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Hybrid origin and differentiation of two tetraploid *Achillea* species in East Asia: molecular, morphological and ecogeographical evidence

Y.-P. GUO,*‡ C. VOGL,† M. VAN LOO* and F. EHRENDORFER*
*Department of Systematic and Evolutionary Botany, Faculty of Life Sciences, University of Vienna, A-1030 Vienna, Austria, †Institute of Animal Breeding and Genetics, University of Veterinary Medicine, A-1210 Vienna, Austria, ‡Faculty of Life Sciences, Beijing Normal University, Beijing 100875, China

Abstract

***Achillea* (Asteraceae-Anthemideae) offers classical models for speciation by hybridization and polyploidy. Here, we test the suspected allotetraploid origin of two species, *Achillea alpina* and *Achillea wilsoniana* between phylogenetically distinct lineages in East Asia. A total of 421 AFLP bands from 169 individuals and 19 populations of five 2x- and two 4x-species were obtained. The data set was analysed with a newly developed model that accounts for polyploidy and assumes lack of recombination between the parental chromosome sets (i.e. disomic inheritance). *A. alpina* and *A. wilsoniana* then appear to be allotetraploids between *Achillea acuminata*-2x (sect. *Ptarmica*) and *Achillea asiatica*-2x (sect. *Achillea*). The two 4x-species share 44% and 48% of their AFLP bands with *A. acuminata*-2x, and 39% and 38% with *A. asiatica*-2x, respectively. Eight plastid haplotypes (A–H) were detected by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analyses. *A. alpina*-4x and *A. wilsoniana*-4x share haplotype F only with *A. asiatica*-2x. This is consistent with the hybrid origin(s) involving the latter as the maternal ancestor. This result corroborates our previous DNA sequence data, where *A. alpina*-4x and *A. wilsoniana*-4x are also placed close to *A. asiatica*-2x. Morphology, ecology, and amplified fragment length polymorphism (AFLP) profiles of the two 2x-species are distinct, whereas the two 4x-species, grouped as *A. alpina* aggregate, form a nearly continuous link between them. Considering all evidence, this 4x-aggregate is regarded as the product of a hybridization between genetically distant 2x-ancestors limited to China and adjacent areas: one *A. acuminata*-like, and the other *A. asiatica*-like. The allopolyploid *A. alpina* agg. exhibits considerable morphological variation and ecological flexibility, and has expanded throughout eastern Asia and to northern North America, far beyond the ranges of their presumed 2x-ancestors.**

Keywords: *Achillea*, AFLP, dominant markers, hybridization, plastid PCR–RFLP, polyploidy

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Introduction

Hybridization plays an important role in evolution (Stebbins 1950, 1959; Barton & Hewitt 1985; Arnold 1997; Rieseberg 1997). In modern angiosperms, hybridization events are estimated to be involved in the origin of 30–80% of species (Rieseberg & Ellstrand 1993; Arnold 2004). Speciation results from interspecific hybridization when postzygotic barriers lead to reproductive isolation between the hybrids and their sympatric parental species (Stebbins 1959; Rieseberg & Wendel 1993; Arnold 1997; Abbott 2003; Rieseberg *et al*. 2003). At the diploid level, recombinational speciation has been documented only in some cases (Grant 1981; Rieseberg & Wendel 1993; Rieseberg 1997); in contrast, hybridization via polyploidy is a major mechanism of speciation in plants (Thompson & Lumaret 1992; Otto & Whitton 2000; Bennett 2004; Soltis 2005). Rapid genetic changes after the formation of allopolyploids, such as genomic rearrangements, gene silencing, and divergence in the

Correspondence: Friedrich Ehrendorfer, Fax: +43 (1) 4277 9541; E-mail: friedrich.ehrendorfer@univie.ac.at

function or expression of duplicated genes (Shaked *et al*. 2001; Skalická *et al*. 2005 and other references cited in Soltis 2005), can produce shifts in morphology and ecological tolerance, and thus greatly increase the potentials for adaptation in polyploid lineages. Well-studied examples are North American *Tragopogon* (Ownbey 1950; Soltis *et al*. 2003, 2004; Pires *et al*. 2004), New Zealand and Australian *Microseris* (Vijverberg *et al*. 2000), Hawaiian *Madiinae* (Baldwin 2003) or arctic taxa of *Draba*, etc. (Brochmann *et al*. 2004). These studies have contributed much to our knowledge of cytogenetic and molecular mechanisms underlying hybrid and polyploid speciation and subsequent adaptive radiation (Thompson & Lumaret 1992; Leitch & Bennett 1997; Ramsey & Schemske 1998, 2002; Soltis & Soltis 1999, 2000; Otto & Whitton 2000; Wendel 2000; Soltis *et al*. 2003; Soltis 2005).

The northern temperate angiosperm genus *Achillea* (Asteraceae-Anthemideae), and in particular its polyploid complex *Achillea millefolium* agg., provides classical models for evolutionary radiation through hybridization and polyploidy (Clausen *et al*. 1948; Ehrendorfer 1959; Hiesey & Nobs 1970; Tyrl 1975; Vetter *et al*. 1996a; Vetter *et al*. 1996b; Guo *et al*. 2004, 2005; Saukel *et al*. 2004). Members of this genus are allogamous and suffruticose or herbaceous perennials. Earlier biosystematic studies and our recent molecular data have revealed several hybrid and polyploid lineages and their considerable ecogeographical radiation (Ehrendorfer 1959; Guo *et al*. 2004, 2005; Saukel *et al*. 2004). Among them, two tetraploid species, *Achillea alpina* L. (= *Achillea sibirica* Ledeb.) and *Achillea wilsoniana* (Heimerl) Heimerl ex Hand.-Mazz., are suspected to have originated by hybridization between two phylogenetically distinct diploid taxa from section *Achillea* (*A. millefolium* agg.) and section *Ptarmica* in northern China (Guo *et al*. 2004, 2005). The two 4x-taxa link their assumed parental 2x-species with respect to morphology, ecology and distribution, but extend far beyond their contact zone into much of East Asia and northern North America. Together with other closely related taxa, placed into section *Ptarmica* by Shih & Fu (1983) and Afanasyev & Bochantsev (1995), they form the enormously variable 4x-complex of *A. alpina* agg. Our earlier DNA sequence data (nrITS and plastid *trnL-F*) have shown that *A. alpina*-4x and *A. wilsoniana*-4x are nested in a poorly resolved clade together with taxa of *A. millefolium* agg. (Guo *et al*. 2004). However, a subsequent amplified fragment length polymorphism (AFLP) study (Guo *et al*. 2005) demonstrates a hybrid genetic bridge formed by *A. alpina*-4x and *A. wilsoniana*-4x between the polyploid complex of *A. millefolium* agg. and *Achillea acuminata* (Ledeb.) Schultz Bip., a 2x-species of section *Ptarmica*. From the *A. millefolium* aggregate, *Achillea asiatica* Serg. was identified as the most likely 2x-progenitor. But the parentage from section *Ptarmica* remains uncertain because no other AFLP profiles from 2x-relatives of *A. acuminata*-2x were available then. Furthermore, the hybrid origin of *A. alpina*-4x and *A. wilsoniana*-4x, tentatively deduced from the distribution patterns of specific AFLP bands, needs to be tested more rigorously.

