The Role of Allotriploidy in the Evolution of *Meconopsis* (Papaveraceae): A Preliminary Study of Ancient Polyploid and Hybrid Speciation

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Abstract: Also known as the Himalayan Poppy, *Meconopsis* is a genus of herbaceous plants growing only in the high elevation habits of the Himalaya and its adjacent plateau and mountain areas. The genus exhibits high morphological and ecological diversity, but the major causes of divergence in *Meconopsis* have not previously been studied. Our recent revised taxonomic classification, based on a molecular phylogeny, divided the genus into four monophyletic sections. Because chromosome number varies among these sections and our previous phylogenetic analyses revealed extensive incongruence between the recovered nrITS and cpDNA trees, possibly due to ancient hybridization, this study focused on evaluating the potential role of ancient polyploidization and hybridization in *Meconopsis*' evolutionary history. Our investigation based on the results of reconstructed ancestral chromosome numbers using a Maximum Likelihood method implemented in chromEvol showed that two extant *Meconopsis* sections (sect. *Grandes* and sect. *Primulinae*) shared a triploid ancestor. We further examined the pattern of hybridization in *Meconopsis* by reconstructing a nuclear marker *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase gene) network. The result, along with morphological, phylogenetic, and cytological evidence, all point to a hybrid nature of the triploid ancestor. Based on the resultant *GAPDH* network, an ancient reticulate evolution scenario in *Meconopsis* is proposed. Overall, this preliminary study shows how an ancient triploid event promoted polyploid evolution in *Meconopsis* and also exemplifies how allotriploidization and successive polyploidization played an important role in diversification of the genus.

Keywords: Himalaya, polyploidization, ancestral chromosome number, hybridization, low-copy marker, speciation mechanism.

Meconopsis is a genus distributed across the high elevation of the Himalaya, the southeast Tibetan Plateau, and the Hengduan Mountains. The genus contains ca. 50 species, and is among the most popular and desirable horticultural groups in British gardens. Although there have been efforts to cultivate most *Meconopsis* species, only a minority of them are able to be grown successfully in gardens. In addition, there has also been extensive crossbreeding for new cultivars (Cobb, 1984). Our phylogenetic study (Xiao, 2013) provided a well-resolved chloroplast (cpDNA) phylogeny for the genus, which led to the recognition and recircumscription of four sections (Fig. 1). Although there has been substantial work on morphology, phylogenetics, and cytology, there is still no coherent framework that synthesizes all the present knowledge into a comprehensive evolutionary explanation of *Meconopsis* diversity. Because various high polyploid levels exist in *Meconopsis* (top right Fig. 1), polyploidization may have had an important impact on the evolution of the genus. A recent report documented a synthesized *Meconopsis* polyploidy (neopolyploid) in Scotland (McNaughton, 2014) which further highlighted the potential role of polyploid evolution. Our interest, therefore, is to understand how different ploidy levels evolved in *Meconopsis* and to decipher if there has been polyploid speciation in the genus. Unlike neopolyploids, however, since ancient polyploidy formation cannot be experimentally re-created, we took the

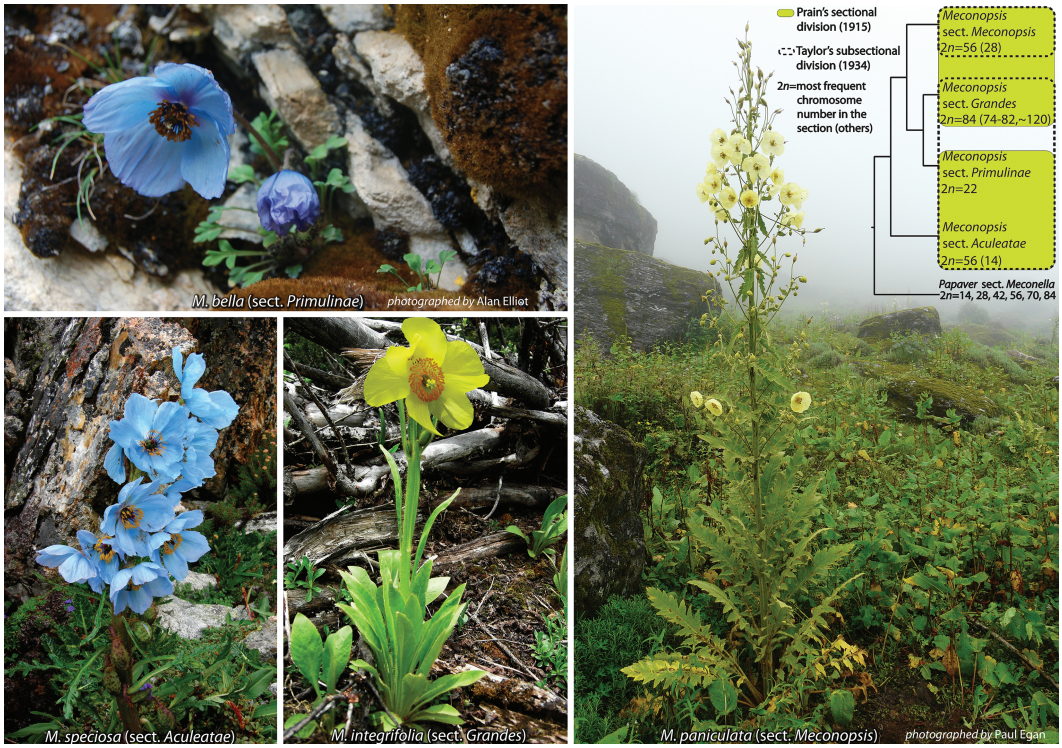

FIG. 1. Overviews of *Meconopsis* sections. Chloroplast phylogeny is shown on top right (Xiao, 2013), along with the traditional taxonomic classifications from Prain (1915) and Taylor (1934).

approach of reconstructing ancestral chromosome numbers on a molecular phylogenetic tree.

