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Evolution of the tetraploid *Anemone multifida* (2n = 32) and hexaploid *A. baldensis* (2n = 48) (Ranunculaceae) was accompanied by rDNA loci loss and intergenomic translocation: evidence for their common genome origin

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• *Background and Aims* In the genus *Anemone* two small groups of taxa occur with the highest ploidy levels $2n = 6x = 48$, belonging to the closely related clades: the montane/alpine Baldensis clade and the more temperate Multifida clade. To understand the formation of polyploids within these groups, the evolution of allohexaploid *A. baldensis* (AABBDD, $2n = 6x = 48$) from Europe and allotetraploid *Anemone multifida* (BBDD, $2n = 4x = 32$) from America was analysed.
• *Methods* Internal transcribed spacer and non-transcribed spacer sequences were used as molecular markers for phylogenetic analyses. Cytogenetic studies, including genomic *in situ* hybridization with genomic DNA of potential parental species as probe, fluorescence *in situ* hybridization with 5S and 18S rDNA as probes and 18S rDNA restriction analyses, were used to identify the parental origin of chromosomes and to study genomic changes following polyploidization.
• *Key Results* This study shows that *A. multifida* (BBDD, $2n = 4x = 32$) and *A. baldensis* (AABBDD, $2n = 6x = 48$) are allopolyploids originating from the crosses of diploid members of the Multifida (donor of the A and B subgenomes) and Baldensis groups (donor of the D subgenome). The A and B subgenomes are closely related to the genomes of *A. sylvestris*, *A. virginiana* and *A. cylindrica*, indicating that these species or their progeny might be the ancestral donors of the B subgenome of *A. multifida* and A and B subgenomes of *A. baldensis*. Both polyploids have undergone genomic changes such as interchromosomal translocation affecting B and D subgenomes and changes at rDNA sites. *Anemone multifida* has lost the 35S rDNA loci characteristic of the maternal donor (B subgenome) and maintained only the rDNA loci of the paternal donor (D subgenome).
• *Conclusions* It is proposed that *A. multifida* and *A. baldensis* probably had a common ancestor and their evolution was facilitated by vegetation changes during the Quaternary, resulting in their present disjunctive distribution.

Key words: 5S rDNA intergenic spacer, *Anemone*, fluorescence *in situ* hybridization, genomic *in situ* hybridization, intergenomic translocation, ITS, polyploidy.

INTRODUCTION

Recent investigations have shown that all seed plants and angiosperms encountered polyploidy or whole-genome duplication events throughout their evolutionary history (Jiao *et al.*, 2011). Polyploidization is accompanied by rapid and dynamic genetic and epigenetic changes, as well as changes in gene expression and phenotypic variation (Cifuentes *et al.*, 2010; Gaeta and Pires, 2010; Parisod *et al.*, 2010). Changes in genome structure range from gene conversion, sequence loss or gain, sequence amplification or reduction, to all known forms of chromosomal rearrangements (Lim *et al.*, 2007*a*, *b*; Weiss-Schneeweiss *et al.*, 2007; Książcyk *et al.*, 2011). Many hybrids and polyploids suffer from numerous rDNA rearrangements, including repeat and locus loss (Lim *et al.*, 2007*a*, *b*; Weiss-Schneeweiss *et al.*, 2007; Kotseruba *et al.*, 2010; Książcyk *et al.*, 2011), interlocus recombination and repeat replacement (Kovarik *et al.*, 2005). The mechanism and consequences of these changes remain largely unknown.

Recent studies of *Tragopogon* and *Brassica* polyploids have provided evidence that homeologous pairing and recombination could be a key mechanism for genome restructuring (Lim *et al.*, 2008; Gaeta and Pires, 2010). Szadkowski *et al.* (2010) showed that the very first meiosis of somatically doubled resynthesized *Brassica napus* already acts as a genome blender, with many of the genetic exchanges between different subgenomes transmitted to the progeny. Moreover, polyploidy formation pathways are shown to have an impact on genetic rearrangements in resynthesized *Brassica napus* as the meiosis of the F_1 interspecific hybrid generated more gametes with recombined chromosomes than did meiosis of the plant produced by somatic doubling (Szadkowski *et al.*, 2011).

The genus *Anemone sensu stricto* consists of approx. 150 species of perennial, low-growing herbs of worldwide distribution and with considerable diversity in morphology (Tamura, 1995). Two basic chromosome numbers ($n = 7$ and $n = 8$), substantial karyotype divergence involving rDNA and

heterochromatin distribution, and the existence of different ploidy levels are prominent features of *Anemone* genome evolution (Baumberger, 1970; Mlinarec *et al.*, 2012). However, polyploidy is unevenly distributed in *Anemone* and is restricted to a few lineages. The most common polyploids are tetraploids, while hexaploidy is rare (Gajewski, 1946; Heimburger, 1961). The only three hexaploids recognized (*A. baldensis*, *A. lithophila* and *A. drummondii*) are found among the Baldensis group with the disjunct areas in the Arctic and the mountains of southern Europe, one the Earth's most polyploidy-rich areas (Brochmann *et al.*, 2004).

Allotetraploid *A. multifida* ($2n = 4x = 32$) is representative of the Multifida group, a small group of species from North America (*A. cylindrica*, *A. virginiana* and *A. multifida*) and one species disjunctly distributed in Europe (*A. sylvestris*). *Anemone multifida* is widely distributed, ranging from the north-eastern part of North America to Alaska and southward to New Mexico and California, and reappearing again in the mountains of southern Chile and Argentina. *Anemone sylvestris* is native to meadows and dry deciduous woodlands of central and western Europe. Allohexaploid *A. baldensis* ($2n = 6x = 48$) is representative of the Baldensis group, a small group of species from the arctic and alpine tundra of North America (*A. parviflora*, *A. lithophila*, *A. drummondii*) and one species disjunctly distributed in Europe (*A. baldensis*). *Anemone baldensis* occurs in the Pyrenees, Alps, Dinnarids and Carpathian mountains of Europe. Hybrids between members of the Multifida group are readily obtained (Heimburger, 1962), suggesting a close relationship between them. Hexaploid *A. janczewskii*, obtained experimentally from the crossing of *A. multifida* with *A. sylvestris*, has existed for more than 120 years in several European botanical gardens (Janczewski, 1892; Gajewski, 1946).

Studies on the origin and evolution of the polyploid *Anemone* species and their relationships with the diploid taxa have been initiated only recently (Mlinarec *et al.*, 2012). Cytogenetic studies to date involve analysis of chromosome number and karyotype as well as rDNA and heterochromatin characterization (Heimburger, 1959; Baumberger, 1970; Marks and Schweizer, 1974; Mlinarec *et al.*, 2006, 2009, 2012). Based on karyotype morphology and additional phylogenetic analyses, Mlinarec *et al.* (2012) proposed a hybrid origin for the hexaploid *A. baldensis* with the diploid *A. sylvestris* as putative progenitor. Similarly, incongruence between the chloroplast and nuclear DNA phylogenetic trees indicated a possible hybridogenous genomic constitution of *A. multifida*, with the presumed parental species among the members of the Baldensis and Multifida groups (Meyer *et al.*, 2010).

