieana*) genetic information only and the ITS gene could not reveal the hybridization event in this case. However, although ten clones were sequenced, it is not possible to completely rule out the possibility that all the ITS types were not completely collected.

*Rosa rubus* and *R. multiflora* have a wide distribution with a large overlapping area. A few putative hybrids between these two species were observed in the field. Two samples (*R. hybrid 1 and R. hybrid 2*) respectively located in Enshi (Hubei, China) and Guiyang (Guizhou, China) were included in this study. In both localities, *Rosa rubus* and *R. multiflora* grow together and have the same flowering time from May to July. In morphology, the putative hybrids possessed lobed stipules that were intermediate between those of *R. rubus* and *R. multiflora*. They had five to seven leaflets per leaf and the size of leaflet was intermediate between the probable parents. In our chloroplast phylogeny (Fig. 1), the putative hybrids were close to *R. rubus* in Clade B or G. Our nuclear phylogenies (Figs. 2–5) were consistent with the hybrid hypothesis for these two individuals. Their nuclear accesses clustered into two different lineages of which one contained *R. rubus* and its close relatives and the other contained *R. multiflora* and its close relatives. All these results suggested that *R. rubus* and *R. multiflora* were probably the parents of the hybrids.

Another putative hybrid (*R. hybrid 3*) that seemed to be morphologically related to sections Synstylae (*R. rubus*) and Chinenses (*R. lucidissima*) was also sampled in this study. Several characters seemed to be intermediate between the two possible parents. *Rosa lucidissima* has bright coriaceous leaves and dark red flowers. *Rosa rubus* has pubescent herbaceous leaves and white flowers. This putative hybrid had slightly coriaceous leaves and light red flowers. Moreover, *Rosa lucidissima* has long (app. 2 mm) glandular bristles on pedicels and branchlets while *R. rubus* only has glands adhering to pedicels. The putative hybrid had short (app. 0.5–1 mm) glandular bristles on pedicels and branchlets. In addition, this individual was located in the overlapping area of *R. lucidissima* and *R. rubus*. Although *R. lucidissima* flowers much earlier (April to May) than *R. rubus* (late April to July), there is an overlap in their flowering time from late April to early May. We obtained two GAPDH accessions for this putative hybrid with one close to sect. Chinenses (*R. lucidissima*) and the other close to *R. rubus* group (Fig. 3). The GAPDH phylogeny confirmed our inference based on morphology. The chloroplast tree (Fig. 1) suggested that *R. rubus* might play the female parent, and *R. lucidissima* might be the male parent. However, the ITS sequences of this sample did not show different copies (Fig. 2). This pattern in the ITS phylogeny is similar to the situation of *R. lichiangensis* mentioned above. As before, we attributed this pattern to homogenization of parental genetic information obscuring hybridization events.

5. Conclusions and perspectives

This study presents phylogenies of *Rosa* sections Chinenses and Synstylae based on three different kinds of genetic markers (chloroplast DNA, nuclear ribosome DNA, and single copy nuclear DNA) with extensive taxon sampling. The two sections were not resolved as monophyletic in any of our analyses. A lot of discordance was observed between chloroplast and nuclear trees. And we conclude that hybridization, rather than incomplete lineage sorting has been the main driving force of the incongruence. The lack of resolution in phylogenies (especially nuclear phylogenies) definitely hindered the acquisition of accurate relationships among species. However, we confirmed the hybrid origin of the species *R. lichiangensis* and other putative hybrids.

Chloroplast markers provided a better backbone for phylogenies, but ignored hybridization because of their maternal inheritance. Insufficient variation among close species was also a problem. The ITS region has been widely used as a nuclear marker in former studies of *Rosa* since it is of biparental inheritance and provides more variation than chloroplast markers. However, concerted evolution and homogenization of parental polymorphism, as discussed above, definitely hampered our understanding of evolution history. Single copy nuclear gene GAPDH provided more variation than chloroplast and ITS genes. In addition, the GAPDH tree provided the most comprehensive and accurate knowledge of the reticulate evolution of *Rosa* although the resolution was low for some nodes. Further studies including more low copy nuclear genes are necessary to better understand the complex evolutionary history of roses (e.g. hybridization and polyploidization). Additionally, population level studies seem more effective for tracking evolutionary history and circumscribing close species (Joly et al., 2006; Liu et al., 2012; Sun et al., 2014). Thus, studies focusing on population level could be critical to the further understanding of sections Chinenses and Synstylae and even the whole genus since a lot of close species (for example *R. soulieana* group, *R. rubus* group, *R. multiflora* group) have undergone a fast diversification, incomplete lineage sorting, or reticulate evolution.

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Appendix A. Supplementary data

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