AFLP analyses demonstrate genetic divergence, hybridization, and multiple polyploidization in the evolution of *Achillea* (Asteraceae-Anthemideae)

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Summary

• *Achillea*, a temperate genus of herbaceous allogamous perennials, is a model for evolutionary radiation through hybridization and polyploidization.

• AFLP analyses were performed on 300 individuals of 66 populations and 27 taxa/cytotypes, mainly from the polyploid *A. millefolium* aggregate and its suspected hybrid links with other clades of the genus.

• The mosaic genetic structure of hybrids and polyploids is revealed by specific AFLP bands shared with their assumed parents. In E Asia, *A. alpina*-4x and *A. wilsoniana*-4x are allotetraploids between *A. acuminata*-2x (sect. *Ptarmica*) and *A. asiatica*-2x (sect. *Achillea*-*A. millefolium* agg.). *A. virescens*-4x is a hybrid species linking *A. nobilis* agg. and *A. millefolium* agg. in S Europe. The hybrid swarm *A. clypeolata*-2x × *A. collina*-4x recently formed in Bulgaria shows no AFLP bands additive to its parents; by contrast, other more ancient allopolyploids exhibit genetic innovations. Relationships within *A. millefolium* agg. are complex. Five 2x-taxa, mostly well separated and regressive, are limited to Eurasia; seven 4x- and 6x-taxa are intimately linked by hybridization, are expansive, and through *A. asiatica*-2x/4x have formed the N American polyploids.

• All these results from AFLPs correspond well to other evidence, and indicate a long history of reticulate evolution in *Achillea*.

Key words: *Achillea*, *Achillea millefolium* agg., AFLP, hybrid speciation, hybridization, polyploidy.

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Introduction

Achillea (Asteraceae-Anthemideae), a large genus of herbaceous allogamous perennials, is a model for remarkable eco-geographical radiation (Guo *et al.*, 2004). How important are hybridization and polyploidization for this process? Within the framework of current multidisciplinary studies on *Achillea*, nrDNA ITS and plastid *trnL-F* sequences have shown rather low variation and limited resolution within the genus, particularly within the complex *A. millefolium* aggregate. These analyses have revealed several instances of conflicts between nuclear and plastid DNA sequences and morphology. Earlier studies have demonstrated numerous cases of polyploidy (2x-4x-6x-8x), transition zones between species, hybridization, and excessive polymorphism (Ehrendorfer, 1959b; Vetter *et al.*, 1996a, 1996b; Saukel *et al.*, 2004). All these data suggest that reticulate evolution is not only involved in recent radiations but must have been active already in the early diversification of *Achillea*. The present paper concentrates on AFLP analyses, which are expected to clarify such complex relationships and evolutionary processes.

The AFLP technique produces large sets of very polymorphic markers that may be used to analyze closely related taxa (Vos *et al.*, 1995; Wolfe & Liston, 1998; Mueller & Wolfenbarger,

1999; Ritland & Ritland, 2000). AFLP bands are dominant markers, which complicates interpretation and statistical analyses (Lynch & Milligan, 1994; Wolfe & Liston, 1998; Holsinger *et al.*, 2002). We note however, that in polyploid species even codominant markers may be insufficient to determine genotypes unequivocally. Another drawback of the AFLP method is that, especially for distantly related taxa, homology between bands may be difficult to establish. However, with large number of loci sampled throughout the whole genome and scrupulous scoring of bands, these problems can be circumvented (Hedrén *et al.*, 2001; Koopman *et al.*, 2001; Kjølner *et al.*, 2004). Thus, AFLP methods have been successfully used to first analyze relationships within closely related groups (Kardolus *et al.*, 1998; Abdalla *et al.*, 2001; Koopman *et al.*, 2001; Després *et al.*, 2003), second investigate gene flow and genomic changes resulting from hybridization and introgression at diploid levels (O'Hanlon *et al.*, 1999; Bensch *et al.*, 2002; Dodd & Afzal-Rafii, 2004), and third estimate genomic contributions from parental to allopolyploid taxa (Vijverberg *et al.*, 2000; Hedrén *et al.*, 2001; Hodkinson *et al.*, 2002).

For the present study, we have selected only a few representatives from the basal clades of *Achillea* and concentrated on the crown group of the genus, the polyploid complex *A. millefolium* agg. and its suspected hybrid links with other clades. We will try to answer the following main questions: first, do results from AFLP analyses correspond to and supplement the ITS and *trnL-F* sequence data, and morphological evidence? Second, can AFLP data help to elucidate the complex and often reticulate relationships among members of the polyploid *A. millefolium* aggregate?

Materials and Methods

Plant diversity and sampling

The species number in *Achillea* may eventually reach 140. Diversity is centred in SE Europe and SW Asia, where all sections occur. Following recent surveys (Saukel *et al.*, 2004; Guo *et al.*, 2004), we accept provisionally six sections (with approximate species numbers in brackets): sect. *Babounya* (*c.* 2), sect. *Arthrolepis* (*c.* 4), sect. *Santolinoideae* (*c.* 36), sect. *Ptarmica* s.str. (*c.* 20), sect. *Anthemoideae* (*c.* 25), sect. *Achillea* s.lat. (incl. sect. *Filipendulinae*; *c.* 50). The last section includes the polyploid *A. millefolium* aggregate (*c.* 24).

Three-hundred individuals from 66 populations of *Achillea* were sampled throughout the N Hemisphere (predominantly Europe), representing 27 taxa/cytotypes and hybrids. Of these, 220 individuals, 49 populations, and 13 species/cytotypes belong to *A. millefolium* agg. (Table 1). For each population, five individuals were analyzed. Only in exceptional cases of very limited population size, this number had to be lowered to between four and one individuals. Vouchers are deposited in the herbarium of the Institute of Botany, University of Vienna, Austria (WU). Some populations were cultivated in the experimental garden of the Institute of Pharmacognosy, University of Vienna (HBPh), and vouchers are kept there.

DNA extraction and AFLP protocols

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

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 (*c.* 0.5 µg) was digested with *Mse*I and *Eco*RI and then ligated to double-stranded adaptor pairs with T4-ligase (MBI Fermentas GmbH, St Leon-Rot, Germany) in a combined restriction-ligation reaction for 2 h at 37°C. Pre-selective 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-labeled primer combinations, *Mse*I-CAG/*Eco*RI-ACT (FAM, blue), *Mse*I-CTT/*Eco*RI-ACC (NED, yellow) and *Mse*I-CAG/*Eco*RI-AGG (HEX, green), 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.

Data analyses

AFLP banding patterns were analysed using 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 60 to 500 bp. To avoid ambiguities, only bands with sufficient florescent intensity were scored and used for the present analyses (data matrix available from the authors on request).

The total data set was organized into two matrices. The first is for a parsimony analysis. To avoid the impact of polyploidy and reticulation on the survey of relationships among taxa, only the 33 diploid populations were included here (Fig. 1). The second is for a Neighbor Joining genetic distance analysis, including 59 diploid and polyploid populations of *A. millefolium* agg. and its hybrid links (Fig. 2). Both parsimony and NJ analyses were performed with PAUP* 4.0b10 (Swofford, 2003). An heuristic search was done initially using 1000 random addition replicates, ACCTRAN optimization, TBR branch-swapping and MulTrees option (no more than 10 trees saved per replicate to minimize swapping on large numbers of suboptimal trees). Bootstrap support (Felsenstein, 1985) was estimated with 1000 bootstrap replicates, TBR branch-swapping and simple sequence addition. A NJ unrooted phylogram was generated with Nei & Li's (1979) genetic distance, and bootstrapped using 1000 replicates.

A multifactor Euclidean distance analysis was conducted for data of the polyploid *A. millefolium* agg. using the algorithm 2D_Euclid (Saukel *et al.*, 2004).

Table 1 *Achillea* taxa and populations studied (sections and groups of taxa are marked by Roman numbers; species and hybrids by Arabic numbers)