To confirm the allopolyploid origin of tetraploid *A. multifida* ($2n = 4x = 32$) and hexaploid *A. baldensis* ($2n = 6x = 48$) and elucidate the parental origin of their chromosomes among species from the Baldensis and Multifida groups, we performed, phylogenetic analyses using internal transcribed spacer (ITS) and non-transcribed spacer (NTS) sequences as molecular markers, cytogenetic studies including genomic *in situ* hybridization (GISH) with genomic DNAs of potential parental species as probe, fluorescence *in situ* hybridization (FISH) with 5S and 18S rDNA as probe and 18S rDNA restriction analyses. We identified three subgenomes (AABBDD) in

A. baldensis and two (BBDD) in *A. multifida*. The A and B subgenomes are closely related to the genomes of *A. sylvestris*, *A. virginiana* and *A. cylindrica*, indicating that these species or their progeny might be the ancestral donors of the B subgenome of *A. multifida* and A and B subgenomes of *A. baldensis*. The D subgenome of *A. multifida* and *A. baldensis* probably originated from the Baldensis group.

MATERIALS AND METHODS

Plant material

Information on all plant material used is given in Table 1. Plants were grown in pots in the Botanical Garden of the University of Zagreb. All species were identified based on morphological and karyological characteristics. For karyological studies, actively growing root-tip meristems were pretreated with 0·05 % colchicine (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) for 4 h at room temperature, fixed in a solution of ethanol and acetic acid (3 : 1) for 24 h at $-20\,^{\circ}$C, and stored in 70 % ethanol at $-20\,^{\circ}$C until use. For cloning as well as for GISH probes, high-quality genomic DNA was isolated from young leaves using the Qiagen mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

PCR amplification and cloning

PCR amplifications of the entire spacer region with the partial 5S rDNA gene as well as ITS region of 35S rDNA were carried out in a 50 μL reaction mixture containing 10 ng template DNA, 0·4 μM of each primer, 200 μM dNTPs, 2·5 U GoTaq DNA Polymerase and corresponding $1\times$ (1·5 mM MgCl$_2$) Green Reaction Buffer (Promega Corp., Madison, WI, USA), using the primer pairs described previously (Besendorfer *et al.*, 2005; Puizina *et al.*, 2008). After an initial denaturing step at 94 °C for 3 min, amplification was carried out using 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 54 °C for 10 s and primer

TABLE 1. *Plant material, accession numbers and chromosome number (2n) for* Anemone

Taxon	Accession no.	Locality	2*n*
Anemone baldensis L.	698G	Hungary, Vácrátót, 1989, Botanical Garden of the University of Zagreb	48
Anemone cylindrica A. Gray	6559B	Germany, seed from the Botanical Garden Chemnitz, Botanical garden of the University of Zagreb	16
Anemone multifida Poir.	12427	Germany, seed from Botanical Garden Chemnitz, Botanical garden of the University of Zagreb	32
Anemone sylvestris L.	1451B	Croatia, Čučerje, Medvednica, Botanical Garden of the University of Zagreb	16
Anemone virginiana L.	11838B	Germany, seed from the Botanical Garden Chemnitz, origin: Canada, Quebec, Country Deux-Montaignes, Oka, 2005, Botanical Garden of the University of Zagreb	16

extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Cloning and transformation were carried out using the InsTAclone PCR Cloning Kit (Fermentas GmbH, Germany) or pGEM-T Easy Vector System (Promega) according to the manufacturers' instructions. Sequencing was carried out by Macrogen Inc. (Seoul, Korea).

Sequence alignment and phylogenetic analysis

For the polyploid taxa we employed 20 NTS and 16 ITS clones of *A. baldensis* and 15 NTS and ten ITS clones of *A. multifida*, while for diploid taxa (*A. sylvestris*, *A. virginiana*, *A. cylindrica*) we employed 3–6 NTS clones and 1–3 ITS sequences. ITS sequences of *Anemone parviflora* (FJ639887), *A. sylvestris* (FJ639894), *A. drummondii* (AY055404), *A. multifida* (AY055405), *A. virginiana* (FJ639898) and *A. lithophila* (FJ639883) were mined from the GenBank databse using keywords (*Anemone*, ITS). These sequences are the result of direct sequencing (Meyer *et al.*, 2010), while NTS and ITS sequences obtained in this study are the result of cloning and

their accession numbers are indicated in Fig. 1. Sequences were aligned with Clustal_x v1·81 (Thompson *et al.*, 1997).

Phylogenetic signal in each dataset was determined from the tree-length distribution of 1000 000 trees, using the g_1-statistic (Hillis and Huelsenbeck, 1992). An unweighted maximum-parsimony (MP) analysis was conducted using PAUP* 4·0b10 (Swofford, 2003). Heuristic searches were performed with 1000 random addition sequence replicates using tree bisection reconnection (TBR) branch swapping. Gaps were treated as missing data. Bootstrap support values (Felsenstein, 1985) from 1000 replicates were calculated using the heuristic search options as above except random addition sequence with 100 replicates. As outgroup taxa we employed the ITS (JF422883) and NTS (JF422865) sequences of the closely related Mediterranean species *A. apennina*.