In the present study, we have generated AFLP data from a considerably larger population sample, focusing on *A. alpina*-4x and *A. wilsoniana*-4x and their possible 2x-parental species. The dominant nature of AFLP markers and the partly polyploid nature of our plant material make data analyses problematic with the existing statistical methods, which are predominately assuming codominant markers and/or diploidy (Lynch & Milligan 1994; Wolfe & Liston 1998; Holsinger *et al*. 2002). In polyploids, even codominant markers (e.g. microsatellites) may give ambiguous results. The AFLP method has the advantage that the large number of loci covers essentially the entire genome (Vos *et al*. 1995), and thus makes it possible to detect the genetic relationships among parental and hybrid taxa (O'Hanlon *et al*. 1999; Bensch *et al*. 2002; Guo *et al*. 2005). Therefore, we propose a new method for analysing AFLP data from populations at different ploidy level with hybrid relationships.

Allopolyploids are often of polyphyletic and polytopic origin (Soltis *et al*. 1995, 2003; Soltis 2005 and references therein). To address this, maternally inherited plastid DNA is commonly used to provide evidence for the maternal lineages of hybrids (Soltis & Soltis 1989; Gross *et al*. 2003). For this purpose, we have additionally employed polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) of plastid DNA to evaluate the single or multiple origins of the assumed allotetraploids, and to identify their maternal parent(s).

By combining our new AFLP and plastid PCR–RFLP analyses with previous results (Guo *et al*. 2004, 2005), and with morphological and ecogeographical evidence, we elaborate a model for allopolyploid speciation between phylogenetically distinct taxa. Two major questions are addressed: Which of the 2x-species contributed to the hybrid 4x-taxa? And what are the evolutionary consequences of allopolyploidization?

Materials and methods

Sampling

Individuals from 19 populations of five diploid species from section *Ptarmica* and section *Achillea* (*A. millefolium* agg.), and of the two assumed allotetraploids linking the two sections were collected (Table 1). In most cases, about (9)10–12(13) individuals were sampled for each population. As the plants studied expand clonally from rhizomes, only individuals located sufficiently distant from each other were collected to avoid samples from the same clone. All vouchers are deposited in the herbarium of the Institute of Botany and the Institute of Pharmacognosy, both in the University of Vienna, Austria (WU).

Table 1 Taxa and populations of *Achillea* sect. *Ptarmica*, sect. *Achillea,* and suspected hybrid derivatives analysed with AFLP and plastid PCR-RFLP

Taxa	Ploidy level*	Pop. code	Pop. no.	Locality and habitat	Collectors & vouchers†‡	Sample size AFLP/RFLP
I. Sect. *Ptarmica* (DC.) W. Koch						
1. *A. ptarmica* L.	2x	K8	329	Ukraine: N Kiev, Romanovka; wet depressions	FE & YG. 2003.07.23	7/2
2. *A. salicifolia* Ledeb. ex Reichenb.	2x	K7	328	Ukraine: N Kiev, Romanovka; mesic grass- and bushland	FE & YG. 2003.07.23	5/–
—	2x	K14	340	Ukraine: Kiev, S Desna mouth into Dnjepr river, *c.* 100 m; alluvial meadows and forest margins	FE & YG. 2003.07.28	6/–
3. *A. impatiens* L.	(2x)	AL9491	355	Russia: Altai, Aktash, 50°22′50″N, 87°37′24″E, 1800–1850 m; grazed forest	AT 9491	6/4
—	(2x)	AL4	151	Russia: Altai, 51°02′52″N, 85°36′47″E, 1700 m; open montane forest	MS 2002.07.31	1/–
4. *A. acuminata* (Ledeb.) Schultz Bip.	2x	CB1	118	China: Jilin, Changbai Mountains, Hancong valley, 680–620 m; in moist grassland	YG & GR 0201	12/5
II. Hybrid species: *A. alpina* agg.						
5. *A. alpina* L.	4x	BJ01	05	China: Beijing, Xiaolongmen, 1700 m; along stream, humid habitat	YG & GR BJ01-1	5/1
—	4x	BJ02	06	China: Hebei, Wuling Mountain, 1500–2000 m; along stream	YG & GR BJ02-5	5/2
—	4x	CB2	119	China: Jilin, Changbai Mountains, Hancong valley, 730–780 m; in grassland	YG & GR 0202	10/4
6. *A. wilsoniana* Heimerl ex Hand.-Mazz.	4x	DL	348	China: Yunnan, Dali, Zhonghesi, 2360 m; in open forest, along stream.	YG & GR 0305	12/–
—	(4x)	GZ	350	China: Guizhou, Daozhen, Natural Conserve of Dasha River, 1750 m; in humid grassland	ZL, 2001.08.06.	12/2
—	4x	HX	349	China: Gansu, Huixian, Jialing, 2000 m; on slope, in humid grassland	YG & GR 0306	12/
—	4x	TB	120/121	China: Shaanxi, Taibai Mountain, Taibai county, 1550 m; on slope, in open grassland	YG & GR 0203	12/4
—	4x	ZD	346/347	China: Yunnan, Zhongdian, Wufeng Mountain & Monastry, 3300 m; on slope under trees and in humid grassland	YG & GR 0301	11/2
III. Sect. *Achillea*: *A. millefolium* agg.						
7. *A. asiatica* Serg.	2x	NM1	122	China: Inner Mongolia, Holhot, Daqing Mountain; in meadow at margin of forest, *c.* 1500 m	YG & GR 0201, 0202	–/4
—	2x	NM2	123	China: Inner Mongolia, Zhayouzhongqi, Daqing Mountain, Huiliang valley; in grassland, *c.* 1600 m	YG & GR 0203, 0204	9/4
—	(2x)	XJ1	124	China: Xingjiang, Yili-Nileke, 850 m	DT 2002.05.15	13/–
—	(2x)	XJ2	125	China: Xingjiang, Urümqi county, Salqiaoke, 1550 m; in dry grassland	DT 2002.06.30	10/2
—	2x	Al1	148	Russia: Altai, 51°02′52″N, 85°36′47″E, 1100 m; in mountain grassland	MS 5302	11/6
—	2x	Al2	149	Russia: Altai, 50°20′46′N, 87°24′34′E, 1100 m; in mountain grassland	MS 2002.07.31	10/–
—	2x	AL9611	353	Russia: Altai, 49°50′70″N, 87°52′59″E, 2280–2330 m, open sandy moist grassland	AT 9611	–/1