Taxa	Ploidy level[1]	Pop. code	Pop. no	Locality and habitat[2]	Collectors & vouchers[3,4]	Sample size
I. Sect. *Santolinoideae* (DC.) O. Hoffmann						
1. *A. wilhelmsii* C. Koch [= *A. santolina* auct. non L.]	(2x)	WhAC	94	Turkey: Anatolia, Cappadocia	FE 2002.03.25	5
–	(2x)	WhAA	95	Turkey: Niğde, Aksaray	FE 2002.03.26	5
II. Sect. *Ptarmica* (DC.) W. Koch						
2. *A. acuminata* (Ledeb.) Schultz Bip.	2x	CB1	118	China: Jilin, Changbai Mt., Hancong Valley, 680–620 m	YG & GR 0201, 2002.07.24	5
III. Sect. *Anthemoideae* (DC.) Heimerl						
3. *A. moschata* Wulfen	(2x)	Mo1	51	Austria: Carinthia, Rotenkogel – Kegelstein	LE 2001.08.28	5
–	(2x)	Mo2	52	Austria: Carinthia, Hafnergruppe	LE 2001.08.24	1
4. *A. oxyloba* (DC.) Schultz Bip.	2x	OX2	49	Austria: Carinthia, Rudnikkofel	LE 2001.08.23	5
IV. Sect. *Achillea* (excl. *A. millefolium* agg.)						
5. *A. ligustica* All.	(2x)	SN	07	Italy: Sicily, Nebrodi Mts.	FE 2001.09.21	5
6. A. clypeolata Sibth. & Sm.	2x	BV	19	Bulgaria: Varna, region of Debir (cult. HBPh)	JS (FE, 2001)	5
–	2x	GBcl	40	Bulgaria: Golo Bardo Mt., near Sofia	JS (FE, 2001)	5
7. *A. crithmifolia* Waldst. & Kit.	(2x)	AB3502	139	Bulgaria: near village Hisaria, 250 m (cult. HBBrno)	A. Vitkova 2002	4
8. *A. nobilis* L.	(2x)	Zn	138	Czech Rep.: Znoimo	LE & FE 2002.07.13	5
V. Assumed hybrids or hybrid taxa[(5)]						
9. *A. alpina* L.	4x	BJ	05/06	China: Beijing, Xiaolongmen; Hebei, Wuling Mt., 1700 m	YG & GR BJ02-5, 2001.10.08	5
–	4x	CB	119	China: Jilin, Changbai Mt., Hancong valley, 730–780 m	YG & GR 0202, 2002.07.24	5
10. *A. wilsoniana* Heimerl ex Hand.-Mazz.	4x	TB	120/121	China: Shaanxi, Taibai Mt., near Taibai county, Wulipo, 1550 m	YG & GR 0203, 2002.07.29	5
11. *A. virescens* (Fenzl) Heimerl	4x	Si10a	146	Slovenia: Sežana, Divača	FE 2002.07.31	5
12. *A. virescens* × *A. collina*	4x	Si10b	147	Slovenia: Sežana, Divača	FE 2002.07.31	5
13. *A. clypeolata* × *A. collina*	4x	GBhy	42	Bulgaria: Golo Bardo Mt., near Sofia (cult. HBPh)	JS (FE, 2001)	5
VI. *A. millefolium* agg.						
14. *A. asiatica* Serg.	2x	MG	20	Mongolia (cult. HBPh)	JS (FE, 2001)	5
–	(2x)	NM1	122	China: Inner Mongolia, Holhot, Daqing Mt., meadow at forest margin	YG & GR 0201, 0202, 2002.08.08	5
–	2x	NM2	123	China: Inner Mongolia, Zhayouzhongqi, Daqing Mt., Huiliang valley, in grassland	YG & GR 0203, 0204, 2002.08.08	4
–	(2x)	XJ1	124	China: Xingjiang, Yili – Nileke, 850 m	D.-Y. Tan 2002.05.15	5
–	(2x)	XJ2	125	China: Xingjiang, Urümqi county, Salqiaoke, 1550 m (ray flowers red)	D.-Y. Tan 2002.06.30	5
–	(2x)	XJ3	126	China: Xingjiang, Urümqi county, Salqiaoke, 1550 m (ray flowers white)	D.-Y. Tan 2002.06.30	5
–	(2x)	XJ4	127	China: Xingjiang, Urümqi city, beside Red-Flag Reservoir, 800 m	D.-Y. Tan 2002.06.30	5
–	2x	Al1	148	Russia: Altai, 51°02′52″, 85°36′47″, 1100 m	MS 5302 (al2–42), 2002.07.30	5
–	2x	Al2	149	Russia: Altai, 50°20′46″, 87°24′34″, 1100 m	MS 2002.07.31	5
15. *A. asiatica* Serg. s.lat.	4x	Al3	150	Russia: Rep. Altai, S Siberian lowland, 220 m	MS 5893, 2002.08.16	5
16. *A. lanulosa* Nutt. ssp. *alpicola* (Rydb.) Keck	4x	US2	132	USA: Washington, Mt. Rainier National Park, 1350–2070 m	PS & AT 7722, 2002.08.18	5
–	4x	US3	133	USA: Washington, Olympic National Park: Grand Pass-Ridge W Grand Lake-Obstruction Peak, *c.* 20 km S Port Angeles 1800–2200 m	PS & AT 7723, 2002.08.18	5

Table 1 *Continued*

Taxa	Ploidy level[1]	Pop. code	Pop. no	Locality and habitat[2]	Collectors & vouchers[3,4]	Sample size
17. *A. ceretanica* Sennen s.str.	2x	10240	16	France: E Pyrenees (cult. HBPh)	FE 2001	2
18. *A. ceretanica* Sennen s.lat.	4x	10222	17	France: Massif Central (cult. HBPh)	JS (FE, 2001)	2
19. *A. setacea* Waldst. & Kit.	(2x)	GR	21	Greece: NE Thessaloniki, drain from lake Limni Koronia	JS (FE, 2001)	2
–	2x	SeAA	92	Turkey: Anatolia, Aksaray (grassy ruderal place)	FE 2002.03.26	5
–	2x	SeAH	93	Turkey: E Ankara, Boğazkale (Hattusas)	FE 2002.03.24	2
–	(2x)	SeRt	106	Austria: Lower Austria, Retz (cult. HBPh)	JS 2002.06.04	5
20. *A. asplenifolia* Vent.	2x	BZ	14/15	Austria: Burgenland, Weiden – Podersdorf	Tod 2001.10.03.	5
–	(2x)	AsWU	61	Hungary: W Szemenye (cult. HBPh)	JS (YG 2002.05.02)	4
–	(2x)	Ha	136	Czech Rep.: S Moravia, Hororanske Louky	LE & FE 2002.07.12	5
–	(2x)	Ta	137	Czech Rep.: S Moravia, Terezin	LE & FE 2002.07.12	5
–	2x	AB3002	141	Bulgaria: Sofia floristic region, near village Opicvet, 660 m	A. Vitkova 2002	3
21. *A. roseoalba* Ehrend.	2x	FW1	03	Austria: Carinthia, Farrendorf	FE & LE 2001.07.21	5
–	2x + 4x	Si3	144	Slovenia: Ljubljana	FE 2002.07.31	5
–	2x + 4x	Si6	145	Slovenia: Ljubljana, Podpec	FE 2002.07.31	5
–	2x	V	162	Italy: Valbruna (Kanaltal), Wiesen an der westlichen Ortseite	JS 2002.07	4
22. *A. collina* J. Becker ex Heimerl	4x	SG	01	Austria: Carinthia, St. Veit/Glan, Längsee	FE (2001).07.22	5
–	(4x)	BS	13	Austria: Burgenland, Seewinkel, SW vs. Illmitz	Tod 2001.10.01	5
–	4x	GBco	41	Bulgaria: Golo Bardo, near Sofia (cult. HBPh)	JS (FE, 2001)	4
–	(4x)	KWc	114	Austria: Kaltenleutgeben, Wiener Hütte (near Vienna)	YG 0201, 2002.06.27	5
–	4x	AB202	140	Bulgaria: Vitosha Mt. 1700 m, in open grassy place	LE & FE 2002.07.13	5
23. *A. pratensis* Saukel & Länger	4x	KA	04	Austria: Carinthia, Kappl	FE & LE 2001.07.18	5
–	(4x)	MiMd	44	Austria: Carinthia, Mölltal, Mühldorf	LE 2001.08.23	5
–	(4x)	SAm2	130	Austria: Salzburg, 1 km N Kaprun, Moossiedlung, mown meadow, *c.* 760 m	LE 2002.08.07	5
–	(4x)	Si2	142	Slovenia: Zg. Jezersko	FE 2002.07.31	5
–	4x	Si8	143	Slovenia: Postojna – Zagor	FE 2002.07.31	5
–	(4x)	Or	160	Austria: Salzburg, Lungau, Mariapfarr, Örmoos, 1150 m	JS 2002.07	5
–	4x	V(a)	162(a)	Italy: Friuli, Valbruna (Kanaltal), meadows west of village	JS 2002.07	1
A. pratensis aff.	4x	S	159	Italy: Val di Seisera (Kanaltal), Weiderasen am Talschluss	JS 2002.07	5
24. *A. styriaca* Saukel & Danihelka ined.	(4x)	StE	59	Austria: Styriaca, Einach, Wald (cult. HBPh)	JS (FE, 2001; YG 2002.05.02)	4
–	(4x)	EI	161	Austria: Styria, Stadl/Mur, Einach	JS 2002.07	5
25. *A. distans* Waldst. & Kit. ex Willd.	(6x)	DiSL	97	Austria: Lower Austria, Baden	JK 96010	3
–	(6x)	DiK(G)F	98	Austria: Lower Austria, Kaltenleutgeben, Kleiner und Großer Flösslberg (cult. HBPh)	JK (YG 2002.05.02)	5
–	6x	S5d	110	Slovakia: S Poprad, Vernarske sedlo	FE 2002.06.16	5
26. *A. millefolium* L. ssp. *millefolium*	6x	S3m	108	Slovakia: Brezno, Pohorela/Hron, 600 m	FE 2002.06.16	5
ssp. *sudetica* (Opiz) Weiss	(6x)	SuO	55	Austria: E Tyrolia, Hohe Tauern, Glorer Hütte, 2350 m	LE 2001.08.29	5
–	(6x)	S6ms	111	Slovakia: Belianske Tatry, 1700 m	FE 2002.06.15–18	5
–	(6x)	STms	128	Austria: Salzburg, Hohe Tauern, Hoher Tenn, Sonnseit-Bratschen, *c.* 2300 m, in natural meadow	PS 2002.08.31	5

[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. [2]For material cultivated in Botanical Gardens (HB): HBPh, Botanical Garden, Institute of Pharmacognosy, University of Vienna. [3]All vouchers are deposited in the herbaria of the Vienna Institute of Botany (WU) and in that of the Institute of Pharmacogonosy. [4]Names of collectors: AT, A. Tribsch; FE, F. Ehrendorfer; GR, G.-Y. Rao; LE, L. Ehrendorfer-Schratt; MS, M. Staudinger; PS, P. Schönswetter; YG, Y.-P. Guo. [5]Only those linking the very polymorphic *A. millefolium* agg. with other clades.