Chromosome preparation and FISH

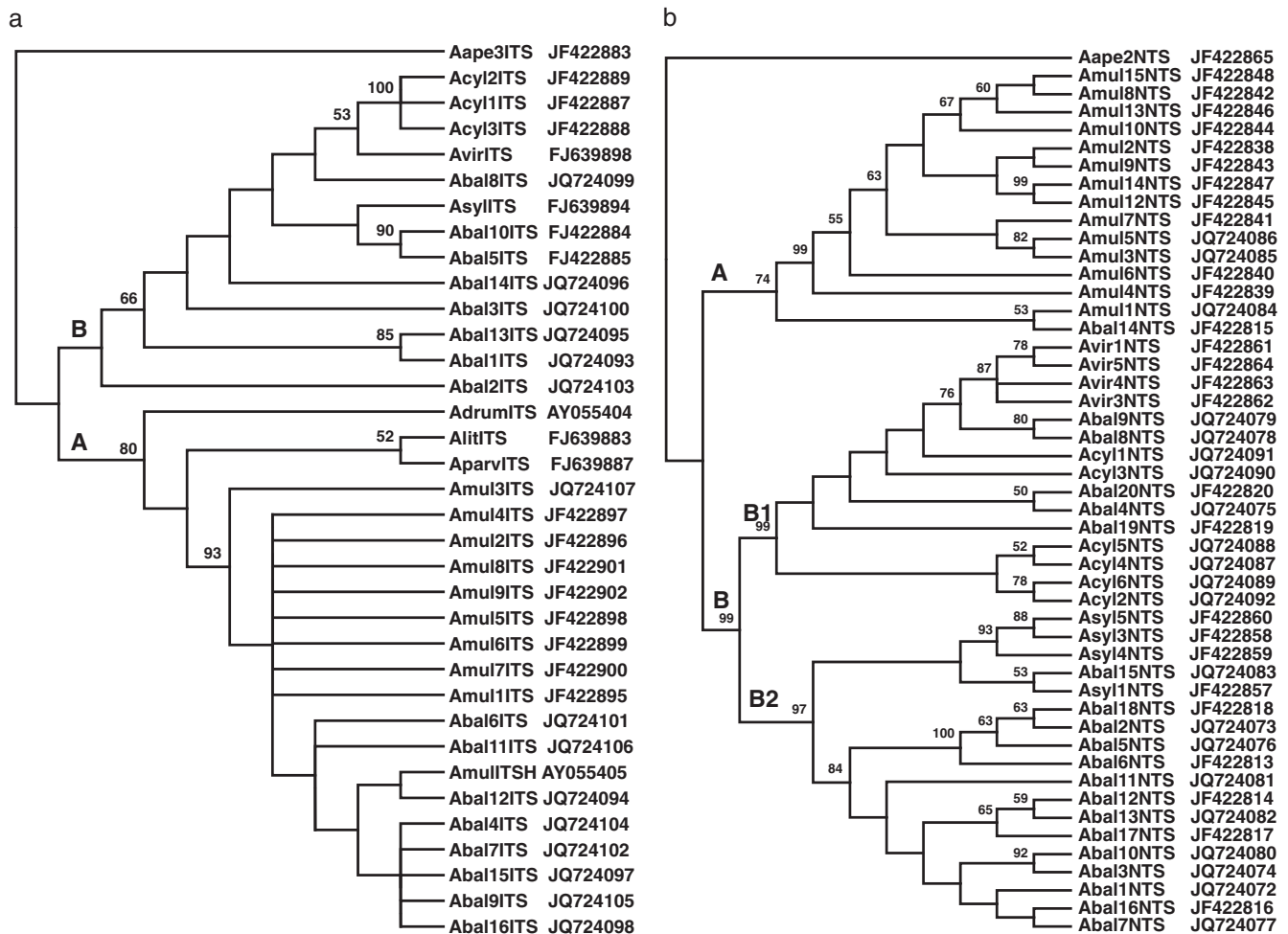Chromosome preparations for FISH and GISH were as described by Mlinarec *et al.* (2006). FISH and GISH

FIG. 1. Phylogenetic relationships among cloned ITS and NTS sequences of *A. baldensis* (Abal), *A. cylindrica* (Acyl), *A. drummondii* (Adrum), *A. lithophila* (Alit), *A. multifida* (Amul), *A. parviflora* (Aparv), *A. sylvestris* (Asyl) and *A. virginiana* (Avir): (a) 50 % majority-rule consensus tree of the 1603 most-parsimonious trees based on ITS sequences, (b) 50 % majority-rule consensus tree of the 478 most-parsimonious trees based on NTS sequences. The A clade (Baldensis clade) and B clade (Multifida clade) are indicated. Bootstrap support values (>50 %) are shown above nodes. Sequences of *A. apennina* were used as outgroup. GenBank sequence accession numbers are indicated next to the name of the particular clone.

experiments were performed according to Mlinarec *et al.* (2012). Clone pTa794, containing the complete 410-bp *Bam*HI fragment of the 5S rRNA gene and the spacer region of wheat (Gerlach and Dyer, 1980), was used as the 5S rDNA probe. The 2·4-kb *Hind*III fragment of the partial 18S rRNA gene and ITS1 from *Cucurbita pepo*, cloned into pUC19 (Torres-Ruiz and Hemleben, 1994), was used as the 35S rDNA probe. The genomic and rDNA probes were directly labelled with Cy3-dCTP (Amersham, GE Healthcare, Little Chalfont, UK) and FITC-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) by using a nick-translation kit according to the manufacturer's instructions (Roche). After overnight hybridization, slides were given a stringent wash in $0·1 \times$ saline sodium citrate resulting in DNA duplexes with an estimated $>85\%$ sequence identity. The preparations were mounted in antifade buffer Vectashield (Vector Laboratories, Peterborough, UK) containing DAPI counterstain (2 µg mL^{-1}) and stored at 4 °C. Signals were visualized and photographs captured on an Olympus BX51 microscope, equipped with a highly sensitive Olympus DP70 digital camera. Images were uniformly processed using Adobe Photoshop for colour contrast and brightness. An average of ten well-spread metaphases was analysed for each individual. Three individuals per taxon were analysed.

Chromosome analyses and construction of ideograms

Acetocarmine staining was performed according to standard protocols (Mlinarec *et al.*, 2006). Chromosome measurements were made on three well-spread chromosome plates of each of three individuals. For chromosome classification, the nomenclature of Levan *et al.* (1964) was followed. Chromosomes were identified according to type, total length, arm ratio, fluorochrome banding pattern and GISH hybridization signal, and were ranked in order of increasing length.

Southern hybridization

Genomic DNA (gDNA) was isolated from 150 mg fresh tissue of five *Anemone* species: *A. cylindrica*, *A. virginiana*, *A. sylvestris*, *A. multifida* and *A. baldensis* using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). gDNA (2·7 µg) was digested overnight with *Eco*RV (New England Biolabs, Ipswich, MA, USA). Digested gDNA (1·35 µg) was loaded per lane on a 1 % (w/v) agarose gel and electrophoretically separated for several hours at 100 V. DNA was blotted onto a positively charged nylon membrane (Roche, Basel, Schwitzerland) for 1 h by using a Model 785 Vacuum Blotter (BioRad, Hercules, CA, USA). Crosslinking was performed for 3 min (0·24 J cm^{-2}) on a UVlink CL508M crosslinker (Uvitec, Cambridge, UK). The 2·4-kb *Hind*III fragment of the partial 18S rRNA gene and ITS1 from *Cucurbita pepo*, cloned into pUC19 (Torres-Ruiz and Hemleben, 1994), was used as a template to generate the DIG-dUTP-labelled probe by using the DIG DNA Labelling and Detection Kit (Roche) according to the manufacturer in combination with the universal M13-forward and M13-reverse primers. The membrane was prehybridized for 1 h and hybridized overnight at 42 °C. Subsequently, the membrane was washed at room temperature and at 68 °C according to the manufacturer's

instructions. DIG was detected using the Anti-DIG-AP, Fab fragments (Roche). The hybridized probe was visualized directly on the membrane by applying the NBT/BCIP solution (Roche) according to the manufacturer's instructions. Strongest signal was found after overnight incubation.

