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Flow cytometry confirms reticulate evolution and reveals triploidy in Central European *Diphasiastrum* taxa (Lycopodiaceae, Lycophyta)

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• *Background and Aims* Interspecific *Diphasiastrum* hybrids have been assumed to be homoploid and to produce well-formed spores serving sexual reproduction. If this were the case, forms intermediate between hybrids and parents or hybrid swarms should be expected. The purpose of this study was: (1) to check whether homoploidy consistently applies to the three hybrids throughout their Central European range; (2) to examine whether their genome sizes confirm their parentage as assumed by morphology; and (3) to perform a screening for detection of ploidy levels other than diploid and variation in DNA content due to backcrossing.
• *Methods* Flow cytometry was used first to measure the relative DNA values [with 4′,6-diamidino-2-phenylindole (DAPI) staining] and ploidy level as a general screening, and secondly to determine the absolute DNA 2C values [with propidium iodide (PI) staining] in a number of selected samples with the main focus on the hybrids.
• *Key Results* A considerable variation of DNA 2C values (5·26–7·52 pg) was detected between the three European *Diphasiastrum* species. The values of the diploid hybrids are highly constant without significant variation between regions. They are also intermediate between their assumed parents and agree closely with those calculated from their putative parents. This confirms their hybrid origin, assumed parentage and homoploid status. Considerably higher DNA amounts (9·48–10·30 pg) were obtained for three populations, suggesting that these represent triploid hybrids, an interpretation that is strongly supported by their morphology.
• *Conclusions* Diploid hybrids have retained their genetic and morphological identites throughout their Central European range, and thus no indications for diploid backcrossing were found. The triploid hybrids have probably originated from backcrossing between a diploid gametophyte of a hybrid (derived from a diplospore) and a haploid gametophyte of a diploid parental species. By repeated crossing events, reticulate evolution patterns arise that are similar to those known for a number of ferns.

Key words: *Diphasiastrum*, Lycopodiaceae, flow cytometry, nuclear DNA content, homoploidy, triploidy, hybridization, reticulate evolution.

INTRODUCTION

The lycophytes are the oldest extant group of spore-producing vascular plants, and their earliest fossil remains are from the Devonian or possibly (the genus *Baragwanathia*) even from the late Silurian period (Hueber, 1983; Garratt *et al.*, 1984; Rickards, 2000; Gensel and Berry, 2001; Hao and Gensel, 2001; Wikström, 2001; Kotyk *et al.*, 2002).

While their extant representatives comprise a number of rather small and inconspicuous herbaceous plants, in the Carboniferous, lycophytes (*Lepidodendron* and *Sigillaria*) grew as tall trees (Stewart and Rothwell, 1993; Berry and Fairon-Demaret, 2001; Pigg, 2001; Taylor *et al.*, 2009) and, in association with the arborescent giant horsetails (Calamitaceae and Archaeocalamitaceae), formed swampland

forests for 40 million years and were important contributors to coal formation.

Reconstructions of relationships for the main lineages of vascular plants that diverged since the Devonian period have revealed a basal dichotomy, separating the lycophytes from the so-called euphyllophytes (Euphyllophytina, all other vascular plants) approx. 400 million years ago (Raubeson and Jansen, 1992; Kenrick and Crane, 1997; Duff and Nickrent, 1999; Pryer *et al.*, 2001, 2004*a*, *b*; Tsuji *et al.*, 2007). The latter comprise two major clades: the spermatophytes (seed plants, Soltis and Soltis, 2004), and the monilophytes (ferns, *sensu* Pryer *et al.*, 2004*b*), including horsetails, whisk ferns and all eusporangiate and leptosporangiate ferns.

The extant lycophytes include members of the homosporous Lycopodiaceae, as well as the heterosporous Selaginellaceae

and Isoëtaceae. Although being extremely diverse and of virtually cosmopolitan distribution, the family of Lycopodiaceae is considered to be monophyletic (Wikström and Kenrick, 1997; Yatsentyuk *et al.*, 2001). While in the past all species of the Lycopodiaceae were placed generally in the single genus *Lycopodium*, in the last several decades the family has been variously divided into 2–16 genera (Øllgaard, 1987; Wagner and Beitel, 1993; Haines, 2003). In Europe, currently four genera are recognized: *Diphasiastrum*, *Huperzia*, *Lycopodium* and *Lycopodiella* (Valentine and Moore, 1993).