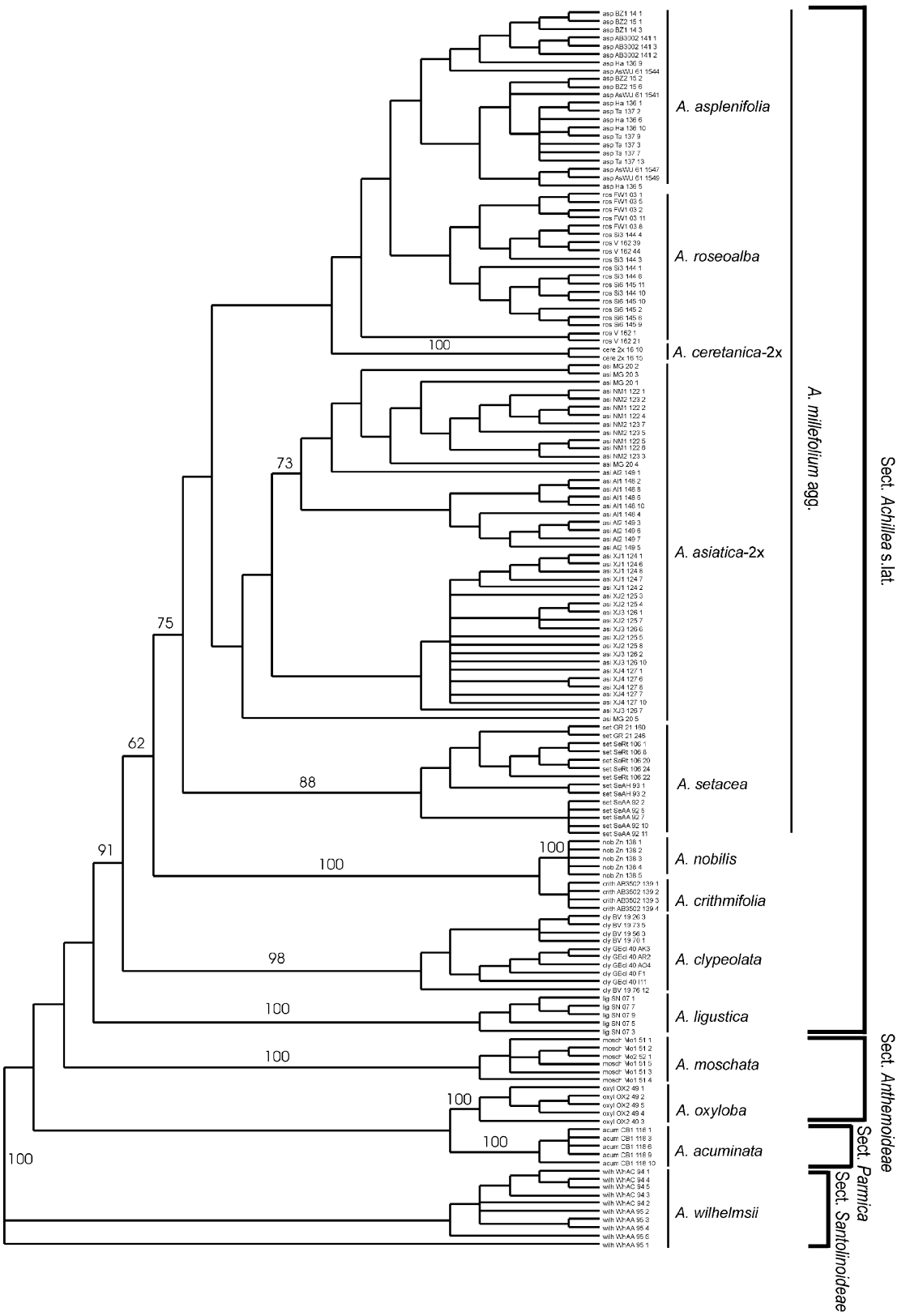

Fig. 1 Strict consensus tree of the 30 equally most parsimonious trees based on 368 AFLP bands for 13 diploid *Achillea* species with 33 populations and 151 individuals (337 pasimony-informative characters; tree length = 1888; CI = 0.1811; RI = 0.7448). The tree is rooted with *A. wilhelmsii* according to previous analyses using nrITS and plastid *trnL-F* sequences (Guo *et al*., 2004). Bootstrap percentages (> 50%) are shown above the branches (those for the terminal branches are left out for better legibility). Populations and individuals are marked with codes.

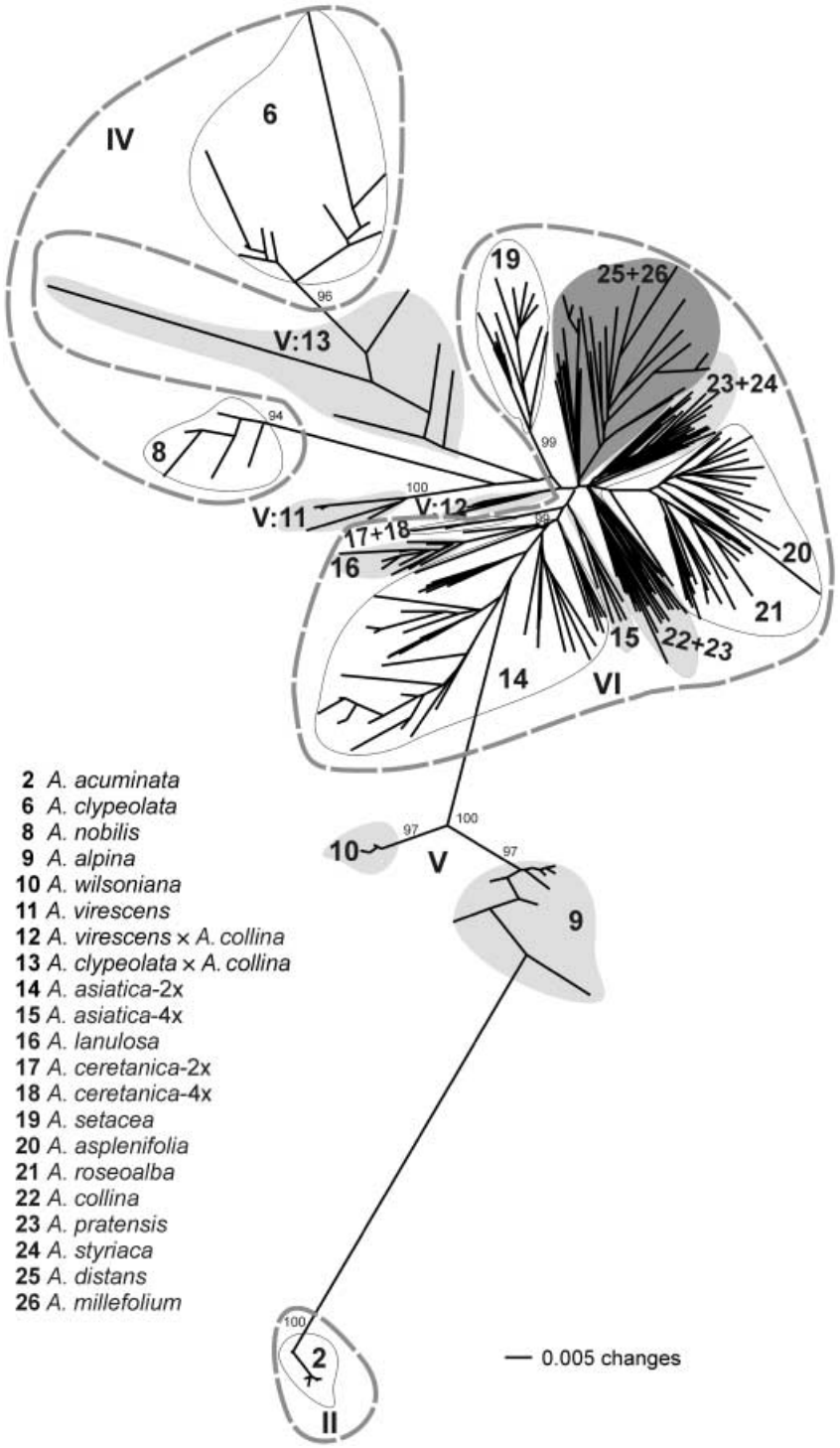

Fig. 2 Unrooted Neighbor Joining phylogram (Nei-Li distance) for 58 populations and 13 taxa of *Achillea millefolium* agg. (VI: 14–26) with hybrid taxa (V: 9–11) and hybrids (V: 12–13) linking to other members of sect. *Achillea* (IV: 6, 8) and sect. *Ptarmica* (II: 2). The tree is constructed from 368 AFLP bands. Bootstrap values (> 95% only) are shown at the nodes of the major branches. Ploidy levels: 2x, unshaded; 4x, light shaded; 6x, dark shaded. Taxa and group numbers correspond to Table 1.

To elucidate hybrid and polyploid reticulation in *Achillea*, we have attempted to trace ± stabilized and exclusive AFLP bands from each diploid species (species group) to the assumed hybrid derivatives. For this purpose, concentration was on species or population fixed bands, that is those stabilized in all the individuals of a species, or at least, in one large population of a species. Thus, rare and not even partly stabilized bands are excluded. Individual bands are designated by letters for the three fluorescent labeled primer combinations (B, blue; Y, yellow; G, green) and numbers (corresponding to the order of bands arranged by their size, from small to large). Arrows indicate the assumed transfer of bands.

For an already well documented hybrid swarm from Bulgaria (Saukel *et al.*, 2004), AFLP band frequencies were calculated for the morphologically differentiated and locally intermingled partial populations: two parental and one hybrid. For this purpose only polymorphic bands with ≥ 60% presence in at least one of the three partial populations were considered and categorized into population specific, shared by pairs of populations, and all-shared. Frequencies of these different categories of bands were calculated for each population and presented as pie-charts.

Results

Three AFLP primer combinations yielded 368 clearly identifiable bands from 300 individuals and 66 populations of *Achillea*, out of which 360 (97.8%) were polymorphic.

Divergence of diploid *Achillea* taxa

A maximum parsimony analysis for 12 diploid *Achillea* taxa generated 30 equally most parsimonious trees; the strict consensus tree is shown in Fig. 1. The tree was rooted with *A. wilhelmsii* (sect. *Santolinoideae*) as suggested by earlier analyses using ITS and *trnL-F* sequences (Guo *et al.*, 2004). In more distal positions this is followed by *A. acuminata* (sect. *Ptarmica*) with *A. oxyloba* and *A. moschata*. The latter two species belong to sect. *Anthemoideae*, which again appears as a grade rather than a clade. Members of sect. *Achillea* s.lat. form the next clade. *A. ligustica* appears as sister to the remaining taxa in sect. *Achillea*, which form a well supported monophylum [Bootstrap Percentage (BP) 91] under the condition that the traditional 'sect. *Filipendulinae*' (with *A. clypeolata*) is included. In this clade, *A. clypeolata* is basal, whereas *A. crithmifolia* and *A. nobilis* form a group sister to the monophyletic crown group with the five 2x members of *A. millefolium* agg. (BP 75). The populations of all these 2x species of *A. millefolium* agg. form monophyletic clades with the exception of *A. roseoalba*. The phylogenetic positions from basal to distal are (*A. setacea* (*A. asiatica* (*A. ceretanica* (*A. roseoalba* + *A. asplenifolia*)))). Within some of the widespread 2x species, even the intraspecific geographic differentiation is resolved: in *A. setacea*, the populations from Anatolia are followed by those from N Greece and Austria; in *A. asiatica*, one can separate the populations from NW China, Altai, and Mongolia + adjacent N China; and in *A. asplenifolia*, the populations from Bulgaria, Moravia and Austria appear quite distinct.

