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Multiple Events of Allopolyploidy in the Evolution of the Racemose Lineages in *Prunus* (Rosaceae) Based on Integrated Evidence from Nuclear and Plastid Data

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Abstract

*Prunus* is an economically important genus well-known for cherries, plums, almonds, and peaches. The genus can be divided into three major groups based on inflorescence structure and ploidy levels: (1) the diploid solitary-flower group (subg. *Prunus*, *Amygdalus* and *Emplectocladus*); (2) the diploid corymbose group (subg. *Cerasus*); and (3) the polyploid racemose group (subg. *Padus*, subg. *Laurocerasus*, and the *Maddenia* group). The plastid phylogeny suggests three major clades within *Prunus*: *Prunus-Amygdalus-Emplectocladus*, *Cerasus*, and *Laurocerasus-Padus-Maddenia*, while nuclear ITS trees resolve *Laurocerasus-Padus-Maddenia* as a paraphyletic group. In this study, we employed sequences of the nuclear loci *At103*, ITS and *s6pdh* to explore the origins and evolution of the racemose group. Two copies of the *At103* gene were identified in *Prunus*. One copy is found in *Prunus* species with solitary and corymbose inflorescences as well as those with racemose inflorescences, while the second copy (II) is present only in taxa with racemose inflorescences. The copy I sequences suggest that all racemose species form a paraphyletic group composed of four clades, each of which is definable by morphology and geography. The tree from the combined *At103* and ITS sequences and the tree based on the single gene *s6pdh* had similar general topologies to the tree based on the copy I sequences of *At103*, with the combined *At103*-ITS tree showing stronger support in most clades. The nuclear *At103*, ITS and *s6pdh* data in conjunction with the plastid data are consistent with the hypothesis that multiple independent allopolyploidy events contributed to the origins of the racemose group. A widespread species or lineage may have served as the maternal parent...
for multiple hybridizations involving several paternal lineages. This hypothesis of the complex evolutionary history of the racemose group in *Prunus* reflects a major step forward in our understanding of diversification of the genus and has important implications for the interpretation of its phylogeny, evolution, and classification.

### Introduction

*Prunus* L. belongs to subfamily Amygdaloideae of the family Rosaceae [1]. It consists of ca. 250–400 species of deciduous and evergreen trees and shrubs widely distributed in the temperate zone of the Northern Hemisphere and in the subtropics and tropics of both the New and Old Worlds [2–4]. *Prunus* is economically important as the source of many temperate fruit and nut crops, such as almonds, cherries, plums, and peaches, as well as for timber and ornamentals [5]. The genus is defined based on a combination of characters including presence of leaf glands, a solitary carpel, superior ovary position, fruit a drupe, and solid pith [2, 6].

The taxonomy of *Prunus* has been controversial, especially concerning the generic delimitation and infrageneric classification [2, 5, 7]. The most widely accepted classification of this genus consists of five subgenera: *Prunus*, *Amygdalus* (L.) Focke, *Cerasus* Pers., *Laurocerasus* Koehne, and *Padus* (Moench) Koehne [2]. However, some treatments segregated the group into multiple genera [8, 9]. Recent phylogenetic studies support a broad circumscription of *Prunus* [1, 3, 4, 10]. The most recent classification of the genus recognized only three subgenera: *Prunus*, *Cerasus*, and *Padus*, with a broader concept of subgenus *Padus* that included *Laurocerasus* and the former genera *Maddenia* Hook. f. & Thoms and *Pygeum* Gaertn. [7].

Several molecular phylogenetic studies [3–6, 10, 11–15] have been conducted to investigate the evolutionary relationships of *Prunus* using both plastid (*rbcL*, *matK*, *ndhF*, *rps16*, *rpl16*, *trnL-L-F*, and *trnS-S-G*) and nuclear (*nrITS* and *s6pdh*) sequences. All previous studies have clearly supported the monophyly of *Prunus* s.l. The plastid data have supported three main clades within *Prunus*, which correspond to three groups that can be identified based on inflorescence structure [4]: (1) the deciduous solitary-flower group, including subg. *Prunus*, *Amygdalus*, and *Emplectocladus*; (2) the deciduous corymbose inflorescence group, referring to subg. *Cerasus*; and (3) the racemose inflorescence group, containing subg. *Laurocerasus*, comprising evergreen species (also including southeast Asian species formerly assigned to the genus *Pygeum* [16]), as well as subg. *Padus* and the former genus *Maddenia*, both comprising deciduous species with temperate distributions [4, 6, 17]. Most taxa of the solitary-flower and corymbose groups are diploid (*2n* = 2x = 16), while taxa of the racemose group usually have higher ploidy levels with *2n* = 4x = 32 or sometimes *2n* = 8x = 64 [4, 11, 16–19].

Plastid phylogenies support the monophyly of the three groups described above and resolve the first two clades as sister to one another [4] (Fig 1A). In contrast, nrITS DNA data have supported a different topology, with one clade consisting of members of subgenera *Prunus*, *Amygdalus*, and *Emplectocladus*, identical to clade A in the plastid phylogeny (the solitary flower group), a second clade including most members of *Cerasus* (the corymbose inflorescence group, clade B in the plastid phylogeny), and species of *Laurocerasus*, *Padus* and *Maddenia* (the racemose inflorescence group) comprising a paraphyletic group of lineages (Fig 1B; also see Fig 4 in Chin et al. [4]), rather than a clade as in the plastid phylogeny (clade C).

The incongruences in relationships among the racemose inflorescence lineages resolved in the maternally inherited plastid phylogeny vs. in the biparentally inherited nuclear ITS phylogeny have led to the suggestion of a hybrid origin of this group [4]. However, a number of molecular genetic processes (e.g., ancient or recent array duplication events, genomic harboring of pseudogenes in various states of decay, and/or incomplete intra- or interarray
homogenization) can impact ITS sequences in ways that may mislead phylogenetic inference [20]. Thus, more nuclear markers, especially low-copy nuclear genes that can track both parents’ genomes in hybrids [21], are needed to test the hypothesis of the allopolyploid origin of racemose Prunus [4].

This study aims to provide further insights into the phylogenetic relationships within Prunus using sequences of the low-copy nuclear At103 [22, 23] and s6pdh [24] genes, as well as data from ITS. The primary goal of this study is to clarify the evolution of the polyploid racemose group using these nuclear sequences, integrating evidence from the established plastid phylogeny.

Materials and Methods

Ethics Statement

Species of Prunus, Prinsepia, Physocarpus and Oemleria sampled in this study do not represent endangered or protected plants. Thus no specific permits were required for the collection of samples, which complied with all relevant regulations. The species information is provided in Tables 1–3. All voucher specimens are deposited in the US National Herbarium (US) or the Herbarium of University of California, Davis (DAV).