*Ploidy levels have been checked during the present study; those only estimated from pollen diameter or taken from the literature are shown in brackets. †All vouchers are deposited in the herbaria of the Institute of Botany (WU) and of the Institute of Pharmacogonosy, both at the University of Vienna. ‡Names of collectors: AT, A. Tribsch; DT, D.-Y. Tan; FE, F. Ehrendorfer; GR, G.-Y. Rao; MS, M. Staudinger; YG, Y.-P. Guo; ZL, Z.-Y. Liu.

AFLP data were obtained from 169 individuals from 19 populations. For plastid PCR–RFLP analyses, 43 individuals from 14 populations and six species were analysed (Table 1).

DNA extraction

Total genomic DNA was extracted from *c*. 0.02 g silica gel desiccated leaf material following the 2 × CTAB (cetyltrimethyl ammonium bromide) protocol of Doyle & Doyle (1987). The DNA concentration was estimated photometrically (UV-160A, Shimadzu).

AFLP protocols and the band scoring

Established procedures (Vos *et al*. 1995) and the PE Applied Biosystems (1996) protocol were followed to generate AFLP profiles with slight modifications as follows: total genomic DNA (≈ 500 ng) was digested with *Mse*I and *Eco*RI and then ligated to double-stranded adaptor pairs with T4-ligase (MBI Fermentas) in a combined restriction–ligation reaction for 2 h at 37 °C. Preselective amplification was performed using a primer pair with one single selective nucleotide, *Mse*I-C/*Eco*RI-A. Selective amplifications were conducted with three fluorescence-labelled primer combinations, *Mse*I-CAG/*Eco*RI-ACT (FAM), *Mse*I-CTT/*Eco*RI-ACC (NED) and *Mse*I-CAG/*Eco*RI-AGG (HEX) which, according to our primer trials, reveal both infra- and interpopulation variations by clear bands. The selective amplification products were run in a 4.5% denaturing polyacrylamide gel on an ABI PRISM 377 Sequencer, scanned and analysed with ABI PRISM GENESCAN® 3.1.2, and scored with GENOGRAPHER (version 1.6, ©Montana State University, 1998; http://hordeum.oscs.montana. edu/genographer/) in a size range from 50 to 500 bp. To avoid ambiguities, only bands with sufficient fluorescent intensity were scored and used as markers for the present analyses.

Genetic distance among the diploid species

To assess the genetic relationships among the assumed parental species and other 2x-taxa, a neighbour-joining (NJ) genetic distance analysis was performed with PAUP* 4.0b10 (Swofford 2003) using Nei & Li's (1979) genetic distance. The dendrogram was bootstrapped with 1000 replicates.

Analysis of population structure with populations of varying ploidy level using dominant markers

The phylogenetic relationships of some taxa, in which reticulate evolutionary events have been involved, cannot be properly represented as a tree, but rather as a network (Grant 1981; Doolittle 1999; Linder & Rieseberg 2004). Earlier efforts where the polyploid *Achillea* taxa were analysed within the framework of phylogenetic trees were rather unsuccessful (Guo *et al*. 2004, 2005). Available results suggest that the 4x-taxa in this study are of hybrid origin(s) between particular 2x-species (Guo *et al*. 2004, 2005). Therefore, instead of forcing the data into a tree framework, we propose the following method to infer the 2x-progenitors of the 4x-taxa.

Assume a set of K 2x-populations, out of which we want to infer a combination of two progenitors of a particular 4x-descendant populations. Denote the probability of each of the i, j, with $1 \le i \le K$ and $1 \le j \le i$, pairwise combinations of parentage with ρ_{ij}. The method of inferring ρ_{ij} consists of two steps, of which the first step is independent from the second. First, we estimate the population allelic proportions separately and independently within K 2x-populations in the presence of diallelic dominant markers assuming Hardy–Weinberg equilibrium using a Markov chain Monte Carlo method. We start with a guess of the population allelic proportions of the dominant allele for a particular locus (p_l). We then sampled the diploid genotype for eac individual separately and conditioned on their respective data. If, for example, the individual is showing the recessive phenotype at this locus, we infer two recessive alleles in all cases; if the individual is showing the dominant phenotype at this locus, we infer one dominant allele with probability proportional to $2(1 - p_l)p_l$, and two dominant alleles with probability proportional to p_l^2. Conditional on these inferred allelic frequencies, we calculate the population allelic proportions p_l. This cycle is repeated until approximate convergence, which is quite fast in this case (within 100 iterations or so).