The second aim of this study was to investigate if ancient hybridization significantly contributed to the early divergence of *Meconopsis*. In our revised classification (Xiao, 2013), the four sections of *Meconopsis* are monophyletic with each exhibiting consistency in morphology and known chromosome numbers. However, the evolutionary relationships between sections indicated by the cpDNA tree largely conflicted with those inferred from previous morphology-based taxonomic classifications (Prain, 1915; Taylor, 1934). A brief overview of how *Meconopsis* was previously treated based on morphology is shown in Fig. 1 (top right). Prain's (1915) sections were based on indumentum variations while Taylor's (1934) subsectional treatment was based on whether the leaf rosette is persistent through winter or not. Comparing these two treatments, it is clear that *M.* sect. *Grandes* is the "controversial" group in the genus. The *Grandes* clade was grouped with sect. *Meconopsis* in Prain's (1915) work, but with *M.* sect. *Aculeatae* and *M.* sect. *Primulinae* species in Taylor's (1934) treatment. It thus appears that morphological affinity of sect. *Grandes* to neither *M.* sect. *Meconopsis* nor *M.* sect. *Aculeatae* is strongly supported. On the other hand, species in *M.* sect. *Primulinae* had always previously been grouped or mixed together with *M.* sect. *Aculeatae* species taxonomically, an arrangement clearly contradicted by our cpDNA phylogenetic topology. Our phylogenetic study (Xiao, 2013) also revealed incongruence between the nrITS and the cpDNA trees at deep nodes, suggesting that ancient hybridizations may have occurred. These morphological and molecular data led us to the question whether ancient hybridizations were involved in the origin of *M.* sect.

Primulinae and *M.* sect. *Grandes*. In order to explore this possibility, we constructed a phylogeny using low-copy nuclear genes with no recombination, or a low recombination rate, to recover the phylogenetic signals from both the parents if reticulation occurred.

Material and Methods

Reconstructing polyploid evolution and ancestral chromosome numbers

Phylogeny reconstruction – We firstly calculated a cpDNA tree to serve as the phylogenetic framework for ancestral chromosome number reconstruction. Because *Meconopsis* is closely related to *Papaver* and *Roemeria* (Xiao, 1913), we included *Papaver* and *Roemeria* species with *Cathcartia* as outgroups, in order to achieve an estimation of rates of dysploidy formation and/or genome duplication rates. This phylogenetic reconstruction employed the concatenated sequences (*trn*L-*trn*F, *mat*K, *ndh*F and *rbc*L) of *Meconopsis* and *Cathcartia* species, obtained from our phylogenetic study of *Meconopsis* (Xiao, 2013) (sequence information in Appendix 1); and 27 previously published *trn*L-*trn*F sequences downloaded from Genbank (sequence information is listed in Appendix 2), mainly including *Papaver* and *Roemeria* species. Sequences were assembled in Geneious 5.5 (Biomatters, New Zealand), and aligned by Geneious Alignment with the default settings. Absent markers were treated as missing characters. Alignments were then refined manually. Bayesian partition analysis was applied using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001), with each marker treated as a partition. The evolutionary models were selected using jModelTest (Posada, 2008). We applied the models most similar to the best-fit models estimated by jModelTest that were available in MrBayes v3.1.2 for each partition: GTR+G for *rbc*L, GTR+I+G for *ndh*F, GTR+G for *mat*K, and GTR+I+G for *trn*L-*trn*F dataset. Prior probability distributions on all parameters were set to the defaults. Stationarity was reached at twenty million generations using a Markov chain Monte Carlo (MCMC) method. Trees were collected every 100th generation. With 25% burn in, a 50% majority-rule consensus tree was calculated.

Previous cytological studies (Ratter, 1968; Kadereit, 1987; Lavania & Srivastava, 1999; Ying et al., 2006; Kumar et al., 2013) have provided chromosome numbers for many species. For estimating ancestral characters, parsimony based methods have been widely adopted but parsimony approaches usually suffer from strong bias due to their inability to incorporate multiple models and to deal with uncertainty. Mayrose et al. (2010) developed a likelihood method implemented in chromEvol, which estimates the probability of a given ploidy level at any internal node in a given tree. In chromEvol, eight different models are available for testing, each representing a different hypothesis by estimating a different set of parameters (Table 1). For example, the model CONST_RATE_DEMI is characterized by parameters of a constant rate for gaining a single chromosome (gainCONSTR), a constant rate for losing a single chromosome (lossCONSTR), and a constant rate for both whole genome duplication (duplConstR) and demi-polyploidization (demiPloidyR). This model thus hypothesizes that dysploidy, whole genome duplication, and demi-polyploidization (change from $2n$ to $3n$, e.g., forming a triploid) all have occurred along a phylogeny at a constant rate. In contrast to the CONST_RATE_DEMI model, some other models do not allow whole genome duplication or demi-polyploidization. Some of these estimate linear rates (instead of constant rates) to implement the hypothesis that chromosome number change rate is dependent on the current chromosome number. Four of these models were also used. Detailed model comparisons can be retrieved from http://www.tau.ac.il/~itaymay/cp/chromEvol/index.html. All the eight models were run to select for the best-fit model. The selected model was used to estimate ancestral chromosome numbers.

TABLE 1. Model test result in chromEvol (sorted by AIC score).

Models	*Model parameters	Log-likelihood	AIC
CONST_RATE_DEMI	1, 2, 5(=6)	−148.2	302.4
CONST_RATE_DEMI_EST	1, 2, 5, 6	−147.2	302.4
LINEAR_RATE_DEMI_EST	1, 2, 3, 4, 5, 6	−147.7	307.4
LINEAR_RATE_DEMI	1, 2, 3, 4, 5(=6)	−148.9	307.8
CONST_RATE	1, 3, 5	−174.9	355.8
LINEAR_RATE	1, 2, 3, 4, 5	−176.0	362.0
CONST_RATE_NO_DUPL	1, 2	−303.9	611.8
LINEAR_RATE_NO_DUPL	1, 2, 3, 4	−305.4	618.8

* 1: gainConstR, 2: gainLinearR, 3: lossConstR, 4: lossLinearR, 5: duplConstR, 6: demiPloidyR