Taxon sampling and outgroup selection

For the At103 gene sequences, 47 species of Prunus representing all five subgenera recognized by Rehder [2] were sampled (Tables 1 and 2). All samples were used in previous studies by Chin et al. [4] and Liu et al. [15]. In addition, two outgroup species, Prinsepia utilis and Physocarpus opulifolius, were selected based on previous phylogenetic studies of Rosaceae [1]. We also included ITS data for 23 of the species for which we obtained sequences of At103 (Table 3), covering the five subgenera defined by Rehder [2]. For the s6pdh data, we included 26 species, also representing all five subgenera of Prunus recognized by Rehder [2] with Oemleria cerasiformis as the outgroup (Table 4). Our samples were collected throughout the range of Prunus including from Asia, Europe, North America, South America and Africa, and represent all three types of inflorescence structures. We especially made sure that the racemose

Fig 1. Summary of phylogenetic relationships in Prunus, based on the plastid DNA data (a), and nuclear sequences (b) (simplified from Chin et al. [4]). A = solitary inflorescence clade, including subgenus Prunus and Amygdalus; B = corymbose inflorescence clade, which refers to subgenus Cerasus; C = racemose inflorescence group, including subgenus Laurocerasus, Padus, Maddenia and Pygeum group.
Table 1. Taxa and At103 gene GenBank accession numbers of Prunus and outgroups sampled for this study. All voucher specimens (except one collection Potter 081118 deposited in DAV) are deposited in the US National Herbarium (US).

| Taxon | Voucher | Location | Gene bank Accession number |
|-------|---------|----------|---------------------------|
| **Subgenus. 1. Prunus L.** | | | |
| *P. armeniaca* L. | Wen 10326 | Russia, Stavropol region, Pjatigorsk | KU525428 |
| *P. armeniaca* L. | Wen 11933 | USA, Virginia, Fairfax | KU525429 |
| *P. divaricata* Ledeb. | Wen 10350 | Russia, Karachaevo—Cherkesskaja | KU525431 |
| *P. glandulosa* Thunb. | Wen 11932 | USA, Virginia, Fairfax | KU525427 |
| *P. mandshurica* (Maxim.) Koehne | Wen 5444 | USA, Arnold Arboretum | KU525436 |
| *P. mume* (Siebold) Siebold & Zucc. | Wen 11942 | USA, Washington D.C. | KU525435 |
| *P. mume* (Siebold) Siebold & Zucc. | Wen 12030 | China, Fujian Prov., Wuyishan | KU525457 |
| *P. salicina* Lindl. | Wen 8032 | USA, Washington D.C. | KU525443, KU525444 |
| *P. salicina* Lindl. | Wen 10894 | USA, Washington D.C., cult. | KU525456 |
| *P. salicina* Lindl. | Wen 11928 | China, Fujian Prov., Xihe Co. | KU525437-KU525442; KU525445-KU525449; KU525453-KU525455; KU525451, KU525516 |
| *P. sibirica* L. | Wen 8532 | China, Beijing Shi, Baihuashan | KU525430 |
| *P. murrayana* Palmer | Wen 7283 | Vietnam, Lao Cai Prov., Sa Pa | KU525432 |
| *P. nigra* Desf. | Wen 9904 | USA, Wisconsin, Dane Co. | KU525434 |
| *P. nigra* Desf. | Wen 11112 | USA, Texas, Kerr Co. | KU525426 |
| *P. rivularis* Scheele | Wen 11988 | USA, Texas, Kerr Co. | KU525426 |
| **Subgenus. 2. Amygdalus (L.) Focke** | | | |
| *P. mira* Koehne | Wen 9207 | China, Tibet, Tzayu Co. | KU525424 |
| *P. persica* (L.) Batsch | Wen 10883 | Vietnam, Lao Cai Prov., Sa Pa | KU525401 |
| *P. tenella* Batsch | Wen 237 | USA, Arnold Arboretum | KU525432 |
| *P. triloba* Lindl. | Wen 11935 | USA, Pensylvania, Swarthmore College, cult. | KU525425 |
| **Subgenus. 3. Cerasus Pers.** | | | |
| *P. campanulata* Maxim. | Wen 11239 | China, Zhejiang Prov., Qingyuan Co. | KU525406 |
| *P. campanulata* (Maxim.) Yü et Li | Wen 12040 | China, Fujian Prov., Wuyishan | KU525459 |
| *P. cerasoides* Buch. -Ham. ex D. Don | Wen 10126 | Indonesia, Java, cult. | KU525415 |
| *P. cerasoides* Buch. -Ham. ex D. Don | Wen 10833 | Vietnam, Lao Cai Prov., Sa Pa | KU525405 |
| *P. cerasoides* Buch. -Ham. ex D. Don | Tibet 3163 | China, Tibet | KU525402 |
| *P. clarofolia* Schneid. | Tibet 2839 | China, Tibet | KU525416 |
| *P. clarofolia* Schneid. | Wen 9211 | China, Tibet, Bomi Co. | KU525417 |
| *P. dielsiana* (Schneid.) Yü et Li | Wen 9295 | China, Hunan Prov., Xinning Co., cult. | KU525414, KU525460 |

(Continued)
inflorescence group was well represented in our sampling, because this group is the most species-rich and morphologically diverse group in the genus, and the one whose phylogenetic relationships are in question and whose origins we sought to clarify.

DNA isolation, amplification, cloning, and sequencing

Total genomic DNA was extracted from silica gel-dried or herbarium material using the Plant DNA Extraction Kit AGP965/960 (AutoGen, Holliston, Massachusetts, U.S.A.) or the DNeasy Plant Mini Kit (Qiagen, Crawley, UK). All PCR amplifications were performed in 25-μL reactions containing 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.4 mM of each primer, 1 U of Taq DNA polymerase (Qiagen), and approximately 10–50 ng of the template DNA.

The PCR primer pair for At103 was "F" (CTTCAAGCCMAAGTTCATCTTC TA) and "R" (TTGGCAATCATTGAGGTACATNGTMACATA) as in Li et al. [23], and the amplification conditions were: 3 min initial denaturation at 95°C, 35 cycles of 30 s denaturation at 94°C, 45 s annealing at 50°C, and 60 s extension at 72°C, followed by a final extension of 5 min at 72°C.

The PCR products were cleaned with ExoSAP-IT (cat. #78201, USB Corporation, Cleveland, Ohio, U.S.A.). Purified products were sequenced with BigDye 3.1 reagents on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, California, U.S.A.) from both directions. The forward and reverse sequences were assembled using Geneious v.8.1.2. (http://www.geneious.com) [25]. Special attention was paid to those sites with overlapping peaks in the chromatograms, because they may indicate intra-individual variation (polymorphisms). If an obviously overlapping signal was detected in both the forward and reverse chromatograms, the site was considered to be putatively polymorphic between alleles or copies. Those samples with polymorphic sites were cloned using the TOPO TA cloning kit (Invitrogen. Carlsbad. Califor-

Table 1. (Continued)

| Taxon              | Voucher | Location                  | Gene bank Accession number |
|--------------------|---------|---------------------------|----------------------------|
| P. discoidea (Yu & Li) Wei & Chang | Wen 11284 | China, Zhejiang Prov., Linan | KU525413                   |
| P. maackii Rupr.   | Wen 11794 | USA, Washington DC        | KU525400                   |
| P. maackii Rupr.   | Wen 5405  | China, Jilin Prov., Tonghua | KU525458                   |
| P. mahaleb L.      | Wen 1372  | USA, Arnold Arboretum     | KU525518                   |
| P. mahaleb L.      | Wen 355   | USA, Arnold Aboretum, cult.| KU525399                   |
| P. nipponica Matsum. | Wen 11797 | USA, Washington DC        | KU525409                   |
| P. serrula Franch. | Wen 9155  | China, Tibet, Gongbujiangda Co. | KU525404                   |
| P. trichostoma (Koe) Yü et Li | Wen 12086 | China, Sichuan Prov., Emeishan | KU525407                   |
| P. subhirtella Miq. | Wen 11795 | USA, Washington DC        | KU525408                   |
| P. takasimensis Nakai | Wen 11937 | USA, Pennsylvania, Swarthmore College | KU525412                   |
| P. tomentosa Thunb | Wen 8091  | China, Chongqing Shi, Chengkou Co. | KU525403                   |

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The nuclear ribosomal ITS regions were amplified using primers “ITS5a” (CCTTATCATTTAGAGGAAGGAG) and “ITS4” (TCCTCCGCTATTGATATGC) as in Stanford et al. [26].
In addition, we used 15 sequences from our previously published studies [4, 11]. The PCR program was as follows: an initial 5 min at 95°C, followed by 38 cycles of 40 s at 94°C, 45 s at 52°C, and 1 min 20 s at 72°C, and a final extension cycle of 7 min at 72°C.