RESULTS

Phylogenetic analysis of ITS and NTS sequences

Phylogenetic analyses were performed with ITS and NTS sequences to determine if they can be useful in evaluation of possible cases of hybridization among different *Anemone* species (Fig. 1). In addition to *A. baldensis* and *A. multifida*, we included in phylogenetic analyses their putative parental species *A. sylvestris*, *A. virginiana* and *A. cylindrica* (members of the Multifida clade) as well as *A. parviflora*, *A. drummondii* and *A. lithophila* (members of the Baldensis clade). *Anemone apennina* was used as outgroup to root the NTS and ITS trees.

For ITS, the alignment of 35 sequences had 549 characters, of which 434 were constant, 60 were parsimony-uninformative and 55 were parsimony-informative. The length–frequency distribution from 1000 000 random trees showed a strong left skew ($g_1 = -0·38$) as compared with the critical value of $g_1 = -0·13$ (at $P = 0·01$) for 25 taxa and 50 characters, indicating the presence of highly significant phylogenetic signal in the dataset. The heuristic search resulted in 1603 equally parsimonious trees with a length of 168 steps (consistency index (CI) = 0·72, retention index (RI) = 0·89, rescaled consistency index (RC) = 0·64).

The NTS dataset included 49 sequences that comprised 538 characters, of which 215 were constant, 138 were parsimony-uninformative and 138 were parsimony-informative. The distribution of lengths for the 1000 000 random trees evaluated was strongly skewed to the left ($g_1 = -0·53$) compared with the critical value of $g_1 = -0·12$ (at $P = 0·01$) for 25 taxa and 100 characters (Hillis and Huelsenbeck, 1992). This g_1 value indicates that the data are significantly more structured than are random data and implies the presence of strong phylogenetic signal in the NTS dataset. An unweighted parsimony analysis yielded 478 most-parsimonious trees of length 708 steps (CI = 0·68, RI = 0·84, RC = 0·57).

Phylogenetic analyses separated the sequences in either the ITS or the NTS tree into two divergent clades: A and B. All sequences originating from *A. multifida* only derived from within clade A, while those generated from *A. baldensis* fell within the two distinct clades A and B (Fig. 1).

In Fig. 1(a), the ITS tree displays a clear rake-like branching pattern containing a number of unresolved polytomies, suggesting a low rate of sequence divergence. Some of the ITS sequences of *A. baldensis* are associated with those of *A. parviflora*, *A. drummondii* and *A. lythophila* in clade A [bootstrap support (BS) = 80 %], named the Baldensis clade, whereas the other *A. baldensis* sequences are closely related to ITS sequences obtained from the diploid taxa of the Multifida group (*A. sylvestris*, *A. cylindrica* and *A. virginiana*) named the Multifida clade (clade B). Two of the *A. baldensis* clones (Abal5ITS and Abal10ITS) showed close affinities to the sequence AsylITS derived from *A. sylvestris*, whereas

another (Abal8ITS) is rather close to those of *A. virginiana* and *A. cylindrica*. Interestingly, all the ITS clones isolated from the tetraploid *A. multifida* formed a well-supported monophyletic group (BS = 93 %) together with the *A. baldensis* ITS sequences within the Baldensis clade (clade A).

In the NTS tree (Fig. 1b), the data confirm the genomic sequence heterogeneity of *A. baldensis*, as observed for ITS sequences. NTS sequences are distributed in two main clades. One clone (Abal14NTS) is related to the *A. multifida* clones in a distinct clade (BS = 74 %) corresponding to the previous Baldensis clade A. All the other NTS clones fall in the Multifida clade B (BS = 99 %). Within the latter, the *A. baldensis* clones are separated into two subsets belonging each to two well-supported sister subclades (B1 and B2). One *A. baldensis* NTS subset is clearly embedded with *A. cylindrica* and *A. virginiana* in subclade B1 (BS = 99 %), while the other is associated with *A. sylvestris* in subclade B2 (BS = 97 %).

To gain further insight from the NTS sequences we more accurately examined sequence alignment of cloned NTS sequences from the five studied *Anemone* species. Analysis of NTS sequences revealed 12 species-specific nucleotide positions for which the sequence origin could be associated with the corresponding *Anemone* species (Supplementary Data Fig. S1). In agreement with the NTS tree, species-specific nucleotide positions in *A. multifida* NTS clones did not reveal similarity to any other analysed clones of other *Anemone* species. Only one *A. multifida* clone, Amul1NTS, showed similarity with one *A. baldensis* clone, Abal14NTS, but only in five of 12 species-specific sequences (ACTC at position 103, TTGTTC at position 146, GGGTAATG at position 162, GGCTCTCA at position 273 and GGC at position 324) (Supplementary Data Fig. S1). By contrast, species-specific positions identical to either *A. sylvestris*, *A. virginiana* or *A. cylindrica* were identified in the NTS clones of *A. baldensis*. The majority of 20 analysed *A. baldensis* clones possessed

species-specific positions that were identical to *A. sylvestris* (36–78 % identity depending on the position), while some were identical to *A. cylindrica* and/or *A. virginiana* (Fig. 2). Note that the NTS sequences of *A. cylindrica* and *A. virginiana* were almost identical (sharing 11 of 12 species-specific sequences) and differed from *A. sylvestris* in ten of 12 species-specific positions (Supplementary Data Fig. S1).

These phylogenetic analyses proved to be useful in revealing the polyploid origin of *A. baldensis*, suggesting that this allohexaploid originates from crosses between the members of the Multifida and Baldensis clades. The highly supported association between the NTS sequences of *A. baldensis* and those from *A. sylvestris*, *A. cylindrica* or *A. virginiana* suggests that all three species could have been implicated in the origin of *A. baldensis*. Furthermore, although the DNA sequence data did not reveal the hybrid origin of *A. multifida*, a strong association of the ITS sequences with the Baldensis group suggests that the potential candidate for one parental species comes from this group.

GISH *in* A. multifida *and* A. baldensis

To determine the origin of the *Anemone* polyploids, labelled gDNA of presumed parental species was hybridized to mitotic chromosomes of *A. multifida* ($2n = 4x = 32$) and *A. baldensis* ($2n = 6x = 48$). GISH of *A. multifida* root-tip metaphases revealed cross-hybridization of *A. virginiana* (Fig. 3a, d), *A. sylvestris* (Fig. 3c) and *A. cylindrica* (data not shown) gDNAs to only one chromosome set of *A. multifida*. The labelled chromosome set was designed as the B chromosome set in *A. multifida* (Fig. 3d).