Hybridization, polyploidy, and differentiation in *Achillea*

The unrooted NJ phylogram (Fig. 2) shows relationships for the 2x, 4x, and 6x populations and taxa of *A. millefolium* agg., and their assumed hybrid links to other 2x taxa of sect. *Achillea* (i.e. *A. clypeolata* and *A. nobilis*) and of sect. *Ptarmica* (i.e. *A. acuminata*). The postulated hybrids or hybrid species appear to be positioned between their parental groups. Furthermore, the joint presence of specific AFLP bands from the suspected parental populations/taxa in the assumed hybrids reveals phylogenetic reticulations in the genus.

1) In E Asia *A. alpina*-4x and *A. wilsoniana*-4x (Fig. 2, V–9 + 10) form a genetic link between *A. acuminata*-2x (Fig. 2, II–2, sect. *Ptarmica*) and *A. asiatica*-2x (Fig. 2, VI–14, sect. *Achillea*-*A. millefolium* agg.). The 2x taxa in sect. *Ptarmica* and sect. *Achillea* are widely separated. (Figs 1 and 2). As shown by Fig. 3, among all the AFLP bands observed in 2x *Achillea* species, 18 are exclusive to and fixed in *A. acuminata*-2x. Out of them, 14 are also present on the 4x level, four in *A. alpina*, one in *A. wilsoniana* and nine in both. Similarly, out of five such bands in *A. asiatica*-2x, two also appear in *A. wilsoniana*, one in *A. alpina*, and two in both. In addition, three bands are specific to the 4x level, two in *A. alpina*, one in *A. wilsoniana* (Fig. 3).

2) In S Europe *A. virescens*-4x (Fig. 2, V–11) forms a cluster linking *A. nobilis*-2x (Fig. 2, IV–8, sect. *Achillea*) and *A. millefolium* agg. (Fig. 2, VI, sect. *Achillea*). Out of 80 AFLP bands scored for *A. virescens*-4x, four are private, and two (B162 & G81) are shared exclusively with *A. nobilis* (Fig. 4). All the other 76 *A. virescens* bands are either widespread in *Achillea* or shared with members of *A. millefolium* agg. only. Four of the latter type (B140, G27, G61, G93) are fixed in many 2x populations, but also occur in 4x and 6x members (Fig. 4; the 6x taxa not shown).

The studied population of *A. virescens*-4x (Si10a_146) grows sympatrically and intermingled with a population originally determined as '*A. collina*-4x' (Si10b_147) in the karst near Trieste. Figure 2 shows that this '*A. collina*' population (V–12) deviates from the true *A. collina*-4x (VI–22) and shares its band G21 exclusively with *A. virescens*-4x (Fig. 4). Therefore, the population Si10b_147 was designated as *A. virescens* × *A. collina* (Table 1, Figs 2 and 4).

3) A postulated hybrid swarm from Mt. Golo Bardo near Sofia, Bulgaria (Saukel *et al.*, 2004), can be separated morphologically into three partial populations: *A. clypeolata*-2x (sect. *Achillea*: '*Filipendulinae*'; Table 1, IV–6: GBcl_40), *A. clypeolata* × *A. collina*-4x (Table 1, V–13: Gbhy_42), and *A. collina*-4x (*A. millefolium* agg.; Table 1, VI–22: GBco_41). Figure 2 demonstrates the considerable genetic distance between the assumed parental taxa (IV–6 and VI–22) and the excessive AFLP variation of the hybrids (V–13). The frequencies of their specific and shared AFLP bands (Fig. 5) show that the apparent hybrid individuals (Gbhy_42) share 39% of bands with the more *A. clypeolata*-like individuals (GBcl_40), and 40% with the more *A. collina*-like plants (GBco_41), but have no private bands.

4) The NJ phylogram (Fig. 2) illustrates the circumscription of the *A. millefolium* agg. native in the N Hemisphere, and its mosaic genetic structure. At the diploid level, taxa again appear well separated with exception of the polyphyletic *A. roseoalba* (see Fig. 1). The latter overlaps so much with *A.*

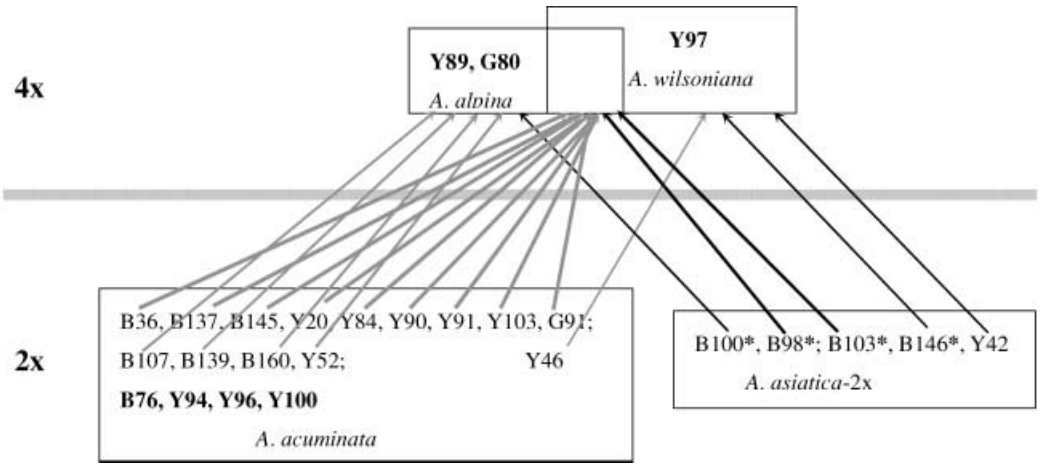

Fig. 3 AFLP bands (population fixed) exclusive to *Achillea acuminata* (sect. *Ptarmica*; grey arrows) and to *A. asiatica* (sect. *Achillea*, *A. millefolium* agg.; black arrows) at the 2x level reappear in *A. alpina*-4x, *A. wilsoniana*-4x, or both, suggesting a hybrid origin of the 4x taxa. Bands private to species on 2x or on 4x level are shown in bold print. *Bands also shared by *A. asiatica*-4x.

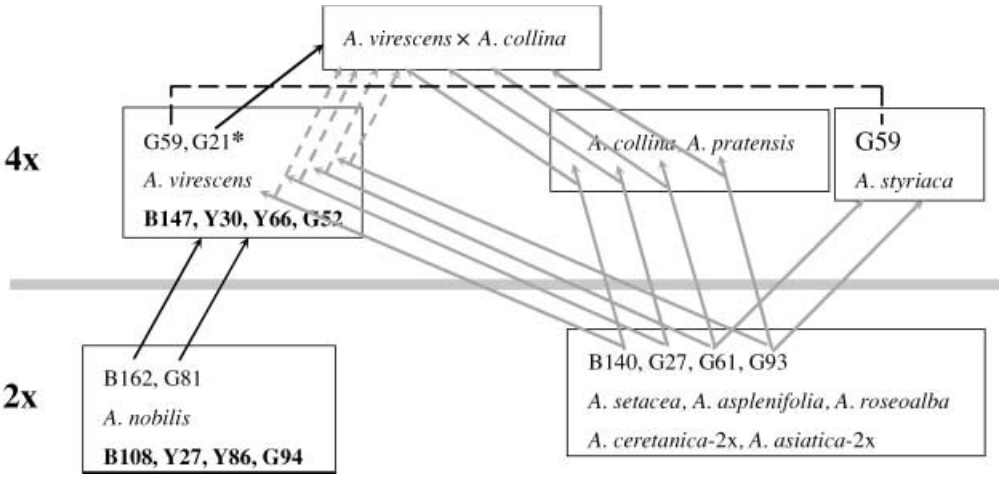

Fig. 4 AFLP bands (population fixed) exclusive to *Achillea nobilis*-2x (*A. nobilis* agg.; black arrows) and to 2x members of *A. millefolium* agg. (regarded as one group; grey arrows) reappear in *A. virescens*-4x, 4x members of *A. millefolium* agg. (*A. collina*, *A. pratensis*), and in the population *A. virescens* × *A. collina*-4x (in the latter possibly from two directions, but arrows with interrupted lines less likely), suggesting hybrid speciation and hybrid introgression within sect. *Achillea*. Bands private to *A. nobilis* and *A. virescens* are shown in bold print. *G21 (black arrow) is a rare band only present in *A. virescens* (1 indiv.) and *A. virescens* × *A. collina* (4 indiv.).

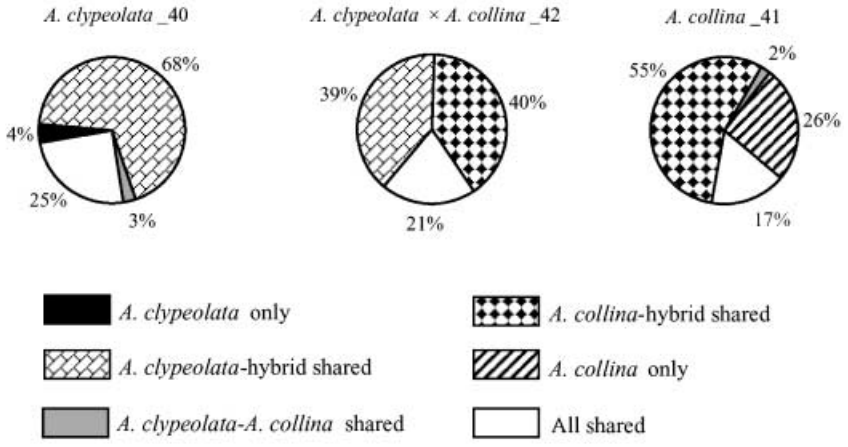

Fig. 5 Frequencies of population specific, pair-shared and all-shared AFLP bands in morphologically defined partial populations of *Achillea clypeolata*-2x (GBcl_40), *A. collina*-4x (GBco_41), and the 4x intermediates (GBhy_42) on Golo Bardo Mt. (Bulgaria) suggest ongoing hybridization. Only polymorphic bands with ≥ 60% occurrence in at least one of the partial populations were considered for the calculation. The assumed hybrid partial population shows no private but large portions of bands from the parental partial populations; by contrast, the two parental populations share only 2–3%.