### Table 2. Taxa and At103 gene GenBank accession numbers of Prunus and outgroups sampled for this study.

All voucher specimens (except one collection Potter 081118 deposited in DAV) are deposited in the US National Herbarium (US).

| Taxon                     | Voucher | Location                        | Gene bank Accession number |
|---------------------------|---------|---------------------------------|----------------------------|
| *P. tomentosa* Thunb      | Wen 11210 | China, Zhejiang Prov., Longquan Co. | KU525411                  |
| *P. trichostoma* Koehne   | Wen 12141 | China, Sichuan Prov., Emei       | KU525410                  |
| *P. yedoensis* Matsum.    | Wen 11796 | USA, Washington DC               | KU525517                  |
| **Subgenus. 4. *Padus* (Moench) Koehne** |         |                                  |                            |
| *P. alabamensis* Mohr.    | Alvin s.n. | USA, Alabama                    | KU525374-KU525376; KU525483-KU525485; |
| *P. buergeriana* Miq.     | Wen 11290 | China, Zhejiang Prov., Anji Co.  | KU525502                  |
| *P. napaulensis* (Ser.) Steud. | Wen 9217 | China, Tibet, Borni Co.          | KU525362-KU525370; KU525503-KU525505; KU525519 |
| *P. padus* L.             | Wen 10319 | Russia, Moscow, Serpukhov       | KU525377-KU525381; KU525495-KU525501; |
| *P. wilsonii* (Schneid.) Koehne | Wen 11174 | China, Zhejiang Prov., cult.     | KU525418-KU525423; KU525490-KU525494 |
| **Subgenus. 5. *Laurocerasus* Koehne** |         |                                  |                            |
| *P. integrifolia* (Sudw.) Sarg. | Wen 8620 | Peru, Oxpampa                    | KU525463                  |
| *P. fordiana* Dunn        | Wen 10845 | Vietnam, LaoCai Prov., Sa Pa     | KU525512                  |
| *P. laurocerasus* L.      | Wen 10366 | Russian Federation               | KU525348, KU525506       |
| *P. tucumanensis* Lillo    | Nee & Wen 53882 | Bolivia            | KU525488                  |
| *P. wallchii* Steud.      | Wen 10877 | Vietnam, LaoCai Prov., Sa Pa     | KU525371-KU525373; KU525486-KU525487; |
| **Pygeum group**          |         |                                  |                            |
| *P. africana* (Hook. f.) Kalkman | Wen 9688 | Madagascar, Toamasina            | KU525361                  |
| *P. arborea* (Blume) Kalkman | Wen 10927 | Vietnam, Ninh Binh Prov.        | KU525359-KU525360; KU525465-KU525469; |
| *P. arborea* (Blume) Kalkman | Wen 10246 | Indonesia, Sulawesi Tenggara, Sanggona | KU525358, KU525514 KU525472-KU525476 |
| *P. costata* (Hemsl.) Kalkman | Wen 10106 | Indonesia, Java., cult.          | KU525356                  |
| *P. grisea* (Blume ex Müll. Berol.) Kalkman | Wen 11037 | Vietnam, Lam Dong Prov.          | KU525349-KU525354;       |
| *P. grisea* (Blume ex Müll. Berol.) Kalkman var. *microphylla* Kalkman | Potter 081118 (DAV) | Indonesia, West Papua Prov., Manokwari District | KU525355, KU525357 |
| *P. lancilimba* (Merr.) Kalkman | Wen 10851 | Vietnam, LaoCai Prov., Sa Pa     | KU525470-KU525471; KU525477-KU525482 |
| *P. lancilimba* (Merr.) Kalkman | Wen 10829 | Vietnam, LaoCai Prov., Sa Pa     | KU525464                  |
| **Maddenia group**        |         |                                  |                            |
| *P. himalayana* Wen       | Tibet 2612 | China, Tibet                    | KU525382-KU525384; KU525386-KU525388; KU525390-KU525398; KU525508-KU525509 KU525515, KU525520 |
| *P. hypoleuca* (Koehne) Wen | Wen 665 | USA, Arnold Arboretum, cult.     | KU525385, KU525389, KU525489, KU525507, KU525510 |
| **Outgroups**             |         |                                  |                            |
| *Physocarpus opulifolius* (L.) Maxim. | Wen 11943 | USA, Virginia, Fairfax Co., cult. | KU525461                  |
| *Prunus utilis* Royle     | Wen 9174 | China, Tibet, Linzhi             | KU525462                  |

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For the *s6pdh* sequences, because intron 1 was highly divergent and difficult to align, we only used the region from the second to the sixth exon. The *s6pdh* sequences from *Prunus* *con- sociiflora*, *P. serotina* subsp. *virens*, *P. napaulesis*, *P. brachypoda*, *P. integrifolia*, *P. myrtifolia*, *P. polystachya*, *P. africana* and *P. integrifolia* were produced by PCR amplification with primers *s6pdh-k* "AGCTCATTACAAGAGTGA AG CAGACGGTGG/s6pdh-p "AGAGTGGTCC TGGATTTCTTATCTA", or with the primer combinations *s6pdh-k* "AGCTCATTACAAGAGTGAAGCAGACGTTGG"/s6pdh-h "AGACCAATGCTGCGAATCTAAGCCC" and *s6pdh-c* "TTTGGAATT CAGACCATGGGCATG/s6pdh-p "AGAGTGGTCC TGGATTTCTTATCTA", which yield overlapping PCR products [12]. In addition, we used 27 sequences from previously published studies [12, 27]. The PCR amplification conditions were as follows: an initial 10 min at 95°C, followed by 35 cycles of 30 s at 95°C, 1 min at 54°C, and 2 min at 72°C, and a final extension cycle of 7 min at 72°C [12]. We also cloned sequences of *Prunus brachypoda*, *P. integrifolia* and *P. polystachya*.  