GISH analysis in *A. baldensis* was more complex due to the presence of three chromosome sets distinguishable by size (Mlinarec *et al.*, 2012). GISH of *A. baldensis* root-tip metaphases showed cross-hybridization of *A. sylvestris* (Fig. 3e, g),
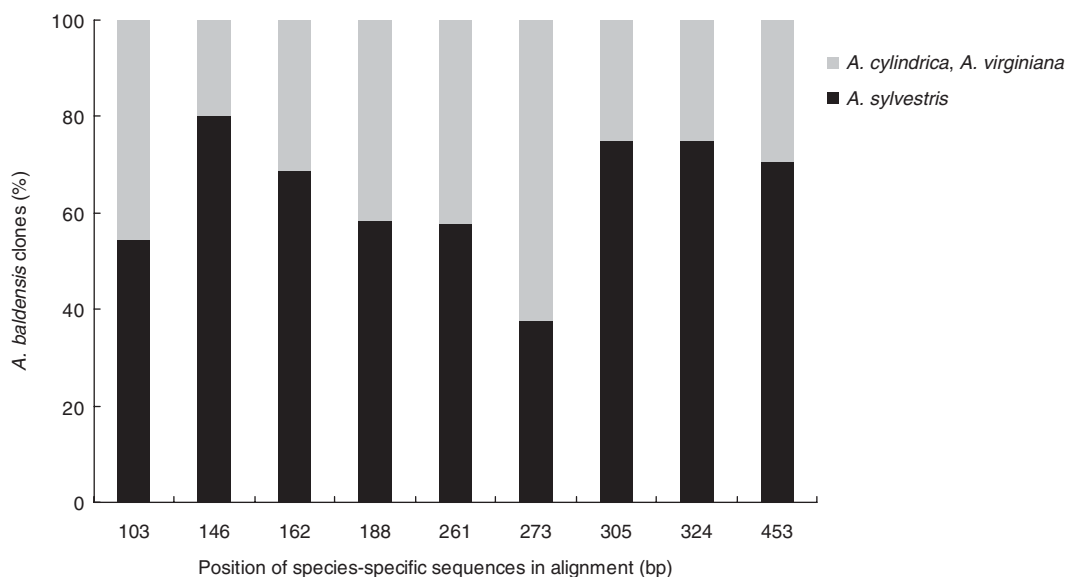

FIG. 2. The proportion of *A. baldensis* NTS clones that possess species-specific sequences identical to *A. sylvestris*, *A. virginiana* and *A. cylindrica*. The species-specific sequences are shown in Supplementary Data Fig. S1.
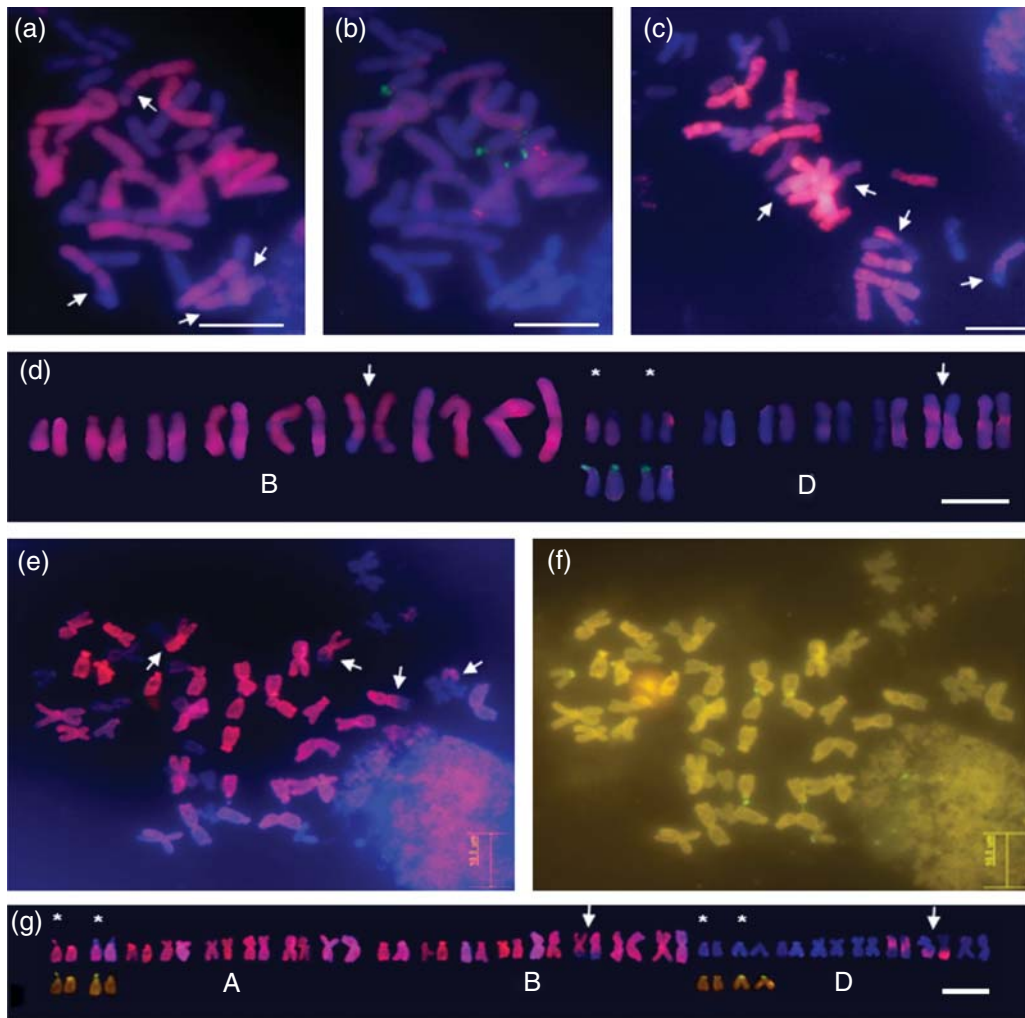
F<small>IG</small>. 3. Karyotype analyses of *A. multifida* (a–d) and *A. baldensis* (e–g). GISH on mitotic chromosomes of *A. multifida* with labelled gDNA of *A. virginiana* (a, d; in red) and *A. sylvestris* (c; in red). GISH on mitotic chromosomes of *A. baldensis* with labelled gDNA of *A. sylvestris* (e, g; in red). The unidentified subgenome shows DAPI-specific staining (in blue). Intergenomic translocation is marked with arrows. 35S rDNA bearing chromosome pairs are marked with asterisks. The chromosome sets of the corresponding subgenomes are marked A, B and D. FISH on *A. multifida* (b) and *A. baldensis* (f) mitotic chromosomes with differentially labelled 35S rDNA (in green) and/or 5S rDNA (in red). Scale bars = 10 μm.