The genus *Diphasiastrum* comprises a relatively small group of lycopods that differ morphologically from the other genera by their leaves being usually decussate (or 4–5-ranked), di- or even trimorphic, and the aerial branchlets being quadrate to flattened. It includes about 25 species (and taxa of hybrid origin) of mainly north temperate and sub-arctic distribution (Wagner and Beitel, 1993). Only a few species occur in the tropics or sub-tropics, where they are restricted to mountain areas (e.g. *D. multispicatum*; see Bennert *et al.*, 2007). The base number is generally accepted to be $x = 23$ (Wagner, 1992), and diploidy prevails by far (Aagaard *et al.*, 2009c).

In Continental Europe, six diploid taxa are recognized, including three parental species (*D. alpinum*, *D. complanatum* and *D. tristachyum*, called 'species' in the following), and three intermediate taxa of putative hybrid origin (*D. × issleri*, *D. × oellgaardii* and *D. × zeilleri*, called 'hybrids' in the following).

It generally has been supposed that these diploid hybrids reproduce sexually via spores, giving rise to F_2 and subsequent generations, but this never has been proven beyond doubt. Also, it is not clear whether backcrosses between hybrids and parent species occur, as would be expected regarding the high crossability of species. Judging from morphology, the parental species seem to retain their identity and no transitions between parents and hybrids have been found so far, with rare putative triploids as a possible exception (see below). A total of seven homoploid hybrids have been reported from North America, Northern Asia and Europe (Wagner *et al.*, 1985).

Flow cytometry was applied in order to check whether (*a*) the known *Diphasiastrum* hybrids in Central Europe are invariably homoploid; (*b*) their genome sizes would be indicative of their origin; and (*c*) cytotypes other than diploid exist.

MATERIALS AND METHODS

Plants of *Diphasiastrum* were identified by their morphological characters. While the species are easy to differentiate, recognition of the hybrids requires advanced experience. Diagnostically useful are the dimensions of the ventral and dorsal leaves and their size in relation to the stem (see Horn, 2006, 2008; Horn and Tribsch, 2009).

For flow cytometric studies, fresh plant material was collected (by K.H., B.Ø., I.J. and R.V.) in Denmark, France, the Czech Republic, the Central German Uplands and northern Italy (Table 1), and kept in the refrigerator prior to analysis for no longer than 2 weeks. Three to five branchlets were collected per colony; discontiguous parts of larger colonies were sampled separately. For all accessions an initial ploidy screening was performed on a Ploidy Analyser (Partec GmbH, Münster,

Germany) using 4′,6-diamidino-2-phenylindole (DAPI)-stained samples (1·6 µg mL^{-1}) isolated in 2·1 % (w/v) citric acid monohydrate, 0·5 % (w/v) Tween-20, together with *Agave sisalana* (5*x*, $2n = 150$, $2C = 20·2$ pg; Zonneveld *et al.*, 2005) as an internal reference standard. Absolute 2C DNA contents were determined for selected accessions (of 2–6 phytogeographic regions with one to several localities each) using propidium iodide (PI) staining. *Diphasiastrum* samples and leaf sections of *Pisum sativum* 'Viktoria, Kifejtö Borsó' (Genebank Gatersleben accession number: PIS 630; $2C = 9·09$ pg; Doležel *et al.*, 2007) as an internal reference standard were simultaneously chopped and stained using the 'CyStain PI absolute P' nuclei extraction and staining kit (Partec GmbH, Münster) according to the manufacturer's instruction. Samples were analysed using either a Partec CyFlow SL flow cytometer (Münster, Germany) or a FACStar^{PLUS} (BD Biosciences, San José, CA, USA). Usually 10 000 nuclei per sample were analysed in at least five replicates per accession. The absolute DNA contents of *Diphasiastrum* species were calculated based on the ratios of the G_1 peak means of sample and reference standard.

Using the 2C DNA contents, a Student's test was performed to test (*a*) for differences between the populations within one taxon and (*b*) for differences between taxa based on all populations studied.

RESULTS AND DISCUSSION

Relative DNA values (DAPI measurements)

Values for relative fluorescence intensity show a 1·38-fold variation between the parent species, with *D. alpinum* possessing the largest and *D. tristachyum* the smallest genome (Table 2). The genome sizes of the three known hybrids are not only in the same range as those of the diploid species, but also intermediate between those of the assumed parents, confirming their diploid status as well as their correct identification. Within the taxa the variation between regions is small and does not exceed the inaccuracy threshold.