Second, we calculate the probability of the 4x-marker phenotype for each locus and individual as follows: we assume that two 2x-populations i and j contribute 2x-genotypes according to their respective allelic proportions in the 2x-Hardy–Weinberg equilibrium. The 4x-phenotype is then a combination of the two independent 2x-genotypes, i.e. disomic inheritance is assumed. The probability of the recessive 4x-phenotype is then for example the product of the probabilities of the recessive phenotype in populations i and j. Assuming independence among loci, the probabilities of the loci are combined, such that we obtain the probability of a 4x-population to have the i, jth combination of the 2x-parents, ρ_{ij}. Iterating between the first and second steps will eventually approximate the posterior distribution. This posterior distribution consists of the posterior probabilities of the $K(K - 1)/2$ combinations of two 2x-taxa being the parents of a 4x-taxon, which we set out to infer.

Identification of specific AFLP bands in 2x- and 4x-taxa

To further illustrate the putative hybrid origin of the two 4x-taxa, we made a two-step procedure to trace the distribution in the tetraploids of specific AFLP bands of

the diploid species. First, we identified AFLP bands that are exclusive to each of the 2x-species with frequencies of 90% or more in at least one of its populations, and then checked for the presence of these bands in the 4x-species. Second, we categorized such exclusive bands of the presumed parental 2x-species as private or shared in different combinations relative to the 4x-species, and calculated their frequencies in each of the 2x-progenitors and the 4x-progenies. Furthermore, bands specific to the 4x-level were also checked.

PCR-based plastid RFLP analyses

Total DNA was used as template in the PCRs for amplifying five plastid DNA fragments with primers *psa*A/*trn*Sr (AS), *trn*H/*trn*K1r (HK), *psb*C/*trn*Sr (CS), *trn*S/*trn*Tr (ST) (Demesure *et al*. 1995), and with *trn*V/*rbc*Lr (VL) (Dumolin-Lapègue *et al*. 1997). Reactions were carried out in a volume of 25 μL containing 20–30 ng of template DNA, 12.5 μL of 2 × buffer, 2 μM of each primer, 0.5 unit of Expand Long Template PCR System (Roche Diagnostics GmbH) and sterile water added to the total volume [2 × buffer (1 mL): 200 μL of 10 × buffer 2 (provided with the kit and containing 2.25 mM of $MgCl_2$), 0.2 mM dNTPs and sterile water up to 1 mL]. Amplifications were conducted in a GeneAmp® PCR System 9700 (Applied Biosystems) with the following cycles: (1) 94 °C, 4 min; (2–41) 93 °C, 45 s; 58 °C, 45 s; 72 °C, 2 min (for ST, HK, and CS) or 4 min (for AS, VL); (43) 72 °C, 10 min. The amplified fragments (5 μL) were digested with restriction enzymes *Mnl*I (for VL fragment and AS fragment), *Hae*III (for CS fragment and ST fragment), and *Alu*I (for HK fragment) in a volume of 20 μL following the manufacturer's instructions, and then separated (5 μL) on a 0.5 mm thick nondenaturing 8% polyacrylamide gel (PAG) in 1% TBE at 500 V for 4–4.5 h. Two gels were run simultaneously under the same conditions on the Dual Vertical Slab Gel System DSG-250 (CBS), along with a cooling unit (14 °C). For visualization of plastid restriction fragments, PAGs were silver-stained (Krystufek 2001).

The PCR–RFLP data were scored as multistate unordered characters. Each clearly visible and recognizable polymorphic restriction band on the polyacrylamide gel (PAG) was regarded as a character and its states as different alleles. The combination pattern of all these alleles within an individual forms a haplotype. Haplotypes were analysed using the NJ genetic distance method (total character difference) with PAUP* 4.0b10 (Swofford 2003).

Results

AFLP analyses

Three AFLP primer combinations yielded 421 clearly identifiable bands from 169 individuals and 19 populations of five 2x-taxa from *Achillea* sect. *Ptarmica* and sect. *Achillea* (*A. millefolium* agg.), and two assumed allotetraploid species. From these AFLP bands, 415 (98.6%) were polymorphic. The two 4x-taxa have more AFLP bands (about 165 bands per individual) than any of the 2x-species studied (about 126 bands per individual).

The NJ analysis (Fig. 1) shows the five 2x-species each to be monophyletic (bootstrap percentage 99/100). *A. asiatica* (sect. *Achillea*) is clearly separated from the four species in section *Ptarmica*. The coherence of the latter as a section, however, is not well supported by bootstrap. Apparently, AFLP variation within populations and species is lower in section *Ptarmica* species than in *A. asiatica*.

The 10 possible pairwise combinations of the five 2x-species (*A. acuminata*, *A. ptarmica*, *A. salicifolia*, *A. impatiens*, and *A. asiatica*) were compared for their potential to generate the two 4x-species *Achillea alpina* and *Achillea wilsoniana*. With the full data set, the posterior probabilities of all these combinations gave a clear result: the parental combination of *A. acuminata*-2x and *A. asiatica*-2x gives a posterior probability of one for both *A. alpina*-4x and *A. wilsoniana*-4x, all others zero (Table 2). This method could thus clearly resolve the parentage.

Table 3 shows the number of exclusive AFLP bands for each of the five 2x-species. Some of them are private to a species, others are shared by one or both of the 4x-species. Bands common to the 2x- and 4x-level can be mostly attributed to *A. acuminata*-2x and *A. asiatica*-2x, whereas exclusive bands of the other three 2x-species from *Achillea* sect. *Ptarmica* are mostly private.

Furthermore, Fig. 2 shows the frequencies of exclusive AFLP bands from the two presumed parental taxa *A. acuminata*-2x and *A. asiatica*-2x in the derived 4x-species. In addition, at the 4x-level there are some novel bands, partly shared but mostly private to either *A. alpina* or *A. wilsoniana*.

Plastid DNA polymorphisms

In total, 32 restriction bands from 5 amplified plastid DNA fragments were obtained: 11 from VL/*Mnl*I, 7 from AS/*Mnl*I, 4 from CS/*Hae*III, 2 from ST/*Hae*III, and 8 from HK/*Alu*I.

The polymorphisms of scored bands from digested fragments VL, AS, CS, HK, and ST correspond to their DNA sequence variation (indels/substitutions) and are expressed on the polyacrylamide gels as different electrophoretic mobility of bands. In addition, two gains/losses of restriction sites were found in HK/*Alu*I digests. These lead to two extra bands in the HK digests of *A. acuminata* when compared to all other taxa analysed.