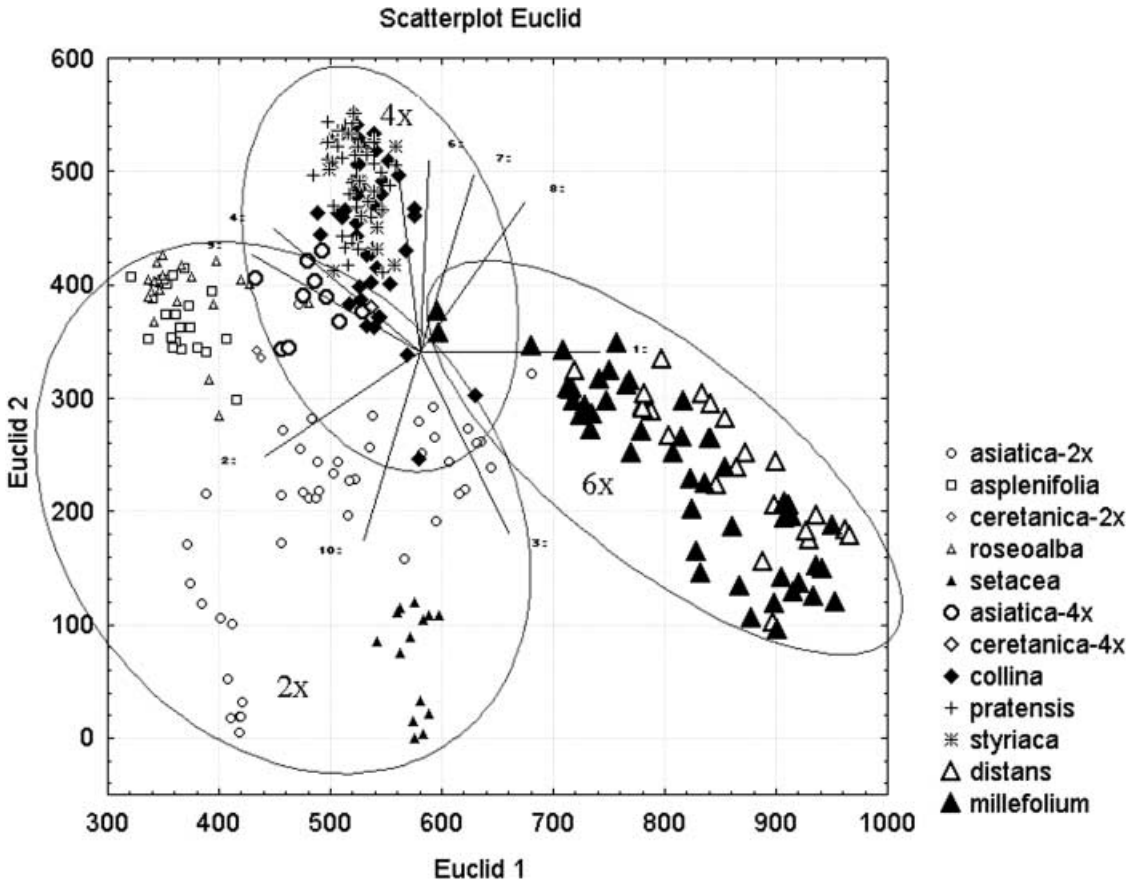

Fig. 6 Scatterplot for 12 Eurasiatic 2x, 4x, and 6x taxa/cytotypes of *Achillea millefolium* agg. The plot is based on 360 polymorphic AFLP bands from 210 individuals and 47 populations, and constructed according to the '2D_Euklid' algorithm (Saukel *et al*., 2004) using 15 PCA factors.

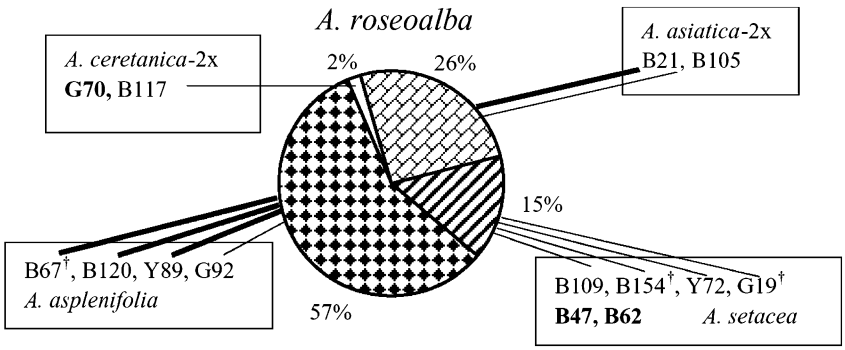

Fig. 7 AFLP bands (population fixed) exclusive to each of four monophyletic and ± relic 2x members of *Achillea millefolium* agg. partly appear in the polyphyletic and expansive *A. roseoalba*-2x also, suggesting its origin through hybrid introgression into *A. asplenifolia*. The central pie chart shows frequencies of these bands in *A. roseoalba*. Thick lines designate bands with frequencies of > 10%, and thin lines of ≤ 10% in *A. roseoalba*. Species private bands are shown in bold print. †Also found in some diploid taxa outside *A. millefolium* agg. and possibly plesiomorphic.

asplenifolia in its AFLP profile (Figs 2 and 6) that we had to combine the two taxa into one group (Fig. 8). By contrast with the diploids, morphologically defined species borders are much more difficult to demonstrate by AFLP analyses for the polyploids. On the 4x level, *A. pratensis* + *A. collina*, and *A. pratensis* + *A. styriaca* each form compact clusters (Fig. 2, VI–22 + 23; 23 + 24). A comparable picture emerges from the Euclidean scatter plot (Fig. 6). On the 6x level *A. millefolium* and *A. distans* form one cluster and their AFLP profiles are hardly distinguishable from each other (Fig. 2, VI–25 + 26; Fig. 6). All these 4x and 6x clusters are positioned between diploid taxa (Fig. 2). This complex scenario is clarified, as shown below, by tracing characteristic AFLP bands from the 2x to 4x, and to 6x level (Figs 7 and 8).

Already on the 2x level, we suspect that hybrid introgression into *A. asplenifoia* was involved in the origin of *A. roseoalba*. This polyphyletic taxon (Fig. 1) exhibits a predominant proportion of bands characteristic of *A. asplenifolia*, but there are also links with other monophyletic 2x taxa in the aggregate (Fig. 7).

Among the taxa of *A. millefolium* agg. on the 4x level, the AFLP profile of the W European *A. ceretanica*-4x appears quite similar to and clearly derived from *A. ceretanica*-2x. According to Fig. 8, none of the characteristic bands of the other 2x members in *A. millefolium* agg. reappear in *A. ceretanica*-4x. Nevertheless, distinction between the 2x and 4x cytotypes of *A. ceretanica* are obvious in spite of very limited sample size: among all their 85 bands scored, 60 are shared, 13 specific to the 2x and 12 to the 4x level.

The AFLP profile of the C European *A. styriaca*-4x (Fig. 8) is characterized by four bands from the *A. asplenifolia* + *A. roseoalba* cluster (B67, G14, G69, Y89), one from *A. ceretanica*-2x (B56), and one from *A. setacea* (B32).

A. pratensis-4x and *A. collina*-4x (C to E Europe) have similar AFLP profiles (Fig. 8) and therefore fall into the same cluster in Figs 2 and 6. Nevertheless, in *A. pratensis*, G35 is private and three other bands have not yet been found in *A. collina*. A major part of their shared bands can be traced back to the 2x level, apparently from *A. setacea* (B134, B13, B32, B101, Y77) and the *A. asplenifolia* + *A. roseoalba* group (B67, B120, G69, Y89). Some less common bands link *A. collina* + *A. pratensis* also with *A. ceretanica*-2x (B56) and with *A. asiatica*-2x (B103, G65).

Additionally, the 4x taxa of *A. millefolium* agg. in Europe exhibit several shared new bands as shown in Fig. 8 by black connecting lines (B71, B112, G44, G90) and in the overlapping box (B102). These bands are missing so far on the 2x level.

The pattern of particular AFLP bands transferred from the 2x to the 4x level is repeated from the 4x to the 6x level, the latter represented by *A. distans* and *A. millefolium* from Europe to Altai. In Fig. 8, we find bands that can be traced from 2x through 4x to 6x levels, for example from *A. asplenifolia* + *A. roseoalba* (G14, G69, B67), from *A. setacea* (B13, B122, Y77), from *A. ceretanica* (B56), and even from *A. asiatica*-2x (B103). In addition, bands that apparently have originated among 4x taxa are also handed on to the 6x level (B71, B102, B112, B156, G44, G90). Nevertheless, it is difficult to identify possible source taxa or precursors for these 6x taxa, because there is so much overlap and sharing of bands at the 4x level. Furthermore, on the 6x level again, the *A. distans* + *A. millefolium* cluster presents specific bands (B68 and G72 in both, B30 in *A. distans* only).

Recent progress in our knowledge of *A. millefolium* agg. in C and E Asia has shown that there is extensive eco-geographical radiation of the 2x and 4x *A. asiatica* (Table 1). As shown by Fig. 8, most of the characteristic bands in *A. asiatica*-2x apparently have not extended beyond *A. asiatica*-4x (G82, B98, B100 and B146). Other bands differentiate *A. asiatica*-4x and can be traced back to *A. setacea* (B13, B32, Y77), *A. asplenifolia* (Y89), and even to *A. ceretanica*-2x.

AFLP data from the only two north-western USA *A. lanulosa*-4x populations are included in the NJ phylogram (Fig. 2). They clearly suggest relationships with the 2x and 4x *A. asiatica* from C Asia. Furthermore, an overall survey (not shown in Fig. 8) has revealed that the species fixed band (G82) of the 2x and 4x *A. asiatica*, and a not fixed band (B63) of *A. asiatica*-2x extend into these *A. lanulosa*-4x populations. On the other hand, several private bands (B157, Y69, G78) limited to the N American populations are evidence for their genetic differentiation from the Eurasian members of *A. millefolium* agg.