Considerably higher values were found in only three accessions, one from the Bavarian Forest and two from the Oberpfälzer Wald. These exceed those of the species by a factor of 1·30–1·80 (average 1·59). Therefore, these three colonies represent plants with a ploidy level other than diploid; they are very probably triploids (see below).

Nuclear DNA C values of the species (PI measurements)

Analogous to the results of the DAPI measurements, a considerable variation (1·42-fold) in nuclear DNA C amounts was found (Table 3), with *D. alpinum* having the largest 2C content (7·49 pg), *D. tristachyum* the smallest (5·26 pg) and *D. complanatum* being intermediate (5·72 pg), but closer to *D. tristachyum*. The intraspecific differences between localities and regions are small (usually <0·1 pg) and statistically not significant ($P < 0·01$).

To our knowledge, there are only very few reports on 2C DNA amounts in *Diphasiastrum* (Table 4). Our estimates exceed both the values published by Aagaard *et al.* (2009c) and those that were extracted from appendix 2 in Aagaard

TABLE 1. Diphasiastrum *samples used for flow cytometric analyses*

Taxon	Accession number	Date of collection	Locality	Region	Type of study
D. alpinum	H 08/01	20/8/2008	Gr. Sonnen-Berg N St. Andreasberg, NI, D	Har	b
D. alpinum	H 09/01, H 09/73	11/9/2009, 18/11/2009	NNW Finsterau, BY, D	BaFo	a, b
D. alpinum	H 09/03, H 09/75	11/9/2009, 18/11/2009	W Finsterau, BY, D	BaFo	a, b
D. alpinum	H 09/09	11/9/2009	SW Altschönau, BY, D	BaFo	a
D. alpinum	H 09/11	11/9/2009	SW Guglöd, BY, D	BaFo	a
D. alpinum	H 09/27	11/9/2009	NE Frauenau, eastern locality, BY, D	BaFo	a
D. alpinum	H 09/30	11/9/2009	NE Frauenau, western locality, BY, D	BaFo	a
D. alpinum	H 09/36	12/9/2009	Kiesruck NE Buchenau, BY, D	BaFo	a
D. alpinum	H 09/65	17/11/2009	Hirschenstein N Böbrach, BY, D	BaFo	a, b
D. alpinum	H 09/68	17/11/2009	Zlíbský vrch N Strázny, C, CZ	BoFo	a, b
D. alpinum	H 09/87b	18/11/2009	Voithenberg, western locality, BY, D	ObWa	a
D. alpinum	H 09/57	1/10/2009	Hundseck SE Bühlertal, BW, D	BlFo	a
D. alpinum	H 09/63	3/10/2009	Kandel N St. Peter, BW, D	BlFo	a
D. alpinum	V 12664, V 12736	29/10/2009	Vexaincourt W Schirmeck, Vosges, F	Vos	a
D. alpinum	V 12647	1/10/2009	SE St. Quirin, Moselle, F	Vos	a
D. alpinum	V 12669–71, V 12681, V 12685	1/10/2009	Champ du Feu W le Hohwald, Bas-Rhin, F	Vos	a
D. alpinum	V 12713	2/10/2009	Col du Schlucht, Haut-Rhin, F	Vos	a
D. alpinum	V 12740	4/11/2009	Forét d'Abreschviller, Moselle, F	Vos	a
D. alpinum	Ho 09/12	19/11/2009	Rosskopf SW Engenthal, Moselle, F	Vos	a
D. alpinum	V ST/01–09	4/8/2010	Martalm, Ridnauntal, South Tyrol, I	ST	a
D. complanatum	BØ 09/01, BØ 09/03	27/10/2009	Nørlund Plantage S Ikast, Midtjylland, DK	Jut	a
D. complanatum	H 08/02	20/8/2008	Gr. Sonnen-Berg N St. Andreasberg, NI, D	Har	b
D. complanatum	H 09/04, H 09/76	11/9/2009, 18/11/2009	W Finsterau, BY, D	BaFo	a, b
D. complanatum	H 09/12	11/9/2009	SW Guglöd, BY, D	BaFo	a
D. complanatum	H 09/17	11/9/2009	E Spiegelau, BY, D	BaFo	a,