From 43 individuals, 14 populations and six species, a total of eight different plastid haplotypes (A to H) was recognized (Table 4). All populations are monotypic, each

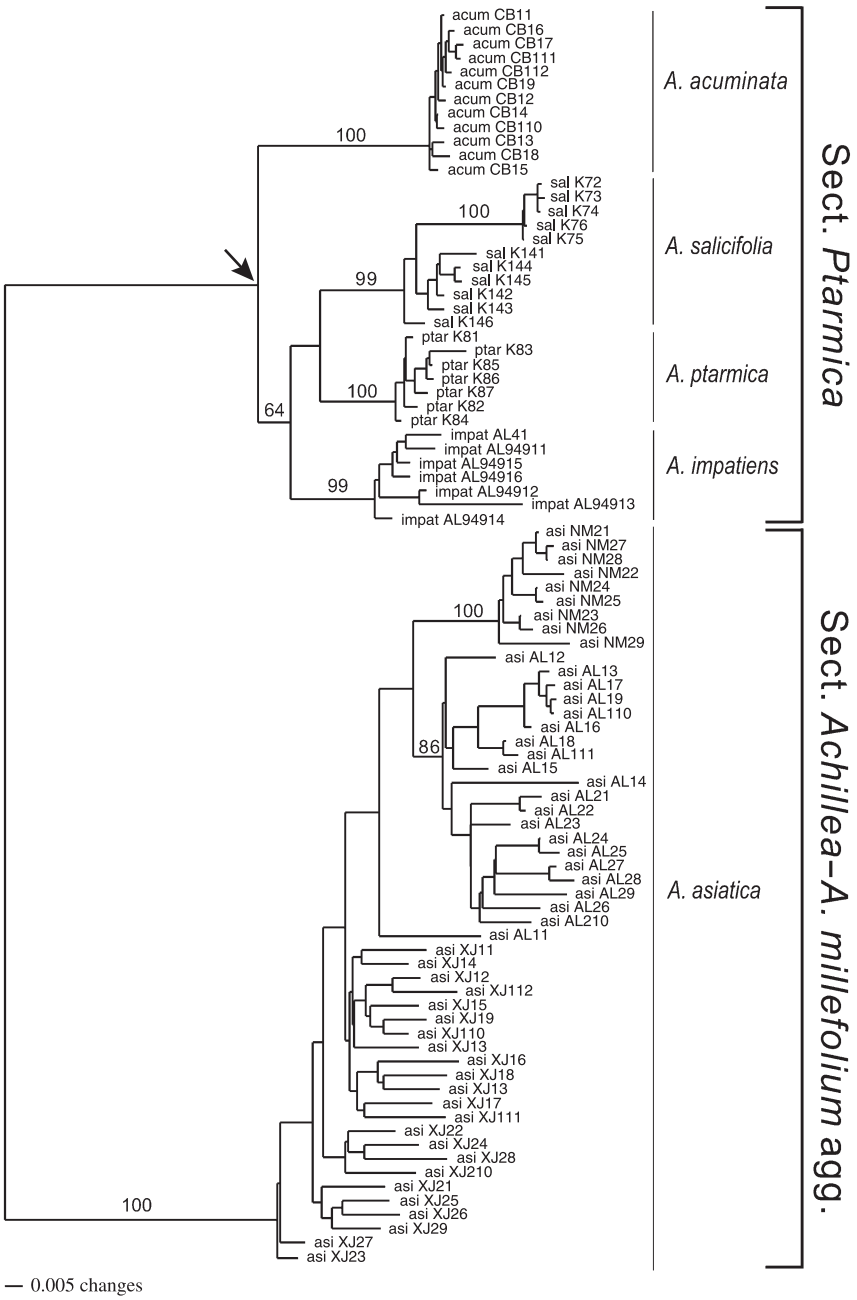

Fig. 1 Neighbour-joining phylogram (Nei–Li distance; midpoint rooting) for 90 individuals from 11 populations and five 2x-species of *Achillea* sect. *Ptarmica* and sect. *Achillea*. The phylogram was constructed with 421 AFLP bands. Bootstrap percentages (> 50%) are shown above branches. A branch that collapses in the > 50% majority-rule bootstrap consensus tree is marked by an arrow.

containing only one haplotype. In the populations of *A. asiatica*-2x four different haplotypes were found (D, E, F, and H), of which F is the most frequent and also occurs in the two 4x-taxa. Haplotype F was identified in two populations of *A. alpina* (BJ01 and CB2) and is common in *A. wilsoniana*. Thus, the 4x-species share haplotype F with *A. asiatica* only. Haplotype G is limited to the population BJ02 of *A. alpina* and has not yet been found in another taxon. An NJ phylogram demonstrates the relationships of the eight plastid haplotypes and shows haplotype G closest to F (Fig. 3).

Discussion

Evidence for the hybrid relationships between genetically distinct Achillea *taxa*

Our previous nrITS and plastid *trnL-F* sequence data support, in principal, the traditional subdivision of the genus *Achillea* into 5–6 sections (Heimerl 1884; Shih & Fu 1983; Afanasyev & Bochantsev 1995; Guo *et al*. 2004; Saukel *et al*. 2004; Ehrendorfer & Guo 2005). Except for *Achillea*