Reconstructing a *GAPDH* phylogenetic network to detect ancient reticulation

Amplification, PCR, cloning and sequencing – We screened the EST library of *Papaver somniferum* and related species to choose genes with a single or low-copy number. Among the candidate genes, *GAPDH* (partial) was selected for its relatively low copy number (we obtained only one copy of the *GAPDH* gene for the accession of *Papaver alpinum*, a diploid taxon), successful PCR amplification, and the possession of four introns (five exons) that are phylogenetically informative. Genomic DNA was extracted from silica-dried leaf materials or herbarium materials using the DNeasy Plant Minikit (Qiagen, Valencia, California, USA). Primers were designed with the reference to the EST sequences of *Papaver somiferum* and other species in Papaveraceae using the EST database from GenBank. The primer pair, forward primer 5′-CACCACCAACTGTCT-TGCTCCCCT-3′ and reverse primer 5′-AGCACCCACACTGAAGAGGGAC-3′, were selected. PCR amplification conditions were optimized and carried out in 25 μL reaction volumes with 20–40 ng DNA, 1.0 unit of Taq polymerase (made by the author), 0.5X Failsafe Buffer B (Epicentre Biotechnologies, Madison, WI, USA), and 2.0 μmol/L primers. Eighteen PCR cycles were performed at 95° C for 30 seconds, 59°C for 40 seconds, and 72° C for 45 seconds for each cycle; followed by 26 cycles of 95° C for 30 seconds, 55°C for 40 seconds, and 72° C for 45 seconds. PCR products were visualized on agarose gel containing Syber Safe DNA gel stain (Invitrogen, Eugene, Oregon, USA). Successfully amplified products were cloned using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA). Thirty to 60 colonies were picked from each plate and amplified by M13 plasmid primers from the cloning kit with the manufacturers' protocols. Successfully amplified products were cleaned using Exo-Sap (Exonuclease I: New England Biolabs Beverly, MA, USA; Shrimp Alkaline Phosphatase: Progema, Madison, WI, USA) with the manufacturers' protocols. Cleaned PCR products were sequenced on an ABI 3730 DNA Analyzer at the Institute for Cell and Molecular Biology Core Facility at The University of Texas at Austin. *GAPDH* sequence information is listed in Appendix 3.

Test for recombinant sequences – A potential difficulty of using nuclear markers is that if *Meconopsis* were a highly polyploid group, multiple copies of the target gene would be amplified simultaneously. Under these circumstances, artificial recombinants could be introduced during PCR when multiple gene copies coexist. We applied an assumption when screening out PCR-mediated recombination that recombinants can only occur once in the raw sequence data but sequences of true genes can reoccur. This assumption is based on the mechanism of PCR-mediated recombination that interruption of complete extension (for example,

Taq polymerase can stop extension when mismatching occurs) during PCR produces an incomplete sequence, which anneals to its homologous gene strand and continues to elongate. We consider these ''interruptions of extension'' as random events which are unlikely to be repeated. We further tested the accuracy of our method by amplifying and examining fragments (< 600 bp) of the *GAPDH* genes, using our designed internal primers (F1_5′-TATTTTCAATCATTTGTTTC-3′, R1_5′-AATCATTGCAT CCGAGAACAA-3′; F2_5′-AACAGTTTAGTTGCCAATTCG-3′, R2_5′-CTCAATAC TGAAAATTTTG CTAG-3′). We applied this test to all of the *Meconopsis* species in this study and all of results confirmed that our method performed accurately.

After excluding PCR recombinants, we used the program Recombination Detection Program (Version RDP2) (Martin et al., 2005; available from http://darwin.uvigo.es/rdp/rdp.html) to exam natural recombination. We used the analysis algorithms RDP, GENECONV, Bootscan/Recscan, MaxChi, Chimaera, SiScan, and 3seq, which are implemented in RDP2. Detected natural recombinants were eliminated from further analysis.

Alignment and phylogenetic analysis – Alignments were performed using Geneious (Biomatters, New Zealand) Alignment 5.5 with the default settings, and then refined manually. The *GAPDH* sequences that lost multiple introns were eliminated from the final alignment. Phylogenetic analysis was carried out by RAxML 7.2.8 (Stamatakis, 2006; Stamatakis et al., 2008) with 1000 bootstrap replications.

Rooting of the tree – We obtained only one copy of *GAPDH* gene from the outgroup species *Papaver alpinum* which belongs to *Papaver* section *Meconella*, the sister group to *Meconopsis*. However, this sequence was difficult to align with *Meconopsis* sequences due to a great deal of sequence divergence and phylogenetic analyses using tentative alignments including this outgroup sequence could not resolve the relationship between the major clades with certainty (bootstrap value < 60 for the node directly shared by the outgroup and some *Meconopsis* species in unrooted trees). Therefore, we chose to use mid-point rooting.

RESULTS

Reconstructing polyploid evolution and ancestral chromosome number

The reconstructed cpDNA Bayesian tree of *Meconopsis*, *Roemeria*, and *Papaver*, rooted by *Cathcartia,* is shown in Fig. 2A, which is consistent with the *Papaver* phylogeny published by Carolan et al. (2006). The partial tree that contains only *Meconopsis* species is expanded in Fig. 2B.

Of the eight models implemented in chromEvol, the four that allowed DEMI (i.e., allowing chromosome number transition from $2n$ to $3n$) all resulted in significantly higher likelihood scores than the other four models without DEMI setting (Table 1). The four DEMI models all generated the same ancestral chromosome numbers, which are shown in Fig. 2A and Fig. 2B. In Fig. 2A, the ancestral chromosome number of the genus *Meconopsis* is $2n=14$ (posterior probability 0.99). The ancestral chromosome numbers for each section, as shown in Fig. 2B, are $2n=21$ for *M.* sect. *Grandes*, $2n=22$ for *M.* sect. *Primulinae*, $2n=28$ for *M.* sect. *Meconopsis*, $2n=14$ for *M.* sect. *Aculeatae*. The chromosome number of the most recent common ancestor shared by *M.* sect. *Grandes* and *M.* sect. *Primulinae* was estimated to be $2n=21$.