D. complanatum	H 09/31	11/9/2009	NE Frauenau, western locality, BY, D	BaFo	a
D. complanatum	H 09/37	12/9/2009	Kiesruck NE Buchenau, BY, D	BaFo	a
D. complanatum	H 09/43, H 09/88	12/9/2009, 18/11/2009	Voithenberg, western locality, BY, D	ObWa	a, b
D. complanatum	H 09/69	17/11/2009	Zlíbský vrch N Strázny, C, CZ	BoFo	a, b
D. complanatum	V ST/10–12	4/8/2010	Martalm, Ridnauntal, South Tyrol, I	ST	a
D. × issleri	H 08/03	20/8/2008	Gr. Sonnen-Berg N St. Andreasberg, NI, D	Har	b
D. × issleri	H 09/02, H 09/74	11/9/2009, 18/11/2009	NNW Finsterau, BY, D	BaFo	a, b
D. × issleri	H 09/05, H 09/77	11/9/2009, 18/11/2009	W Finsterau, BY, D	BaFo	a, b
D. × issleri	H 09/13, H 09/14	11/9/2009	SW Guglöd, BY, D	BaFo	a
D. × issleri	H 09/18	11/9/2009	E Spiegelau, BY, D	BaFo	a
D. × issleri	H 09/24	11/9/2009	Spiegelau, BY, D	BaFo	a
D. × issleri	H 09/32, H 09/33	11/9/2009	NE Frauenau, western locality, BY, D	BaFo	a
D. × issleri	H 06/07, H 09/38	12/10/2006, 12/9/2009	Kiesruck NE Buchenau, BY, D	BaFo	a
D. × issleri	H 09/44, H 09/45, H 09/89	12/9/2009, 18/11/2009	Voithenberg, western locality, BY, D	ObWa	a, b
D. × issleri	H 09/66	17/11/2009	Hirschenstein N Böbrach, BY, D	BaFo	a, b
D. × issleri	H 09/67	17/11/2009	S Haidmühle, BY, D	BaFo	a, b
D. × issleri	H 09/81	18/11/2009	Rindel-Berg E Altschönau, BY, D	BaFo	a, b
D. × issleri	H 09/070	17/11/2009	Zlíbský vrch N Strázny, C, CZ	BoFo	a, b
D. × issleri	H 09/59	1/10/2009	Lochrütte, Feldberg region, BW, D	BlFo	a, b
D. × issleri	H 09/60, V 12698	2/10/2009	Le Tanet, type locality, Haut-Rhin, F	Vos	a, b
D. × issleri	H 09/61	2/10/2009	NE Le Tanet, Haut-Rhin, F	Vos	a, b
D. × issleri	V ST/13–16	4/8/2010	Martalm, Ridnauntal, South Tyrol, I	ST	a
D. × oellgaardii	BØ 09/06	27/10/2009	Præstbjerg Plantage SE Vind, Midtjylland, DK	Jut	a
D. × oellgaardii	H 09/06, H 09/78	11/9/2009, 18/11/2009	W Finsterau, BY, D	BaFo	a, b
D. × oellgaardii	H 09/15	11/9/2009	SW Guglöd, BY, D	BaFo	a
D. × oellgaardii	H 09/20, H 09/83	11/9/2009, 18/11/2009	E Spiegelau, BY, D	BaFo	a, b
D. × oellgaardii	H 09/25	11/9/2009	Spiegelau, BY, D	BaFo	a
D. × oellgaardii	H 09/34	11/9/2009	NE Frauenau, western locality, BY, D	BaFo	a
D. × oellgaardii	H 09/39	12/9/2009	Kiesruck NE Buchenau, BY, D	BaFo	a
D. × oellgaardii	H 09/71	17/11/2009	Zlíbský vrch N Strázny, C, CZ	BoFo	a, b
D. × oellgaardii	H 06/19	16/11/2006	Dreiwappen ENE Althütte, BY, D	ObWa	a
D. × oellgaardii	H 09/46, H 09/47, H 09/90	12/9/2009, 18/11/2009	Voithenberg, western locality, BY, D	ObWa	a, b
D. × oellgaardii	H 09/54	1/10/2009	Beerfelden, HE, D	HeOd	a
D. × oellgaardii	H 09/58	1/10/2009	Hundseck SE Bühlertal, BW, D	BlFo	a
D. × oellgaardii	H 09/62	3/10/2009	Weiherkopf S Münsterhalden, BW, D	BlFo	a
D. × oellgaardii	V 12672, V 12673, V 12676, V 12678, V 12679, V 12686	1/10/2009	Champ du Feu W le Hohwald, Bas-Rhin, F	Vos	a
D. × oellgaardii	V ST/17	4/8/2010	Martalm, Ridnauntal, South Tyrol, I	ST	a
D. tristachyum	BØ 09/02, BØ 09/05	27/10/2009	Nørlund Plantage S Ikast, Midtjylland, DK	Jut	a
D. tristachyum	H 09/07, H 09/79	11/9/2009, 18/11/2009	W Finsterau, BY, D	BaFo	a, b