A. virginiana (data not shown) and *A. cylindrica* (data not shown) gDNAs to two chromosome sets of *A. baldensis*. One labelled chromosome set had chromosomes of similar size, morphology and rDNA FISH pattern to those of *A. sylvestris* and was identified as *A. sylvestris*-like or the A chromosome set (Fig. 3g). The other labelled chromosome set was most similar to *A. cylindrica* and *A. virginiana* (*A. cylindrica* and *A. virginiana* have chromosomes of similar size, morphology and rDNA loci position; Mlinarec *et al.*, 2012) and was designed as the B chromosome set in *A. baldensis*.

Karyotype analysis of *A. multifida* and *A. baldensis* showed that the unidentified chromosome set that showed DAPI-specific staining consists of chromosomes of similar size, morphology and rDNA FISH pattern (Table 2; see Fig. 5 below). The unidentified chromosome set was designed as the D chromosome set in both polyploid species.

To test the affinity between the D subgenomes of *A. multifida* and *A. baldensis*, labelled gDNA of *A. multifida* was hybridized to mitotic chromosomes of *A. baldensis*. Interestingly, GISH of *A. baldensis* root-tip metaphases revealed hybridization of *A. multifida* genomic DNA to all three chromosome sets of *A. baldensis* (Supplementary Data Fig. S2).

Thus, the cytogenetic data obtained in this study are in agreement with the ITS and NTS phylogeny, confirming that *A. multifida* and *A. baldensis* are allopolyploids. The B chromosome set in *A. multifida* and the A and B chromosome sets in *A. baldensis* are closely related to *A. sylvestris*, *A. cylindrica* or *A. virginiana*. The similarity between the complete genomes of *A. multifida* and *A. baldensis*, as revealed by GISH, suggest that these two polyploids, in addition to the A and B subgenome, also have the D subgenome in common. On the basis of these observations, we propose that *A. multifida*

TABLE 2. *Chromosome measurements of* A. baldensis *and* A. multifida

	Chromosome															
	I		II		III		IV		V		VI		VII		VIII	
A. baldensis, 2n = 48	0.8		1.1		0.9		2.1		2.8		3.4		3.7		4.7	
	4.0		4.2		4.3		4.5		3.6		3.9		4.3		4.8	
A	5.0	4.8	3.8	5.3	4.8	5.2	2.1	6.6	1.3	6.4	1.2	7.3	1.2	8.0	1.0	9.5
	1.0		1.2		1.3		2.1		3.9		4.0		4.5		4.7	
	4.5		4.7		4.9		3.9		4.3		4.5		5.2		5.7	
B	4.5	5.5	4.0	5.9	3.8	6.2	1.9	6	1.1	8.2	1.1	8.5	1.2	9.7	1.2	10.4
	0.8		1.0		1.0		1.9		2.5		3		3.2		3.6	
	3.3		3.3		3.4		3.3		3.3		4.0		4.1		4.2	
D	5.1	4.1	3.3	4.3	3.4	4.4	1.7	5.2	1.3	5.8	1.3	7.0	1.3	7.3	1.2	7.8
A. multifida, 2n = 32	0.7		1.0		1.3		2.4		3.8		4.4		4.9		5.1	
	5.0		5.3		5.4		5.0		4.3		4.7		5.8		5.9	
B	7.1	5.7	5.3	6.3	4.2	6.7	2.1	7.4	1.1	8.1	1.1	9.1	1.2	10.7	1.2	11.0
	0.8		1.1		1.0		1.9		2.6		3.1		3.5		3.3	
	3.3		3.3		3.5		3.2		3.3		4.1		3.9		4.5	
D	5.1	4.1	3.0	4.4	3.5	4.5	1.7	5.1	1.3	5.9	1.3	7.2	1.1	7.4	1.4	7.8

The first measurement in each column refers to the short arms; the second, to the long arms; and the third, to the totals. Arm ratios appear at the left. Note that the D chromosome set of *A. baldensis* and *A. multifida* are of similar size and morphology. Chromosome measurements of *A. multifida* are taken from Heimburger (1959).

and *A. baldensis* originate from similar progenitor species or species in closely related taxa.

Genomic changes following polyploidization

In allotetraploid *A. multifida* rDNA from two parental genomes is expected, whereas in allohexaploid *A. baldensis* rDNA from three parental genomes is expected. Surprisingly, detailed ITS sequence analysis revealed only one group of ITS sequences in *A. multifida* and only two groups of ITS sequences in *A. baldensis*. Furthermore, FISH revealed that two pairs of 35S rDNA sites instead of four were detected in *A. multifida* and four instead of six were detected in *A. baldensis* (Fig. 3b, f). Subsequent GISH revealed that the 35S rDNA sequences assigned to the B subgenome of *A. multifida* were not detectable (Fig. 3d, g, asterisk) indicating that the major proportion of 35S rDNA sequences of the B subgenome have been lost in *A. multifida*.

We also observed that in *A. baldensis* and *A. multifida*, two pairs of homeologous chromosomes carry terminal reciprocal translocation, one pair belonging to the B subgenome and the other to the D subgenome. Homeologous translocation occurred among similar homeologous linkage groups B6–D7 in both *A. multifida* (Fig. 3a, c, d, arrows) and *A. baldensis* (Fig. 3e, g, arrows). It was observed in all three tested individuals of *A. baldensis* and *A. multifida*, again suggesting that *A. baldensis* and *A. multifida* originate from similar progenitor species or species in closely related taxa.

Southern blot analysis

As we have shown by GISH analysis that the 35S rDNA of the Multifida-origin subgenome (the B subgenome) was missing in *A. multifida*, we wanted to confirm this by Southern blot analysis (Fig. 4). gDNAs of *A. cylindrica*, *A. virginiana*, *A. sylvestris*, *A. multifida* and *A. baldensis* were restricted and hybridized with the 18S rDNA subunit
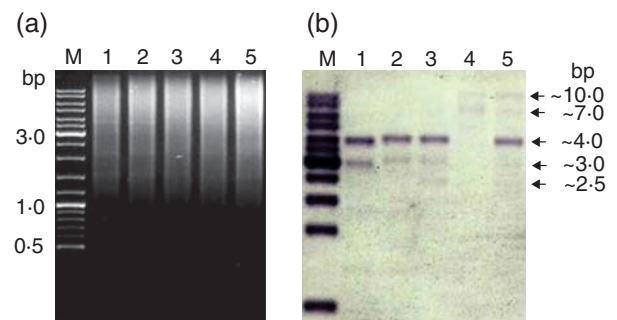