Reconstructing *GAPDH* phylogenetic network

We successfully obtained partial *GAPDH* sequences from 21 *Meconopsis* accessions and one outgroup accession of *Papaver alpinum*. Because *M.* sect. *Primulinae* species are distributed narrowly and endemically, there were few good quality samples for successful PCR amplification. Thus, *M.* sect. *Primulinae* is less well

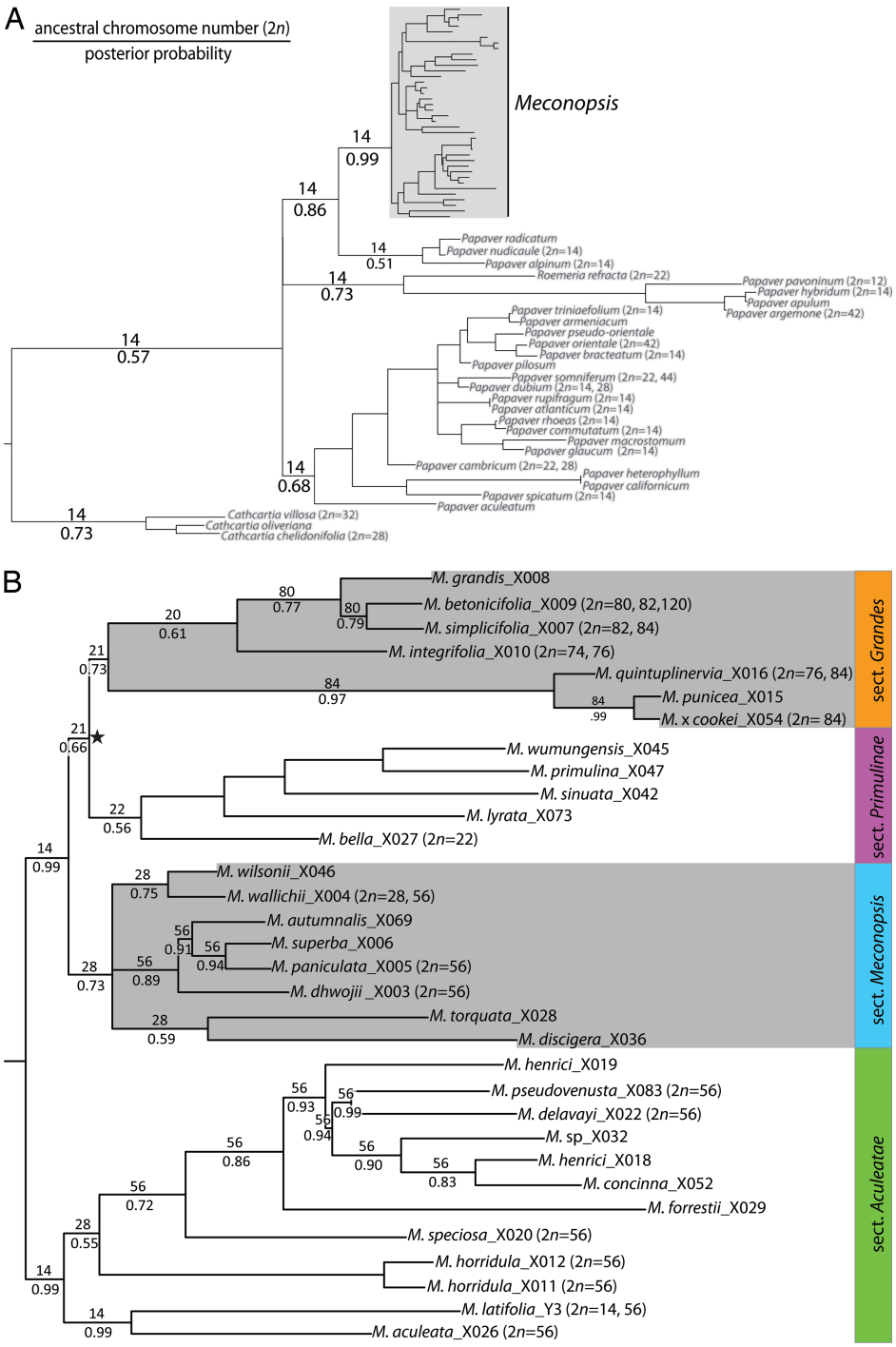

FIG. 2A. The cpDNA Bayesian trees of *Meconopsis*, *Roemeriana*, and *Papaver* rooted using *Cathcartia* with chromosome numbers of extant species at the branch tips. Ancestral chromosome numbers reconstructed using chromEvol are shown with the most probable chromosome number ($2n$ with p value > 0.50) labeled above the tree branches and the posterior probabilities below the branches.

represented in the *GAPDH* network than other sections. On average, 44% of the raw sequences from one PCR reaction were identified as PCR-mediated recombinants. One natural recombination in the sample X022 was detected by program RDP2. After the removal of the recombined sequences, we obtained an average of 2.1 copies of *GAPDH* gene per accession (1.3 in *M.* sect. *Meconopsis;* 2.3 in *M.* sect. *Grandes;* 2.5 in *M.* sect. *Aculeatae;* 2.0 in *M.* sect. *Primulinae*) to reconstruct the network. The average sequence length was 1403 bp with 885 variable sites and an intron/exon ratio of 3.4/1. The ML tree with best likelihood score is shown in Fig. 3 with the bootstrap value labeled above the branch when greater than 50. Three well supported major clades (labeled Clade 1, 2, and 3) are indicated for the ease of discussion (Fig. 3) and each is highlighted by a uniquely patterned branch (left in Fig. 3). Clade 1 comprises only species in *M.* sect. *Aculeatae*; Clade 2 contains species of *M.* sect. *Meconopsis, M.* sect. *Grandes,* and *M.* sect *Primulinae*; and Clade 3 includes sequences from *M.* sect. *Aculeatae*, *M.* sect. *Grandes* and *M.* sect. *Primulinae*. The network shows that *GAPDH* sequences in each *M.* sect. *Aculeatae*, *M.* sect. *Grandes*, and *M.* sect. *Primulinae* have multiple origins. We consequently transformed Fig. 3 to the simplified reticulate structure shown in Fig. 4 in order to illustrate our proposed scenario of ancient reticulate evolution in *Meconopsis.* The patterned branches in Fig. 4 correspond to those in Fig. 3.

DISCUSSION

Hybridization and Allotripolyploid evolution in *Meconopsis*

According to the ancestral chromosome reconstruction (Fig. 2B), there was an ancestor, with $2n=21$ (marked by the ★ in Fig. 2B) that gave rise to two extant ploidy groups in *Meconopsis*, *M.* sect. *Grandes* and *M.* sect. *Primulinae.* In the phylogenetic network using the low-copy nuclear marker *GAPDH* (Fig. 3 in which each *Meconopsis* section is indicated by a different color) the *GAPDH* sequences are clustered into three groups labeled Clades 1, 2, and 3. It is obvious that sequences of *M.* sect. *Grandes* and *M.* sect. *Primulinae* have multiple origins, located in both Clade 2 and Clade 3 on the *GAPDH* network (Fig. 3), which strongly disagrees with the cpDNA tree topology where each section is monophyletic (Fig. 2B). One explanation for this pattern of disagreement is that *M.* sect. *Grandes* and *M.* sect. *Primulinae* had hybrid origin(s). Under this hypothesis, Clade 2, similar to the cpDNA tree (Fig. 2B), could contain the maternal lineage and Clade 3 could represent the paternal lineage for both *M.* sect. *Primulinae* and *M.* sect. *Grandes* (Fig. 3). In addition, *M.* sect. *Primulinae* is morphologically most similar to *M.* sect. *Aculeatae* according to traditional classifications of *Meconopsis* (Prain, 1915; Taylor, 1934) but it is most distant from *M.* sect. *Aculeatae* on the cpDNA tree. A hybrid origin could also explain this morphological divergence pattern as hybrid offspring can sometimes predominantly display the traits of one of its parents.