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T ABLE 1. *Continued*

Taxon	Accession number	Date of collection	Locality	Region	Type of study
D. tristachyum	H 09/10	11/9/2009	SW Altschönau, BY, D	BaFo	a
D. tristachyum	H 09/21	11/9/2009	E Spiegelau, BY, D	BaFo	a
D. tristachyum	H 09/23, H 09/85	11/9/2009, 18/11/2009	Filz-Wald E Spiegelau, BY, D	BaFo	a, b
D. tristachyum	H 09/26	11/9/2009	Spiegelau, BY, D	BaFo	a
D. tristachyum	H 09/28	11/9/2009	NE Frauenau, eastern locality, BY, D	BaFo	a
D. tristachyum	H 09/40	12/9/2009	Kiesruck NE Buchenau, BY, D	BaFo	a
D. tristachyum	H 09/48, H 09/91	12/9/2009, 18/11/2009	Voithenberg, western locality, BY, D	ObWa	a, b
D. tristachyum	H 09/55	1/10/2009	Beerfelden, HE, D	HeOd	a
D. tristachyum	H 09/56	1/10/2009	Olfen, HE, D	HeOd	a
D. tristachyum	H 09/64	3/10/2009	Kandel N St. Peter, BW, D	BlFo	a
D. tristachyum	V 12639, V 12649	1/10/2009	Altdorfkopf WSW Dabo, Moselle, F	Vos	a
D. tristachyum	V 12640, V 12645	1/10/2009	Le Canceley SE Abreschviller, Moselle, F	Vos	a
D. tristachyum	V 12665–67, V 12668, V 12674, V 12677, V 12684, V 12687	1/10/2009	Champ du Feu W le Hohwald, Bas-Rhin, F	Vos	a
D. tristachyum	V 12690	1/10/2009	ENE Moussey, Moselle, F	Vos	a
D. tristachyum	V 1273	12/10/2009	St. Quirin (Tête du Calice), Moselle, F	Vos	a
D. tristachyum	V 12738	27/10/2009	Singristerkopf near Reinhardsmunster, Bas-Rhin, F	Vos	a
D. tristachyum	V 12735	29/10/2009	Vallée de la Sarre Blanche, Moselle, F	Vos	a
D. tristachyum	V 12737	29/10/2009	Kappelbronn N Lutzelhouse, Bas-Rhin, F	Vos	a
D. tristachyum	V 12739	4/11/2009	Forét domaniale de Walscheid, Moselle, F	Vos	a
D. × *zeilleri*	BØ 09/07	27/10/2009	Tihøje ved Røddinglund Plantage WNW Vildbjerg, Midtjylland, DK	Jut	a
D. × *zeilleri*	H 08/04	20/8/2008	Gr. Sonnen-Berg N St. Andreasberg, NI, D	Har	b
D. × *zeilleri*	H 09/08, H 09/80	11/9/2009, 18/11/2009	W Finsterau, BY, D	BaFo	a, b
D. × *zeilleri*	H 09/16	11/9/2009	SW Guglöd, BY, D	BaFo	a
D. × *zeilleri*	H 09/22, H 09/84	11/9/2009, 18/11/2009	E Spiegelau, BY, D	BaFo	a, b
D. × *zeilleri*	H 09/29	11/9/2009	NE Frauenau, eastern locality, BY, D	BaFo	a
D. × *zeilleri*	H 09/35	11/9/2009	NE Frauenau, western locality, BY, D	BaFo	a
D. × *zeilleri*	H 09/41	12/9/2009	Kiesruck NE Buchenau, BY, D	BaFo	a
D. × *zeilleri*	H 09/49, H 09/50, H 09/92	12/9/2009, 18/11/2009	Voithenberg, western locality, BY, D	ObWa	a, b
D. × *zeilleri*	H 09/72	17/11/2009	Zlíbský vrch N Strázny, C, CZ	BoFo	a
D. × *zeilleri*	H 09/52, H 09/53	1/10/2009	Beerfelden, HE, D	HeOd	a, b
D. × *zeilleri*	V 12679, V 12680, V 12682	1/10/2009	Champ du Feu W le Hohwald, Bas-Rhin, F	Vos	a
D. × *zeilleri*	V 12728	9/10/2009	Baerenthal (Lindenkopf), Moselle, F	Vos	a
D. × *zeilleri*	V 12729	9/10/2009	Wingen (Col du Litschhof, Wingen, Bas-Rhin, F	Vos	a
D. × *zeilleri*	V 12732	9/10/2009	Jungenwald, Wingen, Bas-Rhin, F	Vos	a
D. × *zeilleri*	V 12733	9/10/2009	Diebhalt, Climbach, Bas-Rhin, F	Vos	a
D. hybrid, triploid	H 09/19, H 09/82	11/9/2009, 18/11/2009	E Spiegelau, BY, D	BaFo	a, b
D. hybrid, triploid	H 09/51, H 09/86	12/9/2009, 18/11/2009	Voithenberg, eastern locality, BY, D	ObWa	a, b
D. hybrid, triploid	H 09/42, H 09/87a	12/9/2009, 18/11/2009	Voithenberg, western locality, BY, D	ObWa	a, b