FIG. 4. Genomic restriction digests (a) and Southern blotting analyses (b) of 1, *A. cylindrica*; 2, *A. virginiana*; 3, *A. sylvestris*; 4, *A. multifida*; and 5, *A. baldensis* with restriction endonuclease *Eco*RV and probed with 18S rDNA. Asterisks indicate the 35S rDNA of the Baldensis-origin subgenome (the D subgenome), while arrows indicate 35S rDNA of the Multifida-origin subgenome (the A and B subgenomes) in *A. baldensis* and *A. multifida*.

probe (Fig. 4). The quantity of gDNA loaded into each lane was approximately the same for each species (Fig. 4a). Species of the Multifida group (*A. cylindrica*, *A. virginiana* and *A. sylvestris*) generated a similar pattern of bands, having one strong species-specific band of approx. 4 kb and two smaller bands corresponding to approx. 3 and approx. 2.5 kb. Interestingly, these bands were completely absent in *A. multifida*, supporting our previous conclusion that the major proportion of the 35S rDNA from the B subgenome has been lost in *A. multifida*. In addition, two bands corresponding to 7 and 10 kb were detected in *A. multifida* which were also present in *A. baldensis* (Fig. 4b, asterisks). As *A. multifida* and *A. baldensis* share the D subgenome, which is not present in species of the Multifida group, these larger bands probably originate from that subgenome. Furthermore, the remaining bands in *A. baldensis* (Fig. 4b, arrows) were of similar size to those in the lanes of species of the Multifida group, suggesting that they originate from the Multifida-origin subgenome (the A and B subgenomes). The hybridization signals of these bands in *A. baldensis*

were of similar intensity to those in the lanes of species of the Multifida group. Given that *A. baldensis* has inherited two chromosome sets from species of the Multifida group, the similar intensity between these signals indicates a subsequent loss of the 35S rDNA loci of the A or B subgenomes in *A. baldensis*, in agreement with the FISH results.

Phenotype similarities between A. baldensis *and* A. multifida *and their potential parental species* A. sylvestris, A. cylindrica *and* A. virginiana

As polyploids often show new phenotypic characteristics not present in their diploid progenitors we compared some morphological characteristics of *A. baldensis* and *A. multifida* with their putative parental species *A. sylvestris*, *A. cylindrica* and *A. virginiana*. On the basis of leaf morphology, *A. baldensis* represents an intermediate phenotype between those of *A. multifida* and *A. sylvestris*, *A. cylindrica* and *A. virginiana*, while achene fruits of *A. baldensis* resemble those of *A. multifida* and *A. sylvestris*, although they are considerably larger than those of both *A. multifida* and *A. sylvestris* (Supplementary Data Fig. S3).

DISCUSSION

Origin of A. multifida and A. baldensis

The results of this study show that *A. multifida* ($2n = 4x = 32$) and *A. baldensis* ($2n = 6x = 48$) are allopolyploids composed of two (BBDD) and three subgenomes (AABBDD), respectively. In the case of *A. baldensis*, GISH showed cross-hybridization of gDNAs of *A. sylvestris*, *A. virginiana* and *A. cylindrica* to its A and B chromosome sets despite the fact that these chromosome sets are distinguishable by size and morphology (Mlinarec *et al.*, 2012). Such cross-hybridization to *A. baldensis* is probably a result of small intergenomic divergence between members of the Multifida group. That the members of the Multifida clade occur in similar habitats and are interfertile (Heimburger, 1962) provide further support for their relatedness. However, sequence analyses were useful in distinguishing between the two chromosome sets of the Multifida origin in *A. baldensis*. The clustering of NTS sequences of the Multifida origin into two sublades (B1 and B2) suggests that sequences in each subclade could be matched with the sequences of their potential parental species (Kotseruba *et al.*, 2010). Therefore, clustering of NTS sequences of *A. baldensis* with those of *A. sylvestris* within the B2 subclade indicates that *A. sylvestris* might be involved in the origin of *A. baldensis*. The karyotype similarity between the A chromosome set and the chromosome set of *A. sylvestris* indicates that the latter may be a potential donor of the A chromosome set to *A. baldensis*. Accordingly, clustering of NTS sequences of *A. baldensis* with those of both *A. cylindrica* and *A. virginiana* within the B1 subclade suggests that both species are also involved in the origin of *A. baldensis*. Therefore, the B chromosome set of *A. baldensis* could originate from the common ancestor of *A. cylindrica* and *A. virginiana* or it could be of homoploid origin involving *A. cylindrica* and *A. virginiana* as parental species. Both hypotheses are equally possible and could be resolved by the use of single-copy

nuclear genes in phylogenetic analysis (Kelly *et al.*, 2010). Thus, DNA analytical data support our hypothesis that all three species (*A. sylvestris*, *A. cylindrica* or *A. virginiana*) or their progeny could be the ancestral donors of the A and B chromosome sets in *A. baldensis*. This is further supported by the finding that the NTS clones of *A. baldensis* contain species-specific sequences identical to either *A. sylvestris*, *A. cylindrica* or *A. virginiana*.

In *A. multifida*, the ITS sequences did not help in revealing the origin of the B subgenome as the major proportion of the B subgenome-type 35S rDNA repeats have been lost in this species. rDNA loci loss has been reported in many hybrids and allopolyploids such as *Nicotiana tabaccum* (Lim *et al.*, 2000), *Iris versicolor* (Lim *et al.*, 2007b), grass *Zingeria kochi* (Kotseruba *et al.*, 2010) and *Brassica napus* (Książcyk *et al.*, 2011). In addition, we obtained incongruent results from the molecular and cytogenetic analyses of NTS sequences of *A. multifida*. We expected that the major 5S rDNA locus from the B subgenome would have NTS sequences associated with the Multifida group because the B subgenome originates from this group of species. However, all clones derived from *A. multifida* formed a single well-supported clade that differed significantly from the clones of the Multifida group. This finding suggests that the 5S rDNA underwent interlocus homogenization in *A. multifida*.

Previous phylogenetic studies which used plastid DNA sequences as molecular markers place *A. multifida* within the Multifida clade (Meyer *et al.*, 2010). This suggests that in *A. multifida* the Multifida-origin subgenome (the B subgenome) is maternal. Accordingly, we concluded that in the same species, the Baldensis-origin subgenome (the D subgenome) is paternal. Loss of 35S rDNA loci from the maternal parent (donor of the B genome) and maintenance in the paternal parent (donor of the D subgenome) suggest that in *A. multifida* the maternal genome evolves more rapidly than the paternal one. Although the nucleo-cytoplasmic interaction hypothesis of Gill (1991) predicts that the paternal genome should evolve more rapidly than the maternal one, there are more and more examples of genetic changes targeted at the maternal genome donor (this study; Lim *et al.*, 2000; Clarkson *et al.*, 2005; Guggisberg *et al.*, 2008).