### Table 3. Taxa and At103 and ITS GenBank accession numbers of *Prunus* and outgroups sampled for this study. All voucher specimens are deposited in the US National Herbarium (US). Newly generated sequences are indicated by an asterisk (*).

| Taxon | Voucher | Location | GenBank Accession (ITS/At103) |
|-------|---------|----------|------------------------------|
| **Subgenus. 1. *Prunus* L.** | | | |
| *P. armeniaca* L. | Wen 10326 | Russia, Stavropol region, Pjatigorsk | JQ776883; KU525428 |
| *P. divaricata* Ledeb. | Wen 10350 | Russia, Karachaevo—Cherkesskaja | JQ776885; KU525431 |
| *P. mandshurica* (Maxim.) Koehne | Wen 5444 | USA, Arnold Arboretum | JQ776884; KU525436 |
| *P. salicina* Lindl. | Wen 10894 | Vietnam, Lao Cai Prov., Sa Pa | JQ776887; KU525456 |
| **Subgenus. 2. *Amygdalus* (L.) Focke** | | | |
| *P. persica* (L.) Batsch | Wen 10883 | Vietnam, Lao Cai Prov., Sa Pa | JQ776821; KU525401 |
| **Subgenus. 3. *Cerasus* Pers.** | | | |
| *P. discoidea* (Yu & Li) Wei & Chang | Wen 11284 | China, Zhejiang Prov., Linan | JQ776825; KU525413 |
| *P. nipponica* Matsum. | Wen 11797 | USA, Washington DC | JQ776829; KU525409 |
| *P. maackii* (Rupr.) Ledeb. | Wen 11794 | USA, Washington DC | JQ776862; KU525400 |
| *P. yedoensis* Matsum. | Wen 11796 | USA, Washington DC | JQ776833; KU525517 |
| *P. mahaleb* L. | Wen 1372 | USA, Arnold Arboretum | JQ776828; KU525518 |
| **Subgenus. 4. *Padas* (Moench) Koehne** | | | |
| *P. padus* L. | Wen 10319 | Russia, Moscow, Serpuhov | KX013510*; KU525380 |
| *P. buergeriana* Miq. | Wen 11290 | China, Zhejiang Prov., Anji Co. | JQ776852; KU525502 |
| *P. wilsonii* (Schneid.) Koehne | Wen 11174 | China, Zhejiang Prov., cult. | JQ776881; KU525492 |
| **Subgenus. 5. *Laurocerasus* Koehne** | | | |
| *P. laurocerasus* L. | Wen 10366 | Russian Federation | JQ034155; KU525348 |
| *P. tucumanensis* Lillo | Nee & Wen 53882 | Bolivia | KX013511*; KU525488 |
| **Pygeum group** | | | |
| *P. africana* (Hook. f.) Kalkman | Wen 9688 | Madagascar, Toamsina | KX013504*; KU525361 |
| *P. arborea* (Blume) Kalkman | Wen 10927 | Vietnam, Ninh Binh Prov. | KX013505*; KU525466 |
| *P. costata* (Hemsli.) Kalkman | Wen 10106 | Indonesia, Java., cult. | KX013506*; KU525356 |
| *P. grisea* (Blume ex Müll. Berol.) Kalkman | Wen 11037 | Vietnam, Lam Dong Prov. | KX013507*; KU525351 |
| *P. lancilimba* (Merr.) Kalkman | Wen 10829 | Vietnam, LaoCai Prov., Sa Pa | KX013509*; KU525464 |
| **Maddenia group** | | | |
| *P. himalayana* Wen | Tibet 2612 | China, Tibet | KX013508*; KU525508 |
| *P. hypoleuca* (Koehne) Wen | Wen 665 | USA, Arnold Aboretum, cult. | JQ776888; KU525510 |
| **Outgroup** | | | |
| *Physocarpus opulifolius* (L.) Maxim. | Wen 11943 | USA, Virginia, Fairfax Co., cult. | JQ34169; KU525461 |

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The PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA). Amplicons were directly sequenced in both directions using the amplification primers. Cycle sequencing reactions were conducted using the BigDye 3.1 reagents. After being cleaned up by the Sephadex columns, the sequencing products were run on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, California, USA).

### Table 4. Taxa and s6pdh gene GenBank accession numbers of Prunus and outgroups sampled for this study. All voucher specimens are deposited in the US National Herbarium (US) and the Herbarium of University of California, Davis (DAV). Newly generated sequences are indicated by an asterisk (*).

| Taxon | Voucher | Location | GeneBank Accession |
|-------|---------|----------|--------------------|
| **Subgenus. 1. Prunus L.** | | | |
| **P. armeniaca L.** | | | EU056696 |
| **P. cerasifera Ehrh.** | | | EU056697 |
| **P. consociflora C. K. Schneid.** | | | DPRU2457 |
| **P. fremontii S. Watson** | | | AF414986 |
| **P. salicina Lindl.** | | | AF414982 |
| **P. subcordata Benth.** | | | AF414980 |
| **P. mexicana S. Watson** | | | AF414977 |
| **Subgenus. 2. Amygdalus (L.) Focke** | | | |
| **P. fasciculata (Torr.) A. Gray** | | | AF414978 |
| **P. persica (L.) Batsch** | | | AF414988 |
| **Subgenus. 3. Cerasus Pers.** | | | |
| **P. clarofolia C.K. Schneid.** | Wen 9277 (US) | China, Hunan Prov., Xinning Co. | KU525529* |
| **P. emarginata (Douglas ex Hook.) Walp.** | EB139 | USA, California, El Dorado Co. | AF504298, AF504299, AF504300, AF504301, AF504302 |
| **P. emarginata (Douglas ex Hook.) Walp.** | DPRU 2214 | USA, Davis, USDA National Clonal Germplasm Repository | AF414985, AF504303, AF504304 |
| **P. fruticosa Pall.** | | | |
| **Subgenus. 4. Padus (Moench) Koehne** | | | |
| **P. padus L.** | | | AF415007 |
| **P. serotina subsp. virens (Wooton & Standl.) McVaugh** | Beck & Estes s.n. | USA, Texas, Jeff Davis Co. | KU525524* |
| **P. virginiana L.** | | | AF504297 |
| **Subgenus. 5. Laurocerasus Koehne** | | | |
| **P. brachybotrya Zucc.** | Wen 8755 (US) | Mexico, Vera Cruz. | KU525523*, KU525531* |
| **P. caroliniana (Mill.) Alton** | Wen 8620 (US) | Peru, Oxapampa | KU525521*, KU525522* |
| **P. integrifolia Walp.** | Wen 8620 (US) | Peru, Oxapampa | AF414974 |
| **P. ilicifolia (Nutt. ex Hook. & Arn.) D. Dietr.** | | | AF415010 |
| **P. laurisilva L.** | | | AF504296 |
| **P. myrtifolia (L.) Urb.** | Vincent et al. 12720 | Bahamas, Great Abaca Island | KU525530* |
| **P. pygmaea (L.) C. Koch** | | | |
| **P. africana (Hook. f.) Kalkman** | DPRU2557 | Kenya, Trans Nzoia | KU525525* |
| **P. henryi (C. K. Schneid.) Koehne** | Wen 8463 (US) | China, Yunnan Prov. | KU525528* |
| **P. polystachya (Hook. f.) Kalkman** | Wen 8407 (US) | Malaysia, Selangor | KU525526*, KU525527* |
| **Outgroup** | | | |
| **Oemleria cerasiformis (Torr. & A. Gray ex Hook. & Arn.) J. W. Landon** | | | AF415011 |

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Data analyses

Sequences were aligned with MUSCLE [28] and adjusted manually in Geneious v.8.1.2 [25]. For the At103 gene, phylogenetic analyses employed 173 sequences after excluding identical sequences from the clones of the same accession. The analyses were first conducted using maximum likelihood (ML) with PhyML version 3.0 [29]. The best-fit nucleotide substitution model for the dataset was determined based on Akaike Information Criterion (AICc) in jModelTest v.2.1.7 [30, 31]. Nodal robustness on the ML tree was estimated by the nonparametric bootstrap (1000 replicates). To visualize the conflicting evolutionary signals in the At103 data and highlight reticulate evolution, a neighbor net diagram was generated based on uncorrected-P distance matrix, using Splitstree 4.13.1 [32]. Bootstrap support of each group was estimated with 1000 replicates.