Discussion

Phylogeny and eco-geographical radiation of *Achillea*

Reconstruction of major clades. Current knowledge about the phylogeny and systematics of *Achillea* is summarized by Guo *et al.* (2004) based on nrITS and plastid *trnL-F* sequences. Relevant conclusions are supported by the AFLP data (Figs 1 and 2). Members of the sections *Santolinoideae*, *Ptarmica* and *Anthemoideae* appear in basal positions and correspond to the primary eco-geographical radiation of the genus into xeric, hygric and alpine habitats. The following clade is made up of the very diverse taxa of sect. *Achillea* s. lat., which has to include the former sect. *Filipendulinae* (based on ray flower color only). *A. ligustica*, a disjunct Mediterranean montane species, was placed into the *A. nobilis* group by Bässler (1963), but appears isolated and as sister to the remainder of sect. *Achillea*. Instead, *A. nobilis* is found in a well supported SE to C European clade with *A. crithmifolia*. The diploid species of the *A. millefolium* complex, better resolved by AFLPs than by DNA sequences, form the monophyletic crown group of the genus. The clear circumscription of this aggregate and its separation from other *Achillea* taxa so far has been obscured by secondary hybrid and mostly polyploid links, as discussed below.

Hybrid links between *Achillea millefolium* agg. and other clades. A) Up to the present no doubts have ever been expressed about the sharp separation of *Achillea* sect. *Ptarmica* and sect. *Achillea*, which correspond to two well separated clades if diploids only are considered (Fig. 1; Guo *et al.*, 2004). The present AFLP data (Figs 2 and 3) clearly demonstrate an allotetraploid link in E Asia between these two clades, from *A. acuminata*-2x (sect. *Ptarmica*) via *A. alpina*-4x and *A. wilsoniana*-4x to *A. asiatica*-2x (sect. *Achillea*, *A. millefolium* agg.). This is paralleled by a comparable morphological link and enforces a new concept for the taxonomy of *Achillea*. The first monographer of the group, Heimerl (1884), was aware of the similarities of his *A. sibirica* ssp. *wilsoniana* (= *A. wilsoniana*) with *A. millefolium* agg., but attributed them to parallel variation within the two sections. Therefore, he placed the former together with his *A. ptarmica* var. *acuminata* (= *A. acuminata*) and *A. sibirica* ssp. *mongolica* (= *A. alpina*) into sect. *Ptarmica*. In the recent taxonomic literature, for example in *Flora Reipublicae Popularis Sinica* (Shih & Fu, 1983), the three taxa are described under the names used here and also

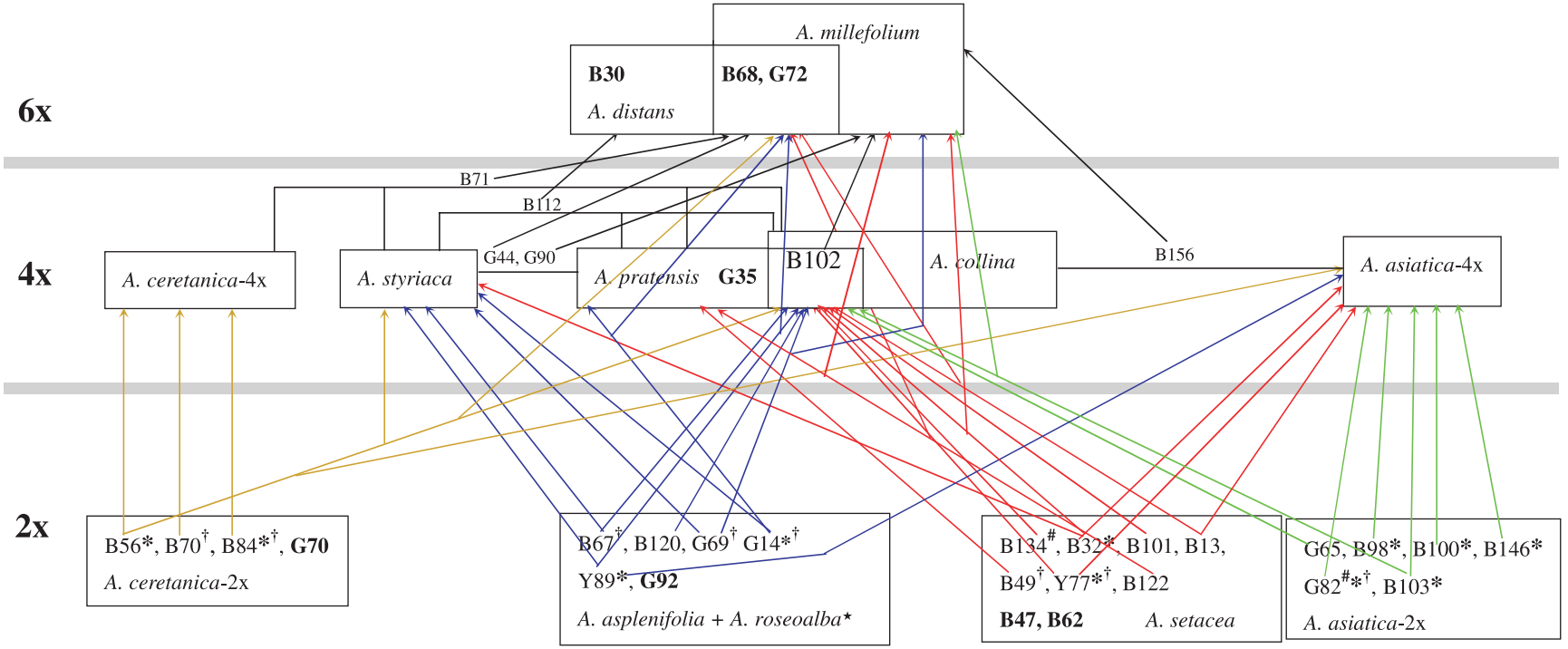

Fig. 8 AFLP bands (2x population exclusive and fixed) link taxa from 2x to 4x and from 4x to 6x levels in Eurasian members of *Achillea millefolium* agg., suggesting several independent lines and different phases of hybridization and polyploid speciation. Colored arrows link exclusive bands from each 2x species (or species group) to taxa on the 4x (and 6x) level. Black arrows link bands limited to the 4x level but also extending to the 6x level. Bands placed into the overlapping boxes and on the horizontal connecting lines are shared by the respective taxa. In addition, private bands of a species (or species group) on all ploidy levels are shown in bold print. ⋆ *A. asplenifolia* and *A. roseoalba* were treated as a species group based on DNA sequences (Guo *et al*., 2004) and the present AFLP data (Figs 1, 2 and 6). #Species fixed band. *Shared also by hybrids or hybrid species which link *A. millefolium* agg. with other diploid taxa outside this aggregate, that is *A. clypeolata* × *A. collina, A. virescens* × *A. collina, A. virescens, A. wilsoniana,* and *A. alpina* (not shown here). †Also found in some diploid taxa outside of *A. millefolium* agg.

placed into sect. *Ptarmica*, whereas *A. asiatica* is listed under sect. *Achillea*.

Further evidence for our concept of an allotetraploid origin of *A. alpina* and *A. wilsoniana* comes from available morphological, karyological and ecological evidence (unpublished data). These data demonstrate a nearly continuous variation in the taxa of the sect. *Ptarmica* – sect. *Achillea* link with respect to leaf shape, capitula size, flower morphology and habitat. They suggest greater affinities of *A. alpina*-4x to *A. acuminata*-2x, and of *A. wilsoniana*-4x to *A. asiatica*-2x. This concept may appear inconsistent with our ITS and *trnL-F* sequence analyses (Guo *et al.*, 2004), which show that *A. alpina* and *A. wilsoniana* together with *A. asiatica* are embedded in the crown clade of the genus with members of *A. millefolium* agg. (sect. *Achillea*), whereas *A. acuminata*-2x appears far removed among other 2x members of sect. *Ptarmica*. These discrepancies in phylogenetic topology can be explained first by the postulated role of *A. asiatica*-2x as maternal parent of the tetraploids (supported by their shared plastid haplotype, different from that of *A. acuminata*; unpublished data), and second by the well known tendency for conversion and homogenization of different ITS types in allopolyploids towards one of the parental taxa, often the maternal one (Chase *et al.*, 2003).

The distribution areas of *A. acuminata*-2x, *A. alpina*-4x, *A. wilsoniana*-4x and *A. asiatica*-2x partly overlap in NE China (Meusel *et al.*, 1991/1992: maps 478c, d, and 479b). Nevertheless, these taxa differentiate as ecological vicariants along a gradient from wet or humid, to mesic and xeric habitats, and therefore, are ecologically ± isolated. *A. alpina* and *A. wilsoniana* share many AFLP bands with *A. acuminata*-2x and *A. asiatica*-2x, each exhibit some specific bands but have no exclusive bands in common (Fig. 3). Nevertheless, it is not yet clear whether they have originated from a single allopolyploidization event with subsequent differentiation or rather from two (or even more) such events followed by later hybrid contacts on the 4x level. Anyway, in comparison with their parental diploids, which have not radiated beyond C and E Asia, the secondary differentiation of the allotetraploid *A. alpina* /*A. wilsoniana* assembly was much more expansive. It includes several other taxa described (e.g. *A. japonica* Schultz Bip., *A. sinensis* Heimerl ex Hand.-Mazz., *A. ptarmicoides* Maxim., *A. camtschatica* Rupr. ex Heimerl, treated as species or subspecies of *A. sibirica* Ledeb.) and has extended throughout northern N America to the Gaspé Peninsula (see map 478d in Meusel *et al.*, 1991/1992).