Here we observed an interesting correlation between *A. multifida* and *A. baldensis*. Both species share genomic changes such as intergenomic translocation between B and D subgenomes and loss of the maternal-type 35S rDNA. Intergenomic reciprocal recombination, observed in *A. multifida* and *A. baldensis*, suggests that chromosome pairing between homeologous chromosomes took place. Accumulating evidence indicates that chromosome pairing between homeologous chromosomes is commonplace in polyploids, notably in neopolyploids such as *Brassica napus* (Nicolas *et al.*, 2007, 2009; Szadkowski *et al.*, 2011) and synthetic allotetraploid tobacco (Petit *et al.*, 2010). The similarity between the complete genomes of *A. multifida* and *A. baldensis*, as shown on both molecular and cytogenetic level, implies that these two polyploids, in addition to the A and B subgenome, also have the D subgenome in common. However, the origin of the D subgenome remains unclear. Its karyotype is unique among diploid *Anemone* species investigated so far regarding chromosome size and position of 5S rDNA (Mlinarec *et al.*, 2012).
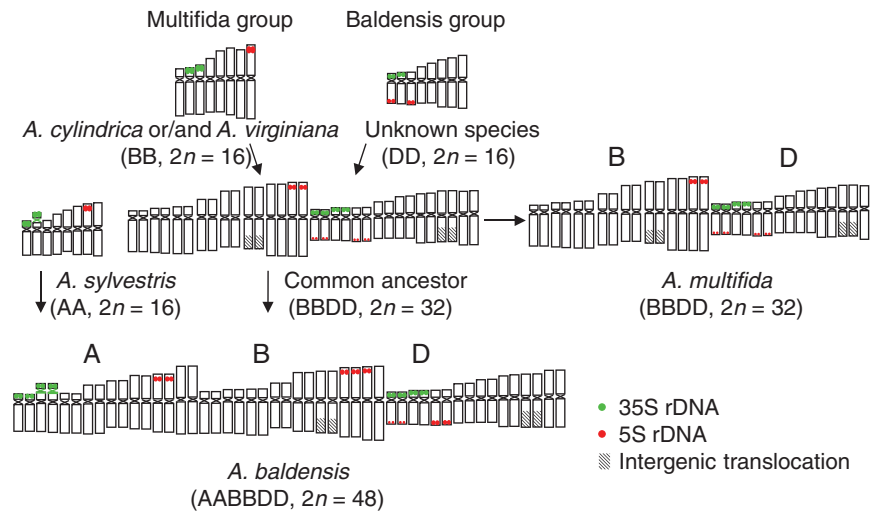
F IG . 5 . Scheme of the evolutionary steps involved in the formation of polyploids *A. multifida* and *A. baldensis*. Ideogram of *A. sylvestris*, *A. cylindrica*, *A. virginiana* and 5S rDNA loci distribution in *A. baldensis* are taken from Mlinarec *et al.* (2012).

However, similar karyological characteristics found in the sister genus *Pulsatilla* (Mlinarec *et al.*, 2012) suggest that the *Pulsatilla* sp. could be the genome donors to the D chromosome set of *A. multifida* and *A. baldensis*. However, we consider this hypothesis to be unlikely because no molecular marker used so far places *A. multifida* and *A. baldensis* close to the *Pulsatilla* clade. By contrast, phylogenetic analyses showed that *A. multifida* and *A. baldensis* are closely related to the Multifida and Baldensis groups (this study; Meyer *et al.*, 2010). This suggests that one of the diploid members of the Baldensis group or its progeny might be the ancestral donors of the D chromosome set to *A. multifida* and *A. baldensis*. *Anemone parviflora*, as the only extant diploid candidate for the genome donor, does not appear likely as a progenitor because no *A. parviflora*-like ITS sequences were found in both polyploids (this study). Alternatively, the possibility that the genome donor of the D subgenome was closely related to the members of the Baldensis group, but went extinct due to dramatic climatic change, cannot be ruled out. Future phylogenetic and karyological studies that include all species of the Baldensis group are expected to reveal the origin of the D subgenome as well as the origin of two other hexaploids, *A. drummondii* and *A. lithophila*.

Biogeographical implications

We propose here that the New World species *A. multifida* and Old World species *A. baldensis* have either the same parental species or parental species in closely related taxa (as shown in a model in Fig. 5). How and when their parental species came into contact is a question that can be tackled on palaeoclimatic grounds.

The early Quaternary Arctic flora was recruited from survivors of the arcto-Tertiary forests, combined with immigrants from temperate mountain ranges (Murray, 1995). One such immigrant, the progenitor of the Multifida clade and probably the most similar to extant *A. virginiana* and *A. cylindrica*, immigrated into the Arctic area in the early Quaternary. Soon after, it crossed with the likely progenitor of the Baldensis clade

(Fig. 5). Successive cycles of divergent evolution among populations isolated in different glacial refugia and migration into deglaciated terrain led to the origin of two allotetraploid taxa, *A. multifida* ($2n = 4x = 32$) and *A. baldensis* ($2n = 4x = 32$), with their distribution centres in North America and Europe, respectively. Indeed, tetraploid *A. baldensis* has been reported in the Arctic area (Baumberger, 1970) but these reports are relatively old and should be taken with caution. In Europe, tetraploid *A. baldensis* ($2n = 4x = 32$) could have crossed with diploid *A. sylvestris* ($2n = 2x = 16$), resulting in the formation of hexaploid *A. baldensis* ($2n = 6x = 48$) (Fig. 5). This hypothesis is also supported by leaf morphology. *Anemone multifida* has a characteristic leaf shape, which is unique among *Anemone* species, while *A. baldensis* is of intermediary type between *A. multifida* and *A. sylvestris* regarding leaf morphology (Supplementary Data Fig. S3). ITS sequence analysis showed that the genome of *A. sylvestris* was involved in the formation of *A. baldensis* before the Pleistocene glaciations. A mutation rate of roughly one mutation in the ITS region every 100 000 years (Kay *et al.*, 2006) suggests that hexaploid *A. baldensis* originated between 3 and 2·5 Mya. Range contraction/expansion cycles during the Quaternary led to subsequent migration of hexaploid *A. baldensis* southward in Europe. In the same period, *A. multifida* could have spread across North America and then migrated to South America along with the glacial range expansion. Postglacially, i.e. during the last 10 000– 15 000 years, *A. baldensis* and *A. multifida* have probably achieved their present disjunctive distribution in the mountains of southern Europe and South America, respectively.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob. oxfordjournals.org and consist of the following. Fig. S1: NTS sequence alignment with species-specific sequences marked. Fig. S2: GISH on partial metaphase plate of *A. baldensis* with labelled gDNA of *A. multifida*. Fig. S3. Achene fruits and leaves of *Anemone* species, except *A. virginiana* where a bud is shown.

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