We combined the At103 and ITS data for 23 samples. Insertions and deletions (indels) were coded as binary characters using the program SeqState [33] with the “simple coding” method [34]. The binary characters were combined with the nucleotide data using the program SequenceMatrix [35]. Bayesian inferences (BI) were conducted in MrBayes v.3.2.5 [36]. The best-fit nucleotide substitution models for ITS, and the exon and intron of At103 were determined using the corrected Akaike information criterion (AICc) in jModelTest v.2.1.7, respectively [31]. In the Bayesian inference, two independent analyses starting from different random trees with three heated and one cold chain were run for 10,000,000 generations, and trees were sampled every 1,000 generations. 10,000 trees from each run were sampled in total. The first 2,500 trees from each run were discarded as burn-in, and the remaining 15,000 trees were used to construct a 50% majority-rule consensus tree and posterior probabilities (PP).

For the s6pdh data, boundaries of the exon2, intron2, exon3, intron3, exon4, intron4, exon5, intron5, and exon6 regions were determined by comparing with the published s6pdh sequence of Prunus subcordata Benth. [12]. Indel coding, selection of best-fitting nucleotide substitution models for each region, and Bayesian inference were performed as described above for At103 and the s6pdh. Rapid bootstrap analysis was conducted with a random number seed and 1000 alternative runs using RAxML v.8.2 [37]. All tree visualizations were achieved with FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

We did not combine the s6pdh with the At103 and ITS data because there were very few samples for which sequences from all three regions were available.

Results

We isolated 212 sequences of the At103 gene from 47 species of Prunus s.l. The length of the At103 ranged from 444 bp to 538 bp. There were 228 variable characters, of which 136 (excluding indel sites) were parsimony-informative in the aligned matrix of 212 sequences. All the At103 gene sequences contained the third exon and the intron between exons 3 and 4. The exon 3 region of the At103 gene was conserved, consisting of 195 bp in the alignment without any indels. The length of the intron ranged from 249 to 343 bp. Modeltest indicated that the best-fit model under AICc was H80+G.

The At103 gene tree generated by maximum likelihood analyses with phyML suggested two major copies of the nuclear At103 gene within Prunus s.l. (herein designated as copy I and copy II), but with weak support (Figs 2–4). Copy I was exhibited by 42 species whereas copy II was only found in 15 species, all belonging to the racemose group (Figs 2–4).

The length of copy I of the At103 gene ranged from 458 to 538 bases. There were 155 variable characters, of which 77 were parsimony-informative in the aligned matrix of 118 sequences. Seven indels were present in the entire gene alignment. The indels consisted of 1–27 nucleotides. Three relatively large ones (a deletion of 27 bp, a deletion of 21 bp, and an
Fig 2. Maximum likelihood (ML) tree inferred from the Atf03 DNA sequences of Prunus. The results of ML bootstrap analysis are shown above the branches. Bootstrap values >50% are shown.

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Fig 3. Maximum likelihood (ML) tree inferred from the At103 DNA sequences of Prunus. The results of ML bootstrap analysis are shown above the branches. Bootstrap values >50% are shown.

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insertion of 50 bp) were found in group A (Prunus-Amygdalus). The length of copy II of the At103 gene ranged from 444 to 498 bases. There were 117 variable characters, of which 75 were parsimony-informative in the aligned matrix of 53 sequences. The alignment of the entire gene had six indels, each consisting of one to six nucleotides.

The copy I sequences supported the monophyly of the Prunus-Amygdalus group (group A), which possess solitary flowers. The ML tree also supported the Cerasus clade (group B), which has corymbose inflorescences. Sequences of the racemose species did not form a monophyletic group, and four subgroups may be identified, and defined by morphology and geography. Subgroups C-1 and C-2 include the species from temperate zone (Padus I-Maddenia and Padus II). Species formerly classified in Maddenia were nested within subgenus Padus. Subgroup C-3 includes the European species P. laurocerasus and the subtropical and tropical Asian species P. wallichii of subgenus Laurocerasus. Subgroup C-4 consists of the tropical species from Southeast Asia belonging to the Pygeum group of subgenus Laurocerasus and the African species Prunus africana.

The copy II sequences were only found in species of the racemose group. The sequences supported the monophyly of the Pygeum group. Also, the Neotropical Prunus integrifolia and P. tucumanensis formed another clade. The Pygeum group was shown to be sister to this

Fig 4. Maximum likelihood (ML) tree inferred from the At103 DNA sequences of Prunus. The results of ML bootstrap analysis are shown above the branches. Bootstrap values >50% are shown.

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Neotropical clade, but with low bootstrap support. Species formerly assigned to *Maddenia* formed a clade. Other relationships within the racemose group were poorly resolved based on the copy II sequences.

A neighbor-net diagram (S1 Fig) suggested two major splits, corresponding to copy I and copy II of the *At103* sequences. The copy I sequences can distinguish three broad groups: group A (corresponding to the *Prunus-Amygdalus* group in Figs 2–4), group B (corresponding to *Cerasus* group in Fig 2) and group C. Group C comprised species of the racemose group with four subgroups supported by copy I sequences, i.e., C-1, C-2, C-3 and C-4 (roughly corresponding to subgroups *Padus* I–*Maddenia*, *Padus* II, *Laurocerasus*, and *Pygeum* in Figs 2–4). Copy II was only possessed by species of the racemose group and it did not provide strong resolution of relationships within the group, although species of the *Pygeum* group were supported to form a cluster (S1 Fig).

The combined *At103*-ITS data set had 1136 characters, of which 283 were variable and 134 were parsimony-informative in the aligned matrix of 23 sequences. Modeltest indicated that under AICc, the best-fit models for ITS and the exon and intron of *At103* were TIM2+I+G, K80 and TPM2uf, respectively. The combined *At103* and ITS sequences supported the monophyly of the *Prunus s.s.*--*Amygdalus* group (PP = 1.00). The Bayesian tree also supported the *Cerasus* clade (PP = 0.97). Sequences of the racemose species were resolved as paraphyletic (Fig 5).

The relationships of *s6pdh* sequences of *Prunus emarginata* were complex. The sequences from the accession EB139 were grouped in two separate clades, with one clone (#4) grouping with *P. lusitanica* of the *Laurocerasus* group, and the other four clones (#1–3, & 5) forming a clade sister to the main *Prunus-Amygdalus-Cerasus* group plus *P. padus*–*P. serotina* subsp. *virens* (Fig 6). The sequences of *P. emarginata* from the second accession (DPRU2214) were shown in three different clades, which were scattered in the *Cerasus* and *Laurocerasus-Padus* groups (sister to *Prunus ilicifolia* of the *Laurocerasus* group; sister to a large clade of the *Laurocerasus-Padus-Pygeum* group; or sister to the *P. fruticosa*–*P. clarofolia* of the *Cerasus* group).

**Discussion**

Two copies of *At103* gene were detected in the species of the polyploid racemose group in *Prunus*. The topologies of the *At103*, the combined *At103*-ITS data, and the *s6pdh* data are generally similar to each other, but clearly different from that of the plastid tree (cf. Figs 1–6 and S1 Fig). The incongruent relationships in the polyploid racemose group in *Prunus*, as also observed in the separate phylogenetic analyses of plastid and nuclear ITS sequences in previous
studies [3, 4, 10, 13, 15], have been hypothesized to be the result of an ancient hybridization event [4].