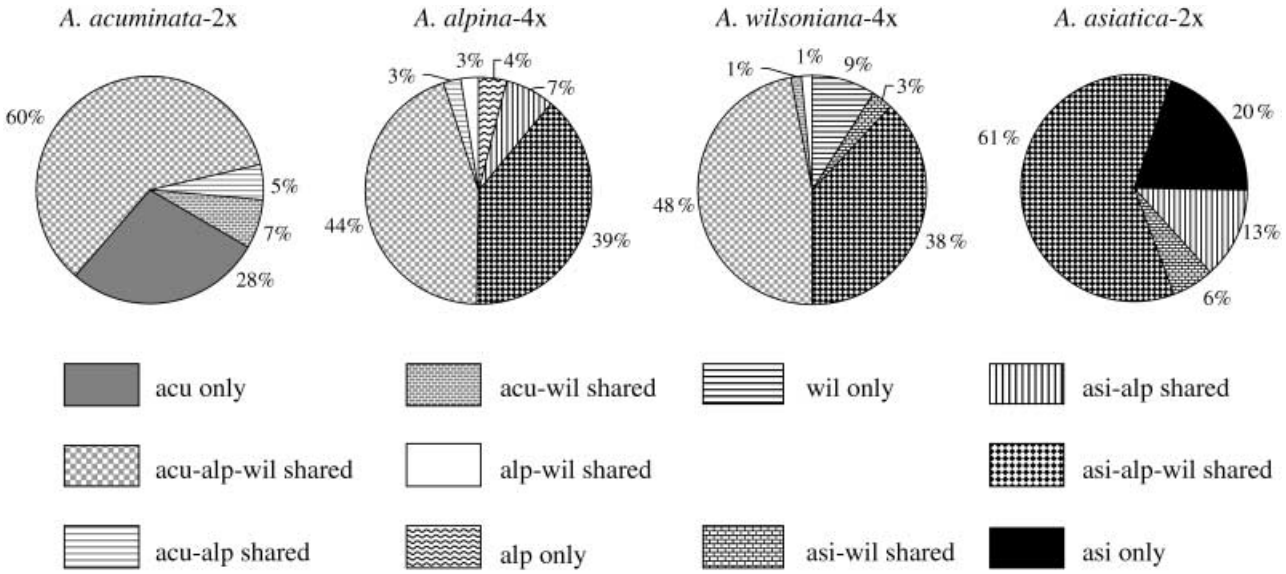

Fig. 2 Frequencies of species-specific and species-shared AFLP bands in populations of *Achillea acuminata*-2x, *Achillea alpina*-4x, *Achillea wilsoniana*-4x, and *Achillea asiatica*-2x in East Asia. The 4x-species share many AFLP bands with the two presumed 2x parental species, but also exhibit a few specific bands.

Table 2 Posterior probabilities for all the possible parentage combinations of *Achillea alpina*-4x and *Achillea wilsoniana*-4x according to AFLP allelic proportions (1000 of a 1000 iterations) assuming lack of recombination between parental chromosome sets

	acu-2x	sal-2x	imp-2x	pta-2x	asi-2x
acu-2x	—				
sal-2x	0	—			
imp-2x	0	0	—		
pta-2x	0	0	0	—	
asi-2x	1	0	0	0	—

Abbreviations: acu, *Achillea acuminata*; imp, *Achillea impatiens*; sal, *Achillea salicifolia*; pta, *Achillea ptarmica*; alp, *Achillea alpina*; wil, *Achillea wilsoniana*; asi, *Achillea asiatica*.

Table 3 Number of exclusive AFLP bands from each of the five 2x-species studied and their occurrence in the 4x-species

		No. of bands			
Species	Total	Private	In alp-4x	In wil-4x	In both 4x-species
acu-2x	36	10	3	4	19
sal-2x	10	9	1	—	—
imp-2x	4	4	—	—	—
pta-2x	11	10	—	—	1
asi-2x	74	19	8	2	45

Abbreviations: acu, *Achillea acuminata*; imp, *Achillea impatiens*; sal, *Achillea salicifolia*; pta, *Achillea ptarmica*; alp, *Achillea alpina*; wil, *Achillea wilsoniana*; asi, *Achillea asiatica*.

asiatica-2x, all taxa treated in the present study have been classified as members of the section *Ptarmica* s.str. This has been verified by the DNA sequence data only for the 2x-members *Achillea acuminata*, *Achillea impatiens*, *Achillea ptarmica*, and *Achillea salicifolia*, whereas the 4x-taxa *Achillea alpina* and *Achillea wilsoniana* do not associate with section *Ptarmica* s.str. but are placed far away into the neighbourhood of *Achillea millefolium* agg., sect. *Achillea* (Guo *et al*. 2004). Subsequent AFLP analyses (Guo *et al*. 2005) have demonstrated that, from all the taxa within *A. millefolium* agg., *A. asiatica*-2x has the closest relationships with *A. alpina*-4x and *A. wilsoniana*-4x. Furthermore, we suggested that these two 4x-taxa form a genetic bridge between *A. acuminata*-2x and *A. asiatica*-2x, i.e. between the sections *Ptarmica* s.str. and *Achillea*. In our former studies, only *A. acuminata* was available as a representative of section *Ptarmica* s.str. Therefore, in this study, we have investigated other taxa of this section which could have figured as the second parent of the two tetraploids.

The present data show that *A. alpina*-4x and *A. wilsoniana*-4x have more AFLP bands than any of the 2x-taxa. This is expected due to the dominant nature of the markers and the higher number of alleles in the polyploids.

The NJ phylogram (Fig. 1) suggests that the 2x-members of section *Ptarmica* are clearly differentiated from each other, with *A. acuminata* slightly set aside, but that internal branches are short and not well supported.

Under the cytogenetic concept, allotetraploids are characterized by disomic inheritance (Stebbins 1950, 1971; Ramsey & Schemske 2002). *A. alpina*-4x and *A. wilsoniana*-4x

Table 4 Eight plastid haplotypes found in 13 populations and six *Achillea* species, three 2x-species of section *Ptarmica* (I), two of the 4x-*A. alpina* agg. (II) and one 2x-species of section *Achillea–A. millefolium* agg. (III), as revealed by restriction fragment length polymorphism from five amplified plastid DNA fragments. Population codes as in Table 1

Taxa	Pop. code	Haplotype
A. acuminata-2x (I)	CB1	A
A. impatiens-2x (I)	AL9491	B
A. ptarmica-2x (I)	K8	C
A. alpina-4x (II)	BJ01	F
—	BJ02	G
—	CB2	F
A. wilsoniana-4x (II)	GZ	F
—	TB	F
—	ZD	F
A. asiatica-2x (III)	NM1	F
—	NM2	F
—	XJ2	E
—	AL1	D
—	AL9611	H