B) In the taxonomic literature (e.g. Bässler, 1963; Richardson, 1976), the tetraploid species *A. virescens* is uniformly treated as a member of *A. nobilis* agg. But already Ehrendorfer (1953, 1959c) advanced morphological and karyological arguments for its hybrid origin from *A. nobilis agg.* and *A. millefolium* agg. Possible candidates are *A. nobilis*-2x and *A. setacea*-2x or *A. collina*-4x, all with widely overlapping areas and similar habitats, particularly in SE Europe (Meusel *et al.*, 1991/1992: maps 479a, c, and d). Discordant plastid and nuclear DNA sequence data (Guo *et al.*, 2004) have given a clue for the hybrid nature of *A. virescens* by showing it distant from *A. nobilis* in the plastid *trnL-F* tree in an unresolved polytomy of *A. millefolium* agg. (incl. *A. collina*) and sister to a Greek population of *A. setacea*. However, the nrITS tree places *A. virescens* together with *A. nobilis* in a subclade, sister to many other members of *A. millefolium* agg. and sect. *Achillea*.

The present AFLP data (Figs 2 and 4) support the postulated hybrid origin of *A. virescens* from *A. nobilis* but can not decide about the other parent from *A. millefolium* agg. Additional evidence now comes from recent plastid RFLP analyses (unpublished data), which show that *A. virescens* shares a particular haplotype with members of *A. millefolium* agg., that is *A. collina*-4x, *A. pratensis*-4x and *A. roseoalba*-2x, whereas *A. setacea* and *A. nobilis* each have different haplotypes. This parallels the *trnL-F* data and makes it very likely that *A. collina* was involved as the maternal and *A. nobilis* with unreduced gametes as the paternal parent in the hybrid origin of *A. virescens*.

From its relatively high proportion of private AFLP bands (a signal of posthybridization recombination or diversification) as well as from its wide distribution from the western Balkans to the southern Italian Peninsula, one has to assume a rather early origin of *A. virescens*. It is remarkable that, in spite of the distinct morphology and obviously strong crossing barriers between *A. virescens*-4x and *A. collina*-4x which grow in true sympatry and flower simultaneously in the open, grassland interspersed karst forest above Trieste near Divača, hybrid introgression is going on between them as shown by the AFLP profiles of their populations (Fig. 4).

C) The finding of very variable and fertile hybrids linking populations of *Achillea clypeolata*-2x and of *A. collina*-4x in Bulgaria was unexpected because of the phylogenetic distance (Fig. 1; Guo *et al.*, 2004) and the different ploidy levels of the parental taxa (Saukel *et al.*, 2004 and references cited there). *A. clypeolata* is basal in all trees within sect. *Achillea* and widely distributed as a xerophyte in the Balkan Peninsula and Romania, where it widely overlaps with the C and E European *A. collina*, a 4x member of *A. millefolium* agg. Multidisciplinary evidence from morphometrics, phytochemistry, karyology, ecology (Saukel *et al.*, 2004), DNA sequences (Guo *et al.*, 2004), and the present AFLP analyses strongly support the concept of extensive hybrid contacts between these taxa and even introgression from *A. clypeolata* into *A. collina* (Fig. 5). Nevertheless, the recent origin of the Golo Bardo hybrids is underlined by the fact that no new private AFLP bands have been found in them. That the 2x/4x barrier has been overcome is obviously due to the function of unreduced gametes. This has been well documented for both the female and male side in *Achillea* (Ehrendorfer, 1959b, 1959c, 1973), and makes hybridization possible between 2x and 4x individuals without the appearance of 3x-F1 hybrids. The origin of 4x-F_1 and later hybrid and backcross generations in the direction of

the 4x parent will result in gene flow from 2x to 4x and may form the basis for new and ± independent taxa.

Differentiation-hybridization-cycles on different ploidy levels in *Achillea millefolium* agg. The *Achillea millefolium* polyploid complex has its natural distribution in the N hemisphere, but its basal diploids are limited to Eurasia (with four species in Europe and three in Asia). The polyploid species are mostly difficult to define, their number varies depending on the authorities and may reach 17 or more. No general survey of the polyploid complex *A. millefolium* agg. is yet available, but relevant information is found in regional floras (e.g. Nobs, 1960; Wagenitz, 1968/1979; Afanasyev & Bochantsev, 1995; Richardson, 1976; Fischer, 1994), chorological analyses (Meusel *et al.*, 1991/1992: maps 479b–d), and in some general contributions (e.g. Ehrendorfer, 1959b; Greilhuber & Ehrendorfer, 1988; Saukel & Länger, 1992a,b; Rauchensteiner *et al.*, 2002; Saukel *et al.*, 2004). Separation of *A. millefolium* agg. from other members of sect. *Achillea* was not possible by ITS and *trnL-F* sequences (Guo *et al.*, 2004). By contrast, the AFLP data clearly characterize the polyploidy complex as a clade, with mostly well separated 2x species (Fig. 1), but with a rather diffuse superstructure of 4x and 6x taxa (Figs 2, 6 and 8).

The primary differentiation of *A. millefolium* agg. into basal 2x taxa has followed a classical eco-geographical vicariance pattern: *A. ceretanica*-2x in subalpine meadows of the E Pyrenees, *A. asplenifolia* in fens and humid grassland of the Pannonian plains from Bulgaria to Austria, *A. setacea* in xeric Pontic steppes from the Ukraine and Anatolia to continental valleys of the Alps, and *A. asiatica*-2x in open montane to alpine grassland from the Altai to Mongolia, NW (Xingjiang) to NE (Heilongjiang) China. In C Europe these 2x taxa appear as disjunct relicts, regressive and under pressure of more competitive and expansive polyploid members of *A. millefolium* agg. Only *A. roseoalba* exhibits considerable fitness and has expanded into the geologically young N Italian plains and adjacent foothills where it occupies mesic forest margins and anthropogenous meadows (Ehrendorfer, 1959a). From its polyphyly (Fig. 1) and its mosaic relationships with other diploid species in the aggregate (Figs 6 and 7), *A. roseoalba* exhibits signs of hybrid introgression into *A. asplenifolia*, even if the direction of possible gene flow (from *A. ceretanica*-2x) is uncertain. Closely corresponding plants were experimentally created from backcrosses of *A. asplenifolia* × *A. setacea*-F_1 with *A. asplenifolia* (Ehrendorfer, 1959b). Thus, *A. roseoalba* probably is a homoploid hybrid species. In any case, it exhibits signs of new genetic differentiation: it has developed a specialized meadow ecotype with a rhythm of double flowering and fruiting, adapted to an early and a late mowing phase during each summer (Ehrendorfer, 1959b).

Striking signs of multiple hybridization and polyploidization become apparent when comparing specific AFLP bands linking 2x and 4x taxa of *A. millefolium* agg. (Fig. 8). There is strong evidence that the diploids *A. asplenifolia* + *A. roseoalba* and *A. setacea* were involved in the origin of the widespread tetraploids *A. pratensis* (mesic anthropogenous meadows, C Europe) and *A. collina* (dry grass- and woodland, C to E Europe). In morphology and ecology, *A. pratensis* is closer to the hygromorphic *A. asplenifolia* + *A. roseoalba* (Saukel & Länger, 1992c) and was originally considered as a 4x cytotype of *A. roseoalba* (Ehrendorfer, 1953), whereas *A. collina* tends much more towards the xeromorphic *A. setacea*. Already in earlier cytogenetic experiments (Ehrendorfer, 1959b), synthetic *A. collina*-4x plants had originated spontaneously among numerous 2x F_2-progeny from an *A. asplenifolia* × *A. setacea* cross and were successfully backcrossed to natural *A. collina*-4x.

As to other tetraploids of the aggregate, first *A. ceretanica*-2x evidently was involved in the origin of *A. ceretanica*-4x (colline to montane grassland, C France), but the appearance of specific AFLP bands in the 2x or 4x populations contradicts a simple autopolyploid relationship between the two cytotypes; second *A. asplenifolia* + *A. roseoalba* and probably also *A. ceretanica*-2x may have contributed to *A. styriaca* (open warm forests, Austria to Bohemia); and third *A. asiatica*-2x together with other 2x taxa (*A. setacea*, etc.) has apparently participated in the development of *A. asiatica*-4x (foothills of Altai). Thus, available AFLP data (Fig. 8) show that the origins of all the 4x taxa of *A. millefolium* agg. must have been due to very complex processes of multiple hybridization and polyploidization, suggesting polyphyletic and polytopic origins. Additionally, the main lines of this reticulate evolution are clearly supported by the distribution of 12 different plastid haplotypes in this polyploid complex. Those specific to each of the 2x taxa have been transferred and reappear in a combined and mosaic fashion in the 4x and 6x taxa (unpublished data).

An important evolutionary aspect of the AFLP profiles from the 4x taxa of *A. millefolium* agg. is the appearance of bands that are obviously lacking among the 2x taxa and exhibit horizontal transfer on the 4x level by hybridization (Fig. 8). We consider all this as evidence for new genetic differentiation in these hybrid 4x taxa during a secondary phase of reorganization, divergent adaptation and geographical expansion. The eco-geographical vicariance pattern separating *A. cerentanica*-4x, *A. styriaca*, *A. pratensis*, and *A. collina* is obviously maintained by selection, in spite of continuing horizontal hybridization, gene flow and strongly overlapping AFLP profiles on the 4x level (Figs 6 and 8). This assumption is supported by crossing experiments, which produce F_1 and further hybrid progenies with hardly any reduction of fertility and vitality (Vetter *et al.*, 1996a,b).

Differentiation-hybridization-cycles have occurred not only on the 4x level but obviously also on the closely attached 6x level of *A. millefolium* agg. (Figs 6 and 8), that is among the ecologically vicariant *A. distans* (warm woodland to subalpine grassland, C to S Europe) and *A. millefolium* (open forests and grassland, temperate to alpine and subarctic, Europe to C

Asia). Numerous AFLP bands link these two 6x taxa with those on the 4x (and 2x) level; others are innovations for the 6x level and imply a new phase of differentiation. That gene flow continues across the 4x/6x barrier is clearly shown by sufficiently fertile and vigorous anorthoploid and aneuploid progeny from experimental F_1, F_2 and backcrosses of *A. collina* × *A. millefolium* (Schneider, 1958), as well as by studies of numerous comparable natural contact zones between 4x and 6x taxa in Europe. Further hybridization occurs on the 6x level between *A. distans* and *A. millefolium*: the two taxa can be easily crossed in the experimental garden and hybridize widely in nature. This has contributed to additional eco-geographical diversity, reflected in several intermediate taxa, even described as species (e.g. *A. stricta* Schleicher, *A. carpatica* Błocki ex O. N. Dubovik).