Given our finding that *Meconopsis* sect. *Primulinae* and *M.* sect. *Grandes* shared a common ancestor of $2n=21$ (★ in Fig. 2B), the formation of this triploid ancestor was probably the result of a hybridization event, which fits the phylogenetic pattern displayed in Fig. 3. Alternatively, it is possible to hypothesize that two or more independent ancient hybridizations in *M.* sect. *Primulinae* and *M.* sect. *Grandes* respectively could also

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FIG. 2B. Reconstructed ancestral chromosome numbers for *Meconopsis* are shown above the branches and their posterior probabilities below the branches. Known chromosome numbers of extant species are shown at branch tips. Each section is labeled with a different color, corresponding to Fig. 3.

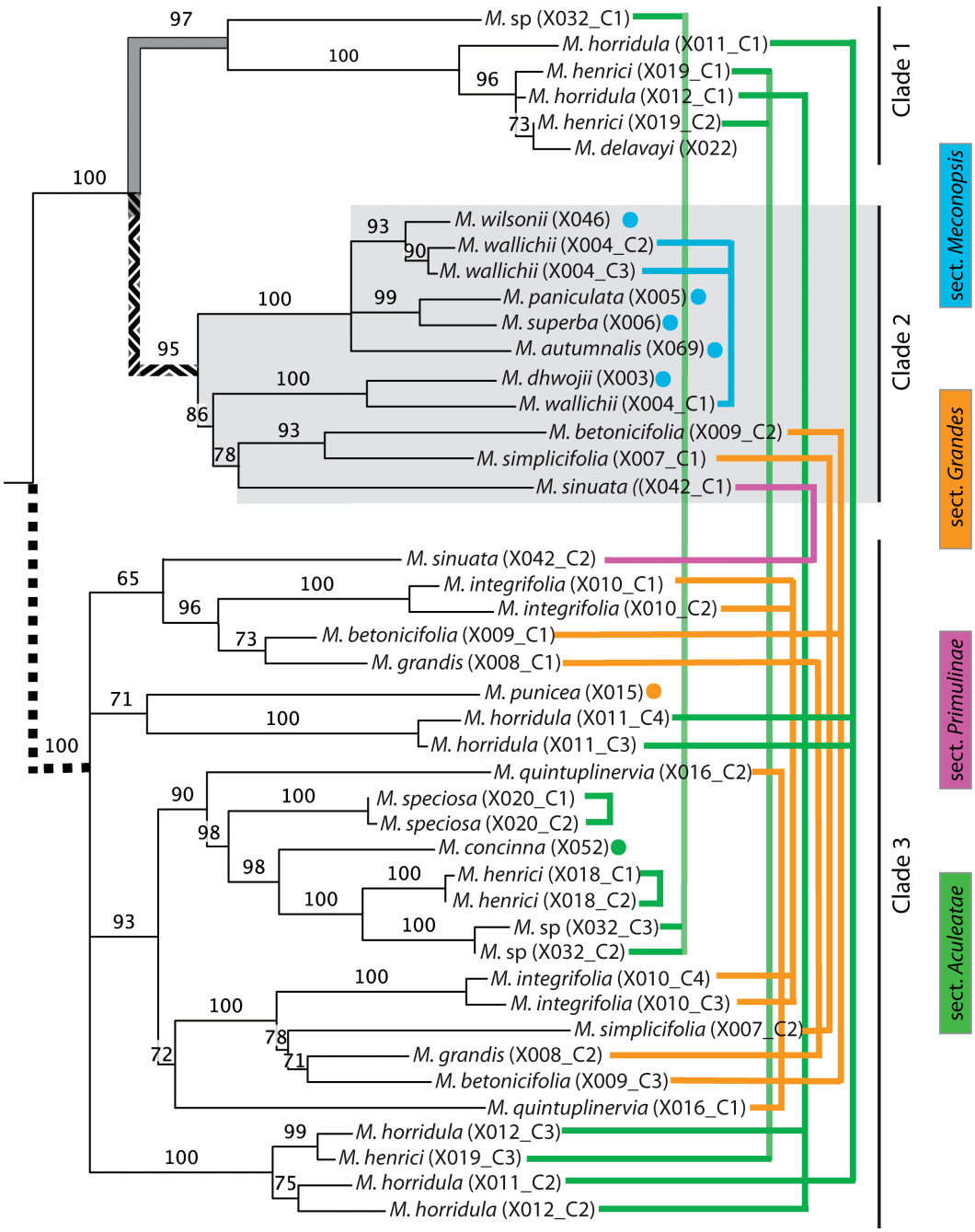

FIG. 3. *GAPDH* phylogenetic network reconstruction in *Meconopsis*. Bootstrap value labeled above the branch when greater than 50. The three different patterns on the branches (left side) that lead to each of the clade (Clades 1-3) are used to symbolize the sequence origin for each clade and also corresponding to those in Fig. 4. Four colors indicate four sections: each of the colored lines (on the right) connects different copies of the same accession, revealing hybridization pattern; colored dots indicate the accessions with only one obtained *GAPDH* sequence.