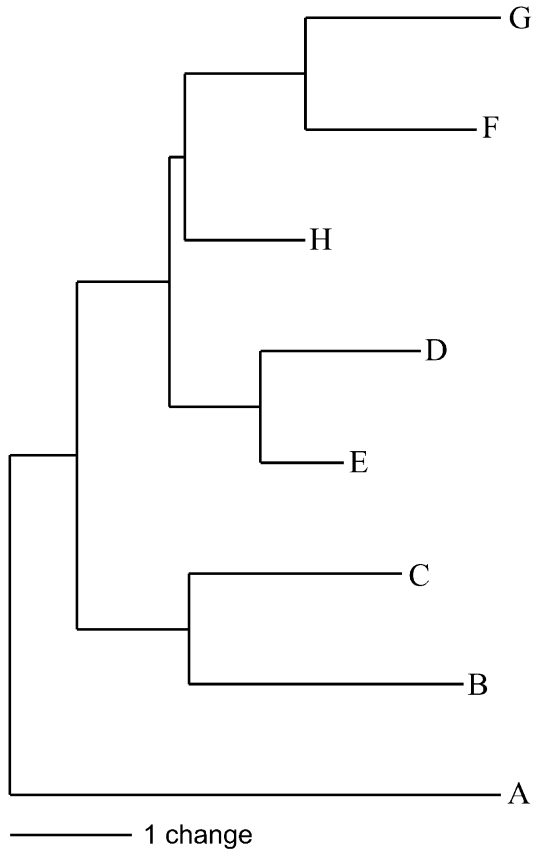

Fig. 3 Relationships of the eight plastid haplotypes found in 2x-taxa of *Achillea* sect. *Ptarmica* (A–C), the suspected allotetraploids of *A. alpina* agg. (F, G), and sect. *Achillea–A. millefolium* agg: *A. asiatica*-2x (D, E, F, H). For populations and taxa, see Table 4. The phylogram is constructed by neighbour-joining genetic distance analyses with total character distances.

were suggested to have originated by hybridization between two phylogenetically distinct diploid taxa (Guo *et al*. 2004, 2005). We thus assume that the chromosome sets of the two 2x-parents do not recombine in the tetraploids, i.e. that they behave as if they were genetically diploid due to the 'fixed heterozygosity' inherited in a disomic manner. With this assumption, resolution was achieved. The analysis of all 2x- and 4x-taxa showed that only the combination *A. asiatica* × *A. acuminata* could have been involved in the origin of *A. alpina* and *A. wilsoniana*, a conclusion supported by a posterior probability of one (Table 2). Therefore, the two 4x-taxa appear as allopolyploids that have kept their two, genetically rather distinct parental chromosome sets relatively intact. Although genomic rearrangements are important consequences of polyploidy, similar cases of limited genomic reorganization or chromosome repatterning in allopolyploids relative to their diploid progenitors are known from other neo- as well as palaeopolyploids (Soltis 2005 and references therein).

Many specific AFLP bands from each of the two presumed 2x-species occur in the 4x-species (Table 3; Fig. 2; Guo *et al*. 2005). In contrast, the two parental species share only few AFLP bands (not shown in Fig. 2). This is consistent with their rather large phylogenetic distance (Guo *et al*. 2004).

Achillea alpina and *A. wilsoniana* share the plastid haplotype F only with *A. asiatica* (Table 4, Fig. 3), indicating the latter as their maternal parent. This parallels our earlier results from the ITS and *trnL-F* sequence analyses (Guo *et al*. 2004). The ITS sequences of *A. alpina* and *A. wilsoniana* are close to those of members of section *Achillea–A. millefolium* agg. This provides an example for the homogenization of ITS through gene conversion in allopolyploids towards one of the parental taxa, often the maternal one (Chase *et al*. 2003). These findings are consistent with the hybrid origin(s) of the allotetraploids involving *A. asiatica*-2x as the maternal parent.

Do morphological data and ecogeographical parameters fit the DNA-based hypothesis of *A. asiatica*-2x and *A. acuminata*-2x as parental taxa of the allotetraploid *A. alpina* and *A. wilsoniana*? The two 2x-species exhibit obvious differences in leaf shape: deeply divided into linear segments and threefold pinnatisect in the former; undivided and dentate in the latter (Fig. 4a, b, g). *A. alpina*-4x and *A. wilsoniana*-4x exhibit a range of leaf shapes that continuously link these two extremes (Fig. 4c–f). *A. acuminata*-2x has few but large flower heads with up to 12 large ligulate flowers (Fig. 4h). In contrast, *A. asiatica*-2x has numerous but much smaller heads and 5(–6) short ligulate flowers (Fig. 4k). Again, the 4x-taxa exhibit inflorescences intermediate between these two extreme states (Fig. 4i, j).

Fig. 4 Morphological differentiation of the series *Achillea acuminata*-2x (a, b, h), *Achillea alpina*-4x (c, d, i), *Achillea wilsoniana*-4x (e, f, j) and *Achillea asiatica*-2x (g, k): lower stem leaves (a–g; natural size) and inflorescences (h–k; h–j ≈ 3/4, k ≈ 1/2 natural size).

Our field observations and herbarium studies have clarified the geographical distribution and ecological differentiation among the 2x- and 4x-taxa in China. *A. asiatica*-2x is widespread in the northern regions (Xinjiang, Inner Mongolia, Hebei, and Heilongjiang) and extends into adjacent Mongolia and Siberia. *A. acuminata*-2x occurs from the northeast (Heilongjiang) to the northwest (Shaanxi, Ningxia), but also reaches eastern Siberia and possibly Mongolia. *A. alpina*-4x is widespread and has a similar distribution in China as *A. acuminata*-2x. In contrast, *A. wilsoniana*-4x is endemic to China and extends from the southwestern region (Yunnan, Guizhou, Sichuan) to the Qin Mountains in the north (Shaanxi and Gansu) and to Shenlongjia Mountain in the east. Thus, distribution areas of the four taxa partly overlap in northern China (as shown by Meusel *et al*. 1991/1992: maps 478b, c, and 479b), where one can postulate the origin of the 4x- from the 2x-species. Nevertheless, we have been unable to confirm the sympatric occurrence of *A. acuminata* and *A. wilsoniana* on Qin Mountain (according to Shih & Fu 1983 and herbarium records).