Chromosome numbers provide further evidence for the possible hybrid origin of the racemose group. The base chromosome number of Prunus is $x = 8$. Most of the species in the solitary flower group (e.g., peach, *P. persica*; almond, *P. dulcis*) and the corymbose group (e.g., sweet cherry, *P. avium*) have the chromosome number of $2n = 2x = 16$. On the other hand, species from the racemose group have been reported to have higher ploidy levels (e.g., $2n = 4x = 32$ for most species; *P. lusitanica* $2n = 8x = 64$, and *P. laurocerasus*, $2n = 22x = 176$) [18]. The higher ploidy levels of these species indicate that polyploidization may have played a role in the origin(s) of the entire racemose group.

It is well documented that hybrid-mediated genome doubling (allopolyplody) has played an important role in plant evolution [4, 38–41]. Speciation involving allopolyplody may have occurred repeatedly in different geographic locations and at different times, which may result in morphological differences between hybrids of the same parentage [42].
Fig 6. Bayesian tree inferred from the s6pdh DNA sequences of *Prunus*. Bayesian posterior probabilities (left) (≥ 0.95) and likelihood bootstrap (right) values (≥ 50%) are given above branches. Dashes represent bootstrap ≤ 50%.

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In the previously generated nuclear ITS tree, the racemose group was resolved as paraphyletic \[3–5, 11\]. The taxa in the racemose group were also not supported to be monophyletic by the \(At103\), \(At103\) and ITS, and \(s6pdh\) trees (Figs 2–6 and S1 Fig), and these taxa did not form a cluster in the neighbor net diagram (S1 Fig). Four subgroups were resolved within the racemose group by copy I of the \(At103\) gene data, corresponding to: (1) the temperate subgenus \(Padus\) (I) and former genus \(Maddenia\); (2) the temperate subgenus \(Padus\) (II); (3) the European and the subtropical Asian members of subgenus \(Laurocerasus\) and (4) the \(Pygeum\) group from Southeast Asia, Africa and Australia (part of subgenus \(Laurocerasus\)) (Figs 2–4 and S1 Fig). The three to four lineages to a large extent have morphological and geographic integrity. Both subgenus \(Padus\) and the former genus \(Maddenia\) are deciduous and distributed in temperate regions. The taxa of subgenus \(Laurocerasus\) are evergreen with axillary inflorescences that are leafless at the base of the rachis, and are distributed in tropical and subtropical regions of both the New and Old Worlds. The \(Pygeum\) group of \(Laurocerasus\) is further characterized by indistinguishable sepals and petals, and is distributed mainly in tropical Asia and Africa with one species in Australia [16].

The phylogenetic trees based on the combined \(At103\)-ITS (Fig 5), and \(s6pdh\) (Fig 6) data are largely congruent with the trees based on separate analyses of ITS or \(At103\) [4, 5, 10]. The \(Prunus\)-\(Amygdalus\) and the \(Cerasus\) groups are nested within a paraphyletic racemose group (\(Padus-Laurocerasus-Pygeum\)) (Figs 5 and 6). In the \(s6pdh\) tree, the taxa of the \(Cerasus\) group did not form a monophyletic group, with each individual of \(Prunus\) \(emarginata\) showing at least two copies (Fig 6).

In most angiosperms, the plastid genome is maternally inherited while the nuclear genome is biparentally inherited [43]. Therefore, the maternal and paternal parent(s) that contributed to the hybrid origin of the racemose group may be inferred by comparing the results of phylogenetic analyses of the plastid DNA [4] with those from the nuclear \(At103\), ITS and \(s6pdh\) DNA sequences. Our data support the hypothesis that allopolyploidy was involved in the origin of the racemose lineages of \(Prunus\), as previously suggested, and further suggest that several independent allopolyploidy events occurred.

The maternal parent(s) of the racemose group must have belonged to an early-diverging lineage of \(Prunus\), as plastid data support three major clades in the genus and resolve the \(Laurocerasus-Padus-Maddenia\) clade (the racemose group) as sister to a clade including the \(Prunus\)-\(Amygdalus\) clade (the solitary flower group) plus the \(Cerasus\) clade (the corymbose groups) [3, 4]. The maternal lineage(s) may have been an extinct widespread species or several species belonging to the same lineage of group C in the \(At103\) gene tree topology.

In contrast, our data suggest that the paternal parents involved in the multiple allopolyploidy events that gave rise to the racemose lineages of \(Prunus\) were more diverged. The \(At103\) phylogeny suggests that some lineages have retained the paternal copy (subgroup C-1, C-2, C-3, C-4), while others have retained the maternal copy (group C in copyII). Collectively, these four subgroups (C-1, C-2, C-3, and C-4) of the racemose group in copy I and the group C in copy II reveal the paternal and maternal ancestral genome donors for the racemose group in \(Prunus\), respectively (Fig 7). Patterns of molecular phylogenetic topologies from the nuclear \(At103\), ITS and \(s6pdh\) and the chloroplast genome and the non-random morphological variations best support the hypothesis of independent events of allopolyploidy in taxa within the racemose group.

In their recent classification of \(Prunus\), Shi et al. [7] proposed that taxa of the racemose group should be treated as only one subgenus \(Padus\). Our hypothesis of independent events of allopolyploidy in taxa within the racemose group argues against recognizing all species of the group as one subgenus (Fig 7). The species with racemose inflorescences may still need to be
treated taxonomically as belonging to several subgenera based on both morphology and the nuclear sequence data.

The time of the first formation of the racemose group was estimated to be 55.4 (45.1–66.3) Myr [4]. Divergence times for subgroups C-1, C-2, C-3, and C-4 of the racemose group were estimated from 37.2 to 14.9 Myr, at different times [4]. Thus the multiple hybridization events may have happened at different times. Furthermore, these multiple allopolyploidy events may have also occurred in different regions, e.g., temperate zone for subgroup C-1 and C-2; the European and subtropical Asian region for subgroup C-3, and Southeast Asian, African tropics and Australia tropics for subgroup C-4. However, the events may have happened so long ago that the diploid ancestral taxa have become extinct, and no extant diploid representatives of the racemose group are known.

The Maddenia group was previously shown to be closely related to subgenera Laurocerasus and/or Padus by phylogenetic studies [3–5, 13, 15]. The At103 gene sequences showed that Maddenia was nested within a subgroup composed of some members of Padus (I) (Prunus padus and P. wilsonii) in copy I, with other species of Padus (II) constituting another subgroup (Fig 5), which is consistent with the phylogenetic results based on sequences of ITS, ndhF, rps16 and rpl16 [11]. The combined At103-ITS sequences also showed that Maddenia was nested within subgroups Padus and Laurocerasus.

Members of the Pygeum group have a perianth without differentiated petals [17]. This group has been shown to be nested within the Laurocerasus-Padus complex based on nuclear and plastid sequences [3, 11]. The At103 neighbornet diagram and the combined At103-ITS data both suggest that species of Pygeum formed a group; however, the phylogenetic position of the African species Prunus africana (also formerly classified in Pygeum) still remains controversial (Figs 2–5 and S1 Fig). Prunus africana possesses some unique characters, such as leaves with incised margins and the glands situated in the margin, but distinct from other taxa of Pygeum. Its position needs to be explored further in future analyses.