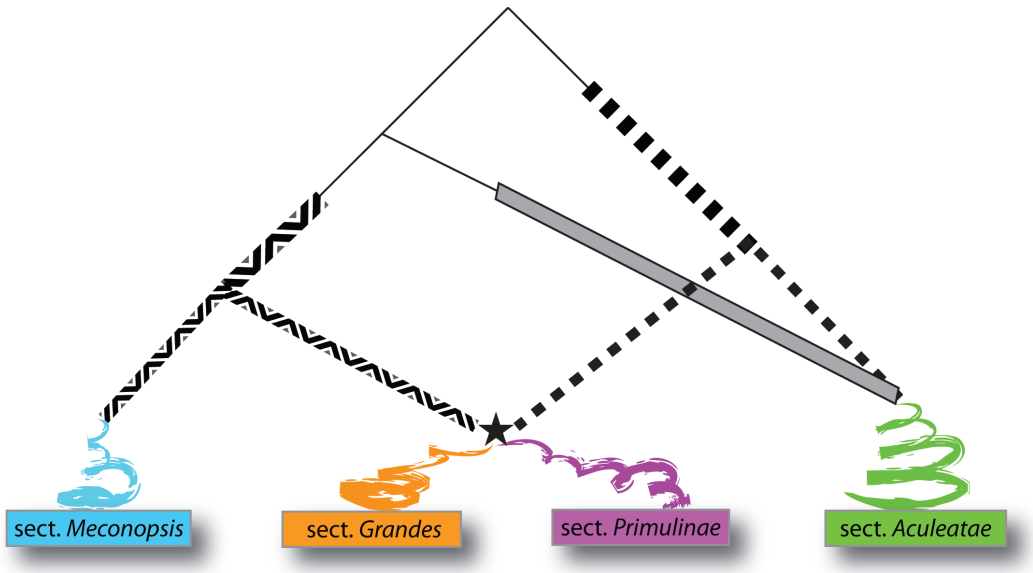

FIG. 4. Summary of ancient reticulate hypotheses in *Meconopsis*. (Note: this graph was transformed from Fig. 3. For example, sect. *Aculeatae* sequences have multiple origins from both Clades 1 & 3, represented by solid grey-patterned branch and dotted-line branch, respectively, in both Figs. 3 & 4).

account for the network structure shown in Fig. 3. However, because, as shown in Fig. 2B, the ancestral chromosome numbers in *M.* sect. *Meconopsis* and *M.* sect. *Aculeatae* are ($2n$) 14, 28 or 56, we think hybridization between these cytological types with any of the deviants from a triploid ($2n=21$) is unlikely.

It is also possible that the structure of *GAPDH* network could also be explained by gene duplication and gene loss. However, we considered this a less likely scenario because it would have to require multiple gene-loss events in the early history of the genus to result in the pattern of the observed *GAPDH* network. Also, the hybridization hypothesis fits the incongruence between nrITS tree and cpDNA tree observed from our phylogenetic study of *Meconopsis* (Xiao, 2013). However, this alternative hypothesis should be tested further in the future.

We therefore reiterate that the most likely scenario is that *Meconopsis* sect. *Grandes* and *M.* sect. *Primulinae* shared a triploid ancestor of $2n=21$ with a $2n=14$ maternal parent (evident when tracing back along the cpDNA phylogeny in Fig. 2B). This triploid ancestor (★ in Fig. 2B) appeared to be transient and later established stable lineages (with even-numbered chromosomes) through different putative pathways as follows:

$$2n=21\rightarrow(21+1)\rightarrow 2n=22\ (\textit{M. bella})$$

$$2n=21\rightarrow(21\times 4,\text{ or }21\times 2\times 2)\rightarrow 2n=84\ (\textit{M. punicea}\ \&\ \textit{M. quintuplinervia})$$

$$2n=21\rightarrow(21-1)\rightarrow 2n=20\rightarrow 2n=\text{various (e.g., }\textit{M. integrifolia}, \textit{M. betonicifolia})$$

Thus our results suggest that the emergence of two *Meconopsis* sections was attributable to a single ancient triploidization event. However, mechanisms of how this triploid hybrid ancestor successfully bypassed the low fertility and reproductive instability associated with aneuploidy and hybrid sterility (Comai, 2005) are unclear. It has been suggested that some reproductive

strategies, e.g., apomixis, or being perennial, can prolong the life cycle and could contribute to higher chances of establishing reproductively stable lineages (Ramsey & Schemske, 1998). Presumably because a polycarpic life cycle (longevity) can increase chances of the occurrence of a mutation that might lead to eventual reproductive success, the polycarpic habit is frequently found associated with allopolyploidy while diploid populations of the same species are predominantly monocarpic (Treier et al., 2009). In *Meconopsis,* the majority of species are strictly monocarpic; polycarpic plants are only found in *Meconopsis* sect. *Primulinae* (i.e., *M. bella*) and *M.* sect. *Grandes* (i.e., *M. betonicifolia, M. grandis*). The long-lived polycarpic habit might be derived from, or related to, an allotriploid origin.

According to McNaughton (2014), fertility was gained over time in *Meconopsis 'Lingholm'*, a cultivar produced by human hybridization between two species with different ploidy levels – apparently followed the sequence of steps given below:

$$M.\ grandis \quad \times \quad M.\ baileyi$$
$$(2n=4x=164)\ \text{(hand crossing)}\ (2n=2x=82)$$
$$\rightarrow\ M.\times sheldonii \ \text{-----}\rightarrow\ M.\ 'Lingholm'$$
$$(2n=3x=123)\quad (38\ \text{years})\quad (2n=6x=246)$$

McNaughton's report (2014) documented the formation of a new hexaploid (*M. 'Lingholm'*) via a sterile triploid (*M.* × *sheldonii*), whose descendant, a few decades later, started to produce viable seeds with double the chromosome numbers of its progenitor. McNaughton (2014) commented that *Meconopsis* triploid cultivars were not uncommon in gardens but that the conversion of a triploid into a fertile hexaploid, as from *M.* × *sheldonii* to *M. 'Lingholm'*, is a unique event, thanks to which, gardeners can now grow their own ''blue heaven'' (*M. 'Lingholm'*). Our results that recovered an ancient triploid viewed in light of the evidence from *M. 'Lingholm'*, suggest that polyploidy was a significant evolutionary force in *Meconopsis* and that polyploidization through triploidy may have occurred more than once through *Meconopsis* history. However, the mechanisms (e.g., somatic doubling, fusion of unreduced gametes) of a triploid's transition to a stable hexaploid or dodecaploid (as in *M.* sect. *Grandes*) a still largely remain hypothetical (Kadereit, 1987; Bretagnolle & Thompson, 1995; Ramsey & Schemske, 1998; Rieseberg & Willis, 2007; McNaughton, 2014).