The four 2x- and 4x-taxa are differentiated ecologically along a gradient from wet to xeric. *A. acuminata*-2x mostly occurs in wet montane habitats. Its population CB1 (Table 1: Changbai Mountains, northeastern China) was found in wet to moist grassland near a stream. In the same general area, the population CB2 of *A. alpina*-4x is much more widespread in drier grassland or even in uncultivated fields. Therefore, their different ecological preferences separate them well in regions of overlap. Generally, *A. alpina*-4x is often seen in the montane region in the north, while, *A. wilsoniana*-4x mostly occurs in the high mountains and the alpine region of southwestern China, where it grows in mesophytic (e.g. population TB) to ± hygrophytic vegetation (e.g. ZD). In contrast to these taxa from wet to mesic and often montane habitats, *A. asiatica*-2x prefers more xeric localities, from lowland to subalpine regions, where it often occurs in rather open xerophytic vegetation dominated by graminoids.

Thus, the available morphological and ecogeographical evidence support our conclusions of the hybrid origin of the *A. alpina*-4x and *A. wilsoniana*-4x, based on nuclear ribosomal and plastid DNA sequences, AFLP, and plastid RFLP data. But did the two 4x-species originate from one or many allopolyploidization events? Three findings from the present study may provide clues for answering this question: first, the two 4x-species share many AFLP bands (86–87%, Fig. 2); second, from the four plastid haplotypes found in *A. asiatica*-2x, only type F has been found in the 4x-taxa; and third, *A. alpina*-4x and *A. wilsoniana*-4x not only occupy widely overlapping areas in China, but they apparently are also connected by intermediate populations. Accordingly, we favour as working hypothesis that the 4x-taxa *A. alpina* and *A. wilsoniana* should be regarded as the monophyletic product of a single allopolyploidization event with a 2x-*A. asiatica*-like ancestor from section *Achillea* as maternal parent, and a 2x-*A. acuminata*-like ancestor from section *Ptarmica* as paternal parent. In view of other possible interpretations (e.g. polyphyletic origins of the 4x-species) which could arise due to a broader sampling strategy, we are planning more studies, including crosses among the putative parental species.

Differentiation and expansion of the allopolyploid species

Post-hybridization genetic differentiation on the 4x-level is suggested by haplotype G which is limited to population BJ02 of *A. alpina*. It might have originated from the similar and common haplotype F (Fig. 3). Our AFLP data show that in addition to the parental bands, a number of private bands are found at the 4x-level only, some specific to *A. alpina* (4%) or *A. wilsoniana* (9%), some in common (3% and 1%, respectively) (Fig. 2). These apparent 4x-novelties could be explained by the limited sampling of the parental species, or by accumulation of genetic changes and a relatively ancient origin of the allotetraploids. Together with gene silencing and divergent expression of the duplicated genes within the polyploid genomes (Ramsey & Schemske 1998, 2002; Soltis & Soltis 1999, 2000; Wendel 2000; Soltis 2005 and references therein), these genetic changes may have stimulated the morphological and ecogeographical differentiation of the 4x-populations.

This differentiation has resulted in a most successful development of the original allo-4x populations into a very polymorphic 4x-complex which has expanded far beyond the distribution of the presumed 2x-ancestral species. We propose to call this complex *A. alpina* aggregate from the oldest name available, i.e. *A. alpina* L. (1753), corresponding to the formerly used *Achillea sibirica* Ledeb. (1811). In addition to *A. wilsoniana* (Heimerl) Heimerl ex Hand.-Mazz. (1936), this complex includes a number of other taxa of dubious specific or infraspecific status, in particular *Achillea mongolica* Fisch. ex Spreng. (1818), *Achillea multiflora* Hook. (1840), *Achillea ptarmicoides* Maxim. (1859), *Achillea pulchra* Koidz. (1918), *Achillea sinensis* Heimerl ex Hand.-Mazz. (1938), *A. sibirica* ssp. *japonica* Heimerl (1884), ssp. *camtschatica* Heimerl (1884), ssp. *subcartilaginea* Heimerl (1884), var. *angustifolia* (Hara) Ohwi 1965, var. *brevidens* (Mak.) Ohwi 1965, etc. Chromosome counts for this 4x-*A. alpina* agg. are available from most of the Chinese populations studied here (Table 1) and from literature references concerning central and eastern Siberian, Mongolian, Japanese and Canadian provenances. The enormous distribution area of 4x-*A. alpina* agg. in the Northern Hemisphere is shown by Meusel *et al*. (1991/1992) on map 478d and reaches from China to Nepal, Tibet, Mongolia, Korea, Japan (Kyushu to Hokkaido), central and eastern Siberia, including Sakhalin and Kamchatka, Alaska, and through northern North America to the Gaspé Peninsula in the east.

Comparable expansions and radiations have also occurred in *Achillea millefolium* agg. (Ehrendorfer 1959; Hiesey & Nobs 1970; Guo *et al*. 2005) and are well documented for many other angiosperm polyploid complexes, e.g. *Senecio* (Harris & Ingram 1992; Abbott *et al*. 2000), Hawaiian *Madiinae* (Baldwin 2003), North American *Tragopogon* (Soltis *et al*. 2003) and Australian *Lepidium* (Mummenhoff *et al*. 2004), etc. All these cases demonstrate the potentials of allopolyploids for ecogeographical radiation and innovative evolutionary differentiation.

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This study was part of Yan-Ping Guo's PhD dissertation work under the supervision of Friedrich Ehrendorfer at the Institute of Botany, University of Vienna. F. Ehrendorfer is generally interested in Seed Plant evolution. At present he concentrates on a research project concerning evolutionary patterns and processes in the genus *Achillea* (Asteraceae) as models for the increase of species diversity through differentiation-hybridization-cycles. Y.-P. Guo's interests include the role of hybridization and polyploidy in the phylogeny of Higher Plants. C. Vogl concentrates on problems of population genetics and M. van Loo at present is a postdoctoral research associate working at the Jodrell Laboratories of Kew Gardens, UK, on hybrid zone persistence in European poplars.