The remarkable tertiary eco-geographical radiation of 4x and 6x taxa of *A. millefolium* agg. in N America has been studied intensively with respect to ecotypes, cytogeography and cytogenetics (see References in Hiesey & Nobs, 1970; Ehrendorfer, 1973). Experimental crosses between N European *A. millefolium*-6x and Californian '*A. gigantea*'-6x are possible but exhibit meiotic disturbances and various degrees of reduced fertility and viability. By contrast, crosses even between widely divergent N American taxa, either on the 4x or the 6x level, are quite normal and vigorous (Ehrendorfer, 1952). Extensive evidence from hybridization between 4x and 6x populations and their disjunct distribution pattern (Hiesey & Nobs, 1970; Tyrl, 1975) have clearly shown that the 6x cytotypes have originated from various 4x populations in a polytopic and polyphyletic way. Thus, the traditional subordination of the numerous N American 4x taxa under the name *A. lanulosa* Nutt., and of the 6x taxa under *A. borealis* Bong., now mostly has been replaced by filing these taxa as varieties under *A. millefolium* s.lat. (Kartesz, 1994). The AFLP pilot data now available from Washington State populations suggest their phylogentic links with C and NE Asiatic 2x and 4x *A. asiatica*, probably via Pleistocene migrations across Beringia. At the same time AFLP profiles attest considerable autonomy of the N American populations, also underlined by deviating plastid types (unpublished data).

Evolutionary patterns and processes in *Achillea*

All taxa of *Achillea* are characterized by perennial and predominantly herbaceous life form, self-incompatibility and allogamous reproduction (Ehrendorfer, 1959b; Vetter *et al.*, 1996a,b), quite uniform diploid karyotypes (Tohidast-Akrad, 1981), delayed formation of crossing barriers as well as strong tendencies towards hybridization and polyploidization (Ehrendorfer, 1959b; Greilhuber & Ehrendorfer, 1988). This evolutionary pattern, classified under 'hybrid polyploid complexes' by Grant (1981) is common in angiosperms. The dynamics of this pattern has been clarified by recent molecular studies on comparable groups, for example *Paeonia* (Sang & Zhang, 1999; Ferguson & Sang, 2001), *Gossypium* (Abdalla *et al.*, 2001), *Nicotiana* (Chase *et al.*, 2003), N American *Tragopogon* (Soltis *et al.*, 2003; Pires *et al.*, 2004), New Zealand and Australian *Microseris* (Vijverberg *et al.*, 2000), Hawaiian *Madiinae* (Baldwin, 2003), or *Dactylorhiza* (Hedrén *et al.*, 2001). With *Achillea* and *A. millefolium* agg. as a model, we will show that the evolutionary dynamics of all these polyploid complexes is based on cycles of differentiation and hybridization (Ehrendorfer, 1959b).

Primary evolutionary differentiation on the diploid level in *Achillea* has followed the well-known eco-geographical vicarious pattern from populations to species, separated by ± effective crossing barriers. This is demonstrated by the present AFLP data and by crossing experiments between different 2x species (Ehrendorfer, 1959b). The floristic literature lists numerous examples for natural *Achillea* hybrids on the 2x level, within or sometimes also between different sections, but most have ± reduced fertility and are of an ephemeral nature. In general, hybridization between species and 2x hybrid swarms are widely studied and discussed in plants as well as in animals (e.g. Stebbins, 1959, 1969; Grant, 1981; Rieseberg & Wendel, 1993; McDade, 1995; Rieseberg, 1995; Arnold, 1997; O'Hanlon *et al.*, 1999; Bensch *et al.*, 2002; Dodd & Afzal-Rafii, 2004; Seehausen, 2004). Cases of homoploid hybrid speciation have been well documented recently for *Helianthus* and other genera (Rieseberg, 1997; Borgen *et al.*, 2003; Rieseberg *et al.*, 2003). Candidates from *Achillea* are *A. roseoalba* (Fig. 7) and the alpine *A. morisiana*, linking *A. moschata* with *A. erba-rotta* in sect. *Anthemoideae* (Heimerl, 1884). Discordant data from plastid and nuclear DNA sequences, and morphology suggest ancient hybridization events between the *Achillea* sections *Santolinoideae* and *Ptarmica* (Guo *et al.*, 2004). Thus, early hybrid reticulation in the evolution of *Achillea* is likely, comparable with the situation among 2x species of *Gossypium* (Rieseberg, 1995).

Polyploidy and hybridization have been essential for massive secondary evolutionary radiation in *Achillea*, particularly in the *A. millefolium* aggregate, as in many other angiosperm clades (Leitch & Bennett, 1997; Wendel, 2000; Soltis *et al.*, 2003). The cytogenetic potentials for the origin of polyploids evidently are considerable in plants (Ramsey & Schemske, 1998). In *Achillea*, spontaneous 3x and 4x individuals of obvious autopolyploid nature are observed interspersed in 2x populations of *A. crithmifolia* (Ehrendorfer, 1959c), *A. roseoalba* (Ehrendorfer, 1953), etc. Spontaneous allopolyploids (4x) were found among experimental 2x F_2-progeny from *A. asplenifolia* × *A. setacea* (Ehrendorfer, 1959b). The common functioning of unreduced gametes in *Achillea* is obvious from karyological observations on ♂ meiosis and from 4x plants in F_1-progenies from experimental crosses between 2x (♀) × 4x (♂) (Ehrendorfer, 1959c). This mechanism leads to regular and multiple gene flow from the 2x to the 4x level, even in the absence of 3x-F_1. Additional polyploidization steps as well as hybridization within and between all higher ploidy levels (Schneider, 1958; Vetter *et al.*, 1996a,b) have increased

complexity of *A. millefolium* agg. enormously as is shown by the diversity of AFLP links (Fig. 8). Finally, polyploidization helps to overcome crossing barriers and makes it possible to successfully establish hybrid progeny, which fails on the diploid level. As a result, hybrid polyploidy has extended genetic links even beyond the aggregate, creating the present scenario in *Achillea* (Fig. 2). Parallel situations can be found in many other polyploid complexes, for example polytopic and polyphyletic origin of polyploids in N. American *Tragopogon* (Soltis *et al.*, 2003; Pires *et al.*, 2004) and *Nicotiana* (Chase *et al.*, 2003), current hybrid contacts between diploid and higher polypoid levels, as well as extensive horizontal gene flow in *Paeonia* (Sang & Zhang, 1999; Ferguson & Sang, 2001) and *Dactylorhiza* (Hedrén *et al.*, 2001).

Such complex evolutionary reticulations have often led to difficulties in the delimitation of polyploid species (as *Achillea collina* and *A. pratensis,* etc.) and the application of the traditional concept of auto- vs allopolyploidy. New combinations of adaptive features and often increased genetic diversity on the 4x (and 6x) level are common in *Achillea*, *Paeonia*, *Dactylorhiza* and other polyploid complexes. These phenomena may be the main reasons for the greater fitness of polyploids compared with their closest 2x relatives, as is obvious from the 4x species link between *Achillea* sect. *Ptarmica* and *A. millefolium* agg., the *A. collina*-4x + *A. pratensis*-4x group, *A. millefolium*-6x, etc. (Figs 2 and 6).

An impressive experimental model, which demonstrats the importance of polyploid hybrid recombination for new ecological radiation was presented by Hiesey & Nobs (1970). They transplanted hundreds of cloned F_2-progenies of a cross between two extremely divergent 6x ecotypes *Achillea borealis* (subarctic) and '*A. gigantea*' (subtropical) to three experimental gardens at altitudes of 30 m, 1400 m, and 3050 m in California. After 2 yr of rigorous climatic selection, a remarkable diversity of transgressive and well adapted new recombinations had become established in each of these gardens. But not only recombination, also new mutations triggered by hybridization and polyploidization should be considered as stimulus for new differentiation. In *Achillea*, this is suggested, i.a., by many new and specific AFLP bands that appear at the 4x and 6x levels but are absent in the 2x taxa (Fig. 8). Even more convincing are results from comparative genetic studies on diploids and their experimental or natural polyploid progeny. Here, one finds nearly always gene silencing or gene activation (Soltis & Soltis, 1999, 2000) and DNA elimination (e.g. Matzke *et al.*, 1999; Shaked *et al.*, 2001; Madlung *et al.*, 2002; Joly *et al.*, 2004) coupled either with relative genome stability (e.g. in *Spartina anglica*: Baumel *et al.*, 2002; N American *Tragopogon*: Soltis *et al.*, 2003; Pires *et al.*, 2004; *Gossypium*: Brubaker *et al.*, 1999; Liu *et al.*, 2001) or with major genome rearrangements (e.g. in *Brassica*: Song *et al.*, 1995; Axelson *et al.*, 2000; *Nicotiana*: Chase *et al.*, 2003; *Triticum*: Feldman *et al.*, 1997; Liu *et al.*, 1998a,b; Eckardt, 2001; Shaked *et al.*, 2001).

Cycles of differentiation and hybridization must have a long history in *Achillea*. The genus probably dates back to the later Tertiary. As in many other angiosperm phylogenies, this is reflected in the branch lengths of trees based on nrITS and plastid *trnL-F* sequences (Guo *et al.*, 2004). DNA sequence and AFLP analyses both show that on the diploid level well separated clades without hybrid contacts and on long branches dominate the basal and older segments of the genus, whereas the actively radiating polyploids appear as unresolved polytomies on shorter branches in a distal position, forming a younger complex network of closely related species. This contrast between stasigenesis and cladogenesis + reticulate radiation can also be demonstrated by comparing older and younger polyploid complexes. In *Achillea* sect. *Achillea* we can confront the relictual complex containing *A. tomentosa* (2x, SW Alps) and *A. chrysocoma* (6x, Balkan Mts.; Saukel *et al.*, 2004) with the fully developed and species-rich complex of *A. millefolium* agg. (2x-4x-6x-8x). Some of its taxa successfully expanded over the N hemisphere and have even become worldwide weeds.

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