In another relevant study, Kadereit (1986, 1987) presented the plausibility of a triploid origin in *Papaver somniferum* ($2n=20$, 22, 44). He proposed a triploid origin of *P. somniferum* by providing several different lines of evidence supporting that *P. somniferum* was derived from a triploid hybrid ($2n=21$). A later molecular study (Nessler, 1994) analyzing the *MLP* (major latex proteins) gene family supported an hypothesis of a triploid hybrid origin of the opium poppy. According to Kadereit (1991), the triploid overcame low fertility and stabilized as even number ploidy through regular bivalent formation. He (1987) also suggested that $2n=22$ is an indication of how a triploid ($2n=21$) establish even-numbered ploids in *Papaver* and its closely related genera based on the chromosome number records of a few unrelated species sharing the same aneuploid number $n=11$: *Meconopsis bella* ($2n=22$), *Papaver somniferum* ssp. *somniferum* ($2n=20$, $2n=22$), *Papaver somniferum* ssp. *setigrum* ($2n=44$), *Papaver aculeatum* ($2n=22$), *Roemeria hybrida* ($2n=22$), *Papaver cambricum* ($2n=14$, 22, 28). The high likelihood scores among the different models calculated by chromEvol (Table 1) with the DEMI parameters (allowing the genome transition from $2n$ to $3n$) support Kadereit's (1986, 1987) conclusion, suggesting that these processes are probably not random and that *Meconopsis-Papaver* species are prone to overcome a triploid block and establish stable lineages by dysploidy formation ($2n=21\rightarrow 2n=20/22$).

Autoploidy within *Meconopsis*

In addition to *Meconopsis* sect. *Primulinae* and *M.* sect. *Grandes*, sequences of *M.*

sect. *Aculeatae* also fell into two different clades (Clade 1 and Clade 3, Fig. 3), suggesting that *M.* sect. *Aculeatae* might also have a hybrid history. The reconstructed polyploid evolutionary scenario suggests that *Meconopsis* sect. *Aculeatae* and *M.* sect. *Meconopsis* acquired chromosome levels of $2n=56$ independently, which is strongly supported by the aberrant chromosome number $2n=28$ in *M.* sect. *Meconopsis* and $2n=14$ in *M.* sect. *Aculeatae* (Fig. 2B). This conclusion contradicts a previous assumption, postulated without any available phylogenetic reference at that time, by Ratter (1968) that species in *M.* sect. *Aculeatae* and *M.* sect. *Meconopsis* were derived from a common ancestor with $2n=56$. However, sequences of *M.* sect. *Meconopsis* are only in Clade 2 with no strong divergence as shown in other *Meconopsis* sections, thus, it is possible that the high ploidy level in *M.* sect. *Meconopsis* resulted via autoploidy. For easy visualization, we simplified *GAPDH* network and displayed it in a reticulate network form (Fig. 4), which illustrates and summarizes our hypotheses of reticulate evolution of *Meconopsis*.

In conclusion, we postulate that there were three major pathways early in the evolution of *Meconopsis*: (1) formation of a triploid hybrid that managed to overcome sterility at the base of the clade that gave rise to *M.* sect. *Primulae* and *M.* sect. *Grandes*; (2) a hybrid origin of *M.* sect. *Aculeatae* with successive polyploidizations; (3) a autoploidization origin of *M.* sect. *Meconopsis.* Future research will be needed to test these hypotheses further, perhaps using next generation sequencing technique to generate more nuclear sequence or transcriptome data. Nonetheless, this is the first hypothesis of ancient hybridization and polyploidy underlying the evolution of *Meconopsis*.

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Appendix 1. Voucher and sequence information (Accession number; ***species name***; voucher (herbarium); (Collecting) COUNTRY: Subdivision; GenBank ID for *mat*K, *ndh*F, *trn*L-*trn*F, *rbc*L. ''-'', denotes a missing sequence).

X003; ***Meconopsis dhwojii*** G. Taylor; UK (cultivated); *W. Xiao RICB9* (E); JX087915, JX087815, JX087755, JX087699. X004; ***Meconopsis wallichii*** Hook.; UK (cultivated); *W. Xiao RICB10* (E); JX087895, JX087821, -, JX087711. X005; ***Meconopsis paniculata*** Prain; UK (cultivated); *W. Xiao RICB5* (E); JX087868, JX087830, JX087743, JX087720. X006; ***Meconopsis superba*** King ex Prain; UK (cultivated); *W. Xiao RICB7* (E); JX087858, JX087851, JX087735, JX087683. X007; ***Meconopsis simplicifolia*** (D. Don) Walp.; NEPAL: Bagmati; *Egan 4* (private collection); JX087891, JX087803, JX087751, JX087700. X008; ***Meconopsis grandis*** Prain; UK (cultivated); *W. Xiao RICB6* (E); JX087873, JX087832, -, JX087695. X009; ***Meconopsis betonicifolia*** Franch.; UK (cultivated); *W. Xiao RICB2* (E); JX087871, JX087806, -, JX087716. X010; ***Meconopsis integrifolia*** (Maxim.) Franch.; CHINA: Yunnan; *W. Xiao 080620* (TEX); JX087901, JX087804, -, JX087701. X011; ***Meconopsis horridula*** Hook.f. & Thomson; CHINA: Sichuan; *Boufford 33724* (GH); JX087905, JX087812, JX087770, JX087712. X012; ***Meconopsis horridula*** Hook.f. & Thomson; CHINA: Yunnan; *W. Xiao 080616* (TEX); JX087898, JX087826, -, JX087729. X015; ***Meconopsis punicea*** Maxim.; CHINA: Sichuan; *Boufford 33684* (GH); JX087862, JX087849, -, JX087718. X016; ***Meconopsis quintuplinervia*** Regel; CHINA: Sichuan; *W. Xiao RICB8* (E); JX087865, JX087831, -, JX087706. X018; ***Meconopsis lancifolia*** Franch. ex Prain; CHINA: Yunnan; *W. Xiao 080621-1* (TEX); JX087857, JX087818, JX087750, JX087731. X019; ***Meconopsis henrici*** Bureau & Franch.; CHINA: Sichuan; *W. Xiao 090722-1* (TEX); JX087913, JX087797, JX087739, JX087724. X020; ***Meconopsis speciosa*** Prain; CHINA: Yunnan; *W. Xiao 090703-2* (TEX); JX087920, JX087829, JX087781, JX087682. X022; ***Meconopsis delavayi*** Franch. ex Prain; UK (cultivated); *W. Xiao 090526* (TEX); JX087866, JX087816, JX087736, JX087688. X024; ***Cathcartia oliveriana*** (Franch. ex Prain) W. Xiao; CHINA: Shaanxi; *J.Z. Xiao 1* (TEX); JX087907, JX087791, JX087765, -. X026; ***Meconopsis aculeata*** Royle; UK (cultivated); *C5255* (E); JX087912, JX087820, -, JX087709. X027; ***Meconopsis bella*** Prain; NEPAL: Kone Khola; *McBeath 1496* (E); JX087919, JX087823, -, JX087723. X028; ***Meconopsis torquata*** Prain; CHINA: Xizang; *Ludlow 9904* (E); JX087875, -, JX087737, JX087696. X029; ***Meconopsis forrestii*** Prain; CHINA: Yunnan; *Fang1154* (Xiang Ge Li La Alpine Garden); JX087853, JX087807, JX087734, -. X032; ***Meconopsis* sp**; CHINA: Sichuan; *Boufford 33308* (GH); JX087903, JX087837, JX087749, JX087710. X034; ***Cathcartia chelidonifolia*** (Bureau & Franch.) W. Xiao; UK (cultivated); *W. Xiao RICB4* (E); JX087897, JX087840, -, JX087690. X036; ***Meconopsis discigera*** Prain; BHUTAN: Upper Mo Chu District; *Bowes Lyon15045* (E); JX087918, JX087824, JX087774, JX087686. X042; ***Meconopsis sinuata*** Prain; INDIA: Sikkim; *ESK 683* (E); JX087890, JX087785, -, JX087725. X045; ***Meconopsis wumungensis*** K.M. Feng; CHINA: Yunnan; *Liu 1990July* (KUN); JX087922, -, -, JX087707. X046; ***Meconopsis wilsonii*** Grey-Wilson; CHINA:

Sichuan; *Boufford 32733* (GH); JX087924, JX087838, JX087740, JX087691. X047; ***Meconopsis primulina*** Prain; BHUTAN: Upper Mo Chu District; *Sargent170* (E); JX087887, JX087843, -, JX087685. X052; ***Meconopsis concinna*** Prain; CHINA: Yunnan; *Boufford 35133* (GH); JX087889, JX087841, JX087759, JX087721. X054; ***Meconopsis*** x ***cookei*** G. Taylor; CHINA: Qinghai; *Long 696* (E); JX087869, JX087827, -, JX087726. X055; ***Cathcartia villosa*** Hook.f.; INDIA: Sikkim; *ESK 205* (E); -, JX087847, -, JX087708. X069; ***Meconopsis autumnalis*** P.A. Egan; NEPAL: Bagmati; *Egan 17* (private collection); JX087872, JX087822, JX087748, JX087714. X073; ***Meconopsis lyrata*** (H.A. Cummins & Prain) Fedde; BURMAR: N.E. upper Burma; *Forrest 25047* (E); -, JX087800, -, -. X083; ***Meconopsis pseudovenusta*** G. Taylor; CHINA: Yunnan; *W. Xiao 090705-2* (TEX); JX087894, JX087796, JX087741, -.

Appendix 2. Species name and Genbank ID for the downloaded *trn*L-*trn*F sequences (previously published by other authors).

Roemeria refracta DC.; DQ251150.1. ***Meconopsis latifolia*** Prain; AY328226.1. ***Papaver pavoninum*** C.A.Mey.; DQ251134.1. ***Papaver argemone*** L.; DQ251149.1. ***Papaver hybridum*** L.; DQ251152.1. ***Papaver apulum*** Ten.; DQ251151.1. ***Papaver heterophyllum*** Greene; DQ251146.1. ***Papaver californicum*** A.Gray; DQ251169.1. ***Papaver aculeatum*** Thunb.; DQ251168.1. ***Papaver spicatum*** Boiss. & Balansa; AY328244.1. ***Papaver cambricum*** L.; DQ251128.1. ***Papaver pilosum*** Sm.; DQ251172.1. ***Papaver armeniacum*** Lam.; DQ251148.1. ***Papaver orientale*** L.; DQ251143.1. ***Papaver bracteatum*** Lindl.; DQ251138.1. ***Papaver pseudo-orientale*** (Fedde) Medw.; DQ251147.1. ***Papaver rupifragum*** Boiss. & Reut.; DQ251165.1. ***Papaver atlanticum*** (Ball) Coss.; DQ251154.1. ***Papaver dubium*** L.; DQ251121.1. ***Papaver somniferum*** L.; DQ251132.1. ***Papaver macrostomum*** Boiss. & A.Huet; DQ251126.1. ***Papaver rhoeas*** L.; FJ626566.1. ***Papaver triniaefolium*** Boiss.; AM397153.1. ***Papaver commutatum*** Fisch., C.A.Mey. & Trautv.; DQ251164.1. ***Papaver glaucum*** Boiss. & Hausskn. ex Boiss.; DQ251159.1. ***Papaver alpinum*** L.; DQ251119.1. ***Papaver nudicaule*** L.; DQ251135.1. ***Papaver radicatum*** Rottb. ex DC.; DQ251113.1.

APPENDIX 3. *GAPDH* sequences information.

Accession No.	Clone No.	GenBank ID
X003		KJ786426
X004	C1	JX394100
	C2	JX394064
	C3	JX394085
X005		JX394082
X006		JX394076
X007	C1	JX394068
	C2	JX394078
X008	C1	JX394072
	C2	JX394080
X009	C1	JX394095
	C2	JX394089
	C3	JX394091
X010	C1	JX394062
	C2	JX394098
	C3	JX394101
	C4	JX394079
X011	C1	JX394096
	C2	JX394086
	C3	JX394067
	C4	JX394065
X012	C1	JX394074
X012	C2	JX394094
	C3	JX394092
X015	C1	JX394087
X016	C1	JX394063
	C2	JX394081
X018	C1	JX394066
	C2	JX394083
X019	C1	JX394097
	C2	JX394088
	C3	JX394061
X020	C1	JX394070
	C2	JX394075
X022		JX394077
X032	C1	JX394073
	C2	JX394071
	C3	JX394084
X042	C1	KJ786428
	C2	KJ786427
X046		JX394090
X052		JX394069
X059		JX394099
X069		JX394093