Fig 7. Hypothesized evolutionary history of Prunus, highlighting independent allopolyploidy events in subgroup Padus I-Maddenia (C-1C-1CC), Padus II (C-2C-2CC), Laurocerasus (C-3C-3CC), and Pygeum (C-4C-4CC). Photographs (top to bottom): Prunus mume; P. yedoensis; P. laurocerasus.

A = solitary flower group; B = corymbose inflorescence group; C = racemose inflorescence group.

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Allopolyploidy in *Prunus* resulting from the fertilization of unreduced female gametes has been reported between diploid and tetraploid species with the evidence for gametophytic apospory in the genus [44]. Future work on the genus needs to investigate this aspect of *Prunus* reproductive biology to gain insights into the mechanisms of allopolyploidy.

*Prunus emarginata* has been treated as a member of subgenus *Cerasus* [2]. The *s6pdh* sequence data suggest a highly complex pattern in the species (Fig 6). Our sequences were from two individuals (specimen vouchers: EB139 and DPRU 2214). Each individual has at least two copies of the *s6pdh* gene, suggesting that hybridization may have been involved in the origin of the species. Individuals of *P. emarginata* vary considerably in the habit, size and shapes of leaves and inflorescences. Its inflorescence is intermediate between that of the *Cerasus* group and the *Padus* group. The *s6pdh* sequences also place it either with the *Prunus-Amgygdalus-Cerasus* group or with the racemose group. Fertile hybrids between *P. emarginata* and naturalized *P. avium* [45], *P. emarginata* and *P. pensylvanica* [46] have been reported. Clearly our *s6pdh* data support a highly complex genetic profile of these species involving reticulate evolution. Unfortunately, the chromosome number of the species is unknown and should be studied.

In conclusion, the hypothesis of multiple events of allopolyploidy in the evolution of the racemose lineages in *Prunus* is supported by our combined evidence from nuclear and plastid markers. A widespread early diverged lineage of *Prunus* is suggested to have served as the maternal parent(s) for multiple allopolyploidy events involving several paternal lineages. This hypothesis of the evolutionary history of the racemose group in *Prunus* reflects a major step forward in our understanding of *Prunus* diversification. Further analyses using more nuclear DNA sequences via next-generation sequencing [47, 48] are needed to produce a robust nuclear phylogeny for the interpretation of the evolutionary diversification of this economically important genus.

**Supporting Information**

S1 Fig. NeighborNet diagram based on uncorrected P distances of nuclear *At103* DNA sequences of *Prunus*. The dash lines indicate the discrimination of two potential copies of *At103* gene. The solid lines indicate seven major lineages of *Prunus*. The red and black numbers indicate the species and bootstrap support values, respectively. Each species is designated with a number as follows: 1. *P. armeniaca*; 2. *P. divaricata*; 3. *P. glandulosa*; 4. *P. mandshurica*; 5. *P. mume*; 6. *P. salicina*; 7. *P. sibirica*; 8. *P. murrayana*; 9. *P. rivularis*; 10. *P. nigra*; 11. *P. miera*; 12. *P. persica*; 13. *P. triloba*; 14. *P. tenella*; 15. *P. tomentosa*; 16. *P. trichostoma*; 17. *P. serrula*; 18. *P. dielsiana*; 19. *P. cerasoides*; 20. *P. trichostoma*; 21. *P. campanulata*; 22. *P. clarafoila*; 23. *P. discoidea*; 24. *P. maackii*; 25. *P. mahaleb*; 26. *P. nipponica*; 27. *P. subhirtella*; 28. *P. takesimensis*; 29. *P. yedoensis*; 30. *P. padus*; 31. *P. wilsonii*; 32. *P. himalayana*; 33. *P. hypoleuca*; 34. *P. alabamen- sis*; 35. *P. napaulensis*; 36. *P. wallchii*; 37. *P. laurocerasus*; 38. *P. africana*; 39. *P. arborea*; 40. *P. costata*; 41. *P. grisea*; 42. *P. pullei*; 43. *P. buergeriana*; 44. *P. integrifolia*; 45. *P. fordiana*; 46. *P. lancilimba*; 47. *P. tucumanensis*; 48. *Physocarpus opulifolius*; 49. *Prinsepia utilis*. (TIF)

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Author Contributions
Conceived and designed the experiments: JW. Performed the experiments: LZ XWJ YJZ RH DP JW. Analyzed the data: LZ XWJ YJZ RH DP JW. Contributed reagents/materials/analysis tools: LZ XWJ YJZ RH DP JW. Wrote the paper: LZ XWJ YJZ XLL SWC RH DP ZYC JW.

References
1. Potter D, Eriksson T, Evans RC, Oh S, Smedmark JEE, Morgan DR, et al. (2007) Phylogeny and classification of Rosaceae. Plant Syst Evol 266: 5–43.
2. Rehder A (1940) Manual of cultivated trees and shrubs hardy in North America exclusive of the subtropical and warmer temperate regions, 2nd ed. New York: MacMillan.
3. Wen J, Berggren ST, Lee CH, Ickert-Bond S, Yi TS, Yoo KO, et al. (2008) Phylogenetic inferences in Prunus (Rosaceae) using chloroplast ndhF and ribosomal ITS sequences. J Syst Evol 46: 322–332.
4. Chin SW, Shaw J, Haberle R, Wen J, Potter D (2014) Diversification of almonds, peaches, plums and cherries—molecular systematics and biogeographic history of Prunus (Rosaceae). Mol Phylogenet Evol 76: 34–48. doi:10.1016/j.ympev.2014.02.024 PMID: 24631854
5. Lee S, Wen J (2001) A phylogenetic analysis of Prunus and the Amygdaloideae (Rosaceae) based on ITS sequences of nuclear ribosomal DNA. Am J Bot 88: 150–160. PMID:11159135
6. Chin SW, Lutz S, Wen J, Potter D (2013) The bitter and the sweet: inference of homology and evolution of leaf glands in Prunus (Rosaceae) through anatomy, micromorphology, and ancestral character state reconstruction. Int J Plant Sci 174: 27–46.
7. Shi S, Li JL, Sun JH, Yu J, Zhou SL (2013) Phylogeny and classification of Prunus sensu lato (Rosaceae). J Integr Plant Biol 55: 1069–1079. doi:10.1111/jipb.12095 PMID: 23945216
8. Yü TT, Lu LT, Ku TC, Li CL, Chen SX (1986) Rosaceae (3), Prunoideae. In: Yü TT, Lu LT, Ku TC, Li CL, Chen SX, editors. Flora Reipublicae Popularis Sinicae. Beijing: Science Press. Volume 38: 1–133.
9. Lu LL, Gu CZ, Li CL, Alexander C, Batholomew B, Brach AR, et al. (2003) Rosaceae. In: Wu ZY, Raven PH, Hong DY, editors. Flora of China. vol 9. Beijing: Science Press, St. Louis: Missouri Botanical Garden Press. Volume 9: 46–434.
10. Bortiri ES, Oh H, Jiang JG, Baggett S, Granger A, Weeks C, et al. (2001) Phylogeny and systematics of Prunus (Rosaceae) as determined by sequence analysis of ITS and the chloroplast trnL-trnF spacer DNA. Syst Bot 26: 797–807.
11. Liu XL, Wen J, Nie ZL, Johnson G, Liang ZS, Chang ZY (2013) Polyphyly of the Padus group of Prunus (Rosaceae) and the evolution of biogeographic disjunctions between eastern Asia and eastern North America. J Plant Res 126: 351–361. doi:10.1007/s10265-012-0535-1 PMID: 23239308
12. Bortiri ES, Oh H, Gao FY, Potter D (2002) The phylogenetic utility of nucleotide sequences of sorbitol 6-phosphate dehydrogenase in Prunus (Rosaceae). Am J Bot 89: 1697–1708. doi:10.3732/ajb.89.10.1697 PMID: 21665596
13. Bortiri E, Vanden Heuvel B, Potter D (2006) Phylogenetic analysis of morphology in Prunus reveals extensive homoplasy. Plant Syst Evol 259: 53–71.
14. Shaw J, Small RL (2005) Chloroplast DNA phylogeny and phylogeography of the North American plums (Prunus subgenus Prunus section Prunocerasus, Rosaceae). Am J Bot 92: 2011–2030. doi: 10.3732/ajb.92.12.2011 PMID: 21646120
15. Chin SW, Wen J, Johnson G, Potter D (2010) Merging Maddenia with the morphologically diverse Prunus (Rosaceae). Bot J Linn Soc 164: 236–245.
16. Kalkman C (1965) The Old World species of Prunus subgenus Laurocerasus including those formerly referred to Pygeum. Blumea 13: 1–115.
17. Wen J, Shi WT (2012) Revision of the Maddenia clade of Prunus (Rosaceae). Phytokeys 11: 39–59. doi:10.3897/phytokeys.11.2825 PMID: 22577333
18. Watkins R (1976) Cherry, plum, peach, apricot and almond. Prunus spp. In: Simmons NW, editor. Evolution of Crop Plants. London: Longman. pp. 242–247.
19. Dickinson TA, Lo E, Talent N (2007) Polyploidy, reproductive biology, and Rosaceae: understanding evolution and making classifications. Plant Syst Evol 266: 59–78.
20. Alvarez I, Wendel JF (2003) Ribosomal ITS sequences and plant phylogenetic inference. Mol Phylogenet Evol 29: 417–434. PMID: 14615184
21. Zimmer EA, Wen J (2012) Using nuclear gene data for plant phylogenetics: progress and prospects. Mol Phylogenet Evol 65: 774–785. doi: 10.1016/j.ympev.2012.07.015 PMID: 22842093
22. Rzeznicka K, Walker CJ, Westergren T, Kannangara CG, von Wettstein D, Merchant S, et al. (2005) Xantha-1 encodes a membrane subunit of the aerobic Mg-protoporphyir IX monomethyl ester cyclase involved in chlorophyll biosynthesis. Proc Natl Acad Sci USA 102: 5886–5891. PMID: 15824317

23. Li M, Wunder J, Bisson G, Scarponi E, Gazzani S, Barbaro E, et al. (2008) Development of COS genes as universally amplifiable markers for phylogenetic reconstructions of closely related plant species. Cladistics 24: 727–745.

24. Yamaki S, Ishikawa K (1986) Roles of four sorbitol related enzymes and invertase in the seasonal alteration of sugar metabolism in apple tissue. J Amer Soc Hort Sci 111: 134–137.

25. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647–1649. doi: 10.1093/bioinformatics/bts199 PMID: 22543367

26. Stanford AM, Harden R, Parks CR (2000). Phylogeny and biogeography of Juglans (Juglandaceae) based on matK and ITS Sequence data. Am J Bot 87: 872–882. PMID: 10860918

27. Rohrer JR, O’Brien MA, Anderson JA (2008) Phylogenetic analysis of North American plums (Prunus sect. Prunocerasus: Rosaceae) based on nuclear LEAFY and s6pdh sequences. J Bot Res Inst Texas 2: 401–414.

28. Edgar RC (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. Nucl Acids Res 32: 1792–1797. PMID: 15034147

29. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Syst Biol 59: 307–21. doi: 10.1093/sysbio/sys010 PMID: 20525638

30. Posada D. (2008) jModelTest: phylogenetic model averaging. Mol Biol Evol 25: 1253–1256. doi: 10.1093/molbev/msn083 PMID: 18397919

31. Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: More models, new heuristics and parallel computing. Nat Methods 9: 772.

32. Huson DH, Bryant D (2006) Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23: 254–267. PMID: 16221896

33. Müller K (2005) SeqState: primer design and sequence statistics for phylogenetic DNA datasets. Appl Bioinformatics 4: 65–69. PMID: 16000015

34. Simmons MP, Ochoterena H (2000) Gaps as characters in sequence-based phylogenetic analyses. Syst Biol 49: 369–381. PMID: 12118412

35. Vaidya G, Lohman DJ, Meier R (2011) SequenceMatrix: concatenation software for the fast assembly of multi-gene dataset with character set and codon information. Cladistics 27: 171–180.

36. Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, et al. (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61: 539–542. doi: 10.1093/sysbio/sys029 PMID: 22357727

37. Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30: 1312–1313. doi: 10.1093/bioinformatics/btu033 PMID: 24451623

38. Soltis PS, Soltis DE (2009) The role of hybridization in plant speciation. Annu Rev Plant Biol 60: 561–588. doi: 10.1146/annurev.plant.043008.092039 PMID: 19575590

39. Wood TE, Takebayashi N, Barker MS, Mayrose I, Greenspoon PB, Rieseberg LH (2009) The frequency of polyploid speciation in vascular plants. Proc Natl Acad Sci USA 106: 13875–13879. doi: 10.1073/pnas.0811575106 PMID: 19667210

40. Jiao Y, Wickett NJ, Ayyampalayam S, Chandler AS, Landherr L, Ralph PE, et al. (2011) Ancestral polyploidy in seed plants and angiosperms. Nature 473: 97–113. doi: 10.1038/nature09916 PMID: 21478875

41. Triplette JK, Clark LG, Fisher AE, Wen J (2014) Independent allopolyploidization events preceded speciation in the temperate and tropical woody bamboos. New Phytol 204: 66–73. doi: 10.1111/nph.12988 PMID: 25103958

42. Hegarty MJ, Hiscock SJ (2005) Hybrid speciation in plants: new insights from molecular studies. New Phytol 165: 411–423. PMID: 15720652

43. White TL, Adams WT, Neale DB (2007) Forset genetics. Wallingford, UK and Cambridge, MA, USA: CABI Publishing.

44. Tavaud M, Zanetto A, David JL, Laigret F, Dirlewanger E (2004) Genetic relationships between diploid and allotetraploid cherry species (Prunus avium, Prunus x gondoiiunii and Prunus cerasus). Heredity 93: 631–638. PMID: 15354194

45. Jacobson AL, Zika PF (2007) A new hybrid cherry, Prunus x pugetensis (P. avium × emarginata, Rosaceae), from the Pacific northwest. Madrona. 54: 74–85.
46. Hosie RC (1969) Native trees of Canada. Can For Ser Dept Fish For, Queen’s Printer for Canada, Ottawa.

47. Wen J, Liu JQ, Ge S, Xiang QY (J), Zimmer EA (2015) Phylogenomic approaches to deciphering the tree of life. J Syst Evol 53: 369–370.

48. Zimmer EA, Wen J (2015) Using nuclear gene data for plant phylogenetics: Progress and prospects II. Next-gen approaches. J Syst Evol 53: 371–379.