Abbreviations used for accession numbers: H, K. Horn; V, R. Viane; Ho, P. Holveck; BØ, I.S. Bisgaard Jakobsen and B. Øllgaard. For localities: D, Germany; BW, Baden-Württemberg; BY, Bavaria; HE, Hesse; NI, Lower Saxony; ST, Sachsen-Anhalt; CZ, Czech Republic; C, Budejovicky Kraj; DK, Denmark; F. France; I, Italy. For regions: BaFo, Bavarian Forest; BlFo, Black Forest; BoFo, Bohemian Forest; Har, Harz Mountains; HeOd, Hessischer Odenwald; Jut, Jutland; ObWa, Oberpfälzer Wald; ST, South Tyrol; V, Vosges Mountains. For types of study: a, flow cytometry by DAPI; b, flow cytometry by PI.

et al. (2009*b*) by about 1 pg. The use of different methods, Feulgen densitometry (Aagaard *et al.*, 2009*b*, *c*) compared with flow cytometry (present study), cannot be responsible for these differences (see Doležel *et al.*, 1998; Vilhar *et al.*, 2001). However, the use of frozen or dried samples (as done by Aagaard *et al.* 2009*b*, *c*) has to be considered with caution as, due to an altered accessibility of the stain to the DNA, an apparent nucleic acid loss cannot be excluded, especially after long-term storage (Suda and Trávníček, 2006; Aagaard *et al.*, 2009*b*, *c*).

Data on genome size in other Lycopodiaceae are scarce. Omitting those of Bouchard (1976) because of large discrepancies compared with more recent studies (see Bennett and

Leitch, 2001), there seem to be only two reports on members of the Lycopodiaceae. *Lycopodium clavatum* was determined to have a 2C DNA content of 5·72 pg (Hanson and Leitch, 2002), which is in the same range as our results for *Diphasiastrum*. In *Huperzia lucidula* a higher 2C DNA content of 11·40 pg was found (Wang *et al.*, 2005), probably reflecting the high base number ($x = 67$ or 68) in this genus (Wagner and Beitel, 1993).

Nuclear DNA C values in the diploid hybrids (PI measurements)

The three diploid *Diphasiastrum* hybrids yielded 2C DNA values which are similar to those of the (diploid) non-hybrid

TABLE 2. *Relative fluorescence intensity (DAPI measurements) for the* Diphasiastrum *taxa studied, summarized by the phytogeographic regions*

Taxon	Genome composition	Ploidy level	Region	No. of sampled colonies	Relative fluorescence intensity (mean $\pm$ s.d.)	Overall mean ($\pm$ s.d.)
D. alpinum	AA	$2x$	BaFo	7	0·321 $\pm$ 0·006	
			BlFo	2	0·319 $\pm$ 0·006	
			BoFo	1	0·313	
			Vos	6	0·317 $\pm$ 0·006	
			ST	9	0·319 $\pm$ 0·006	0·317 $\pm$ 0·006
D. complanatum	CC	$2x$	BaFo	5	0·255 $\pm$ 0·009	
			BoFo	1	0·243	
			Jut	2	0·253 $\pm$ 0·009	
			ObWa	1	0·260	
			ST	3	0·248 $\pm$ 0·002	0·249 $\pm$ 0·011
D. tristachyum	TT	$2x$	BaFo	7	0·235 $\pm$ 0·012	
			BlFo	1	0·226	
			HeOd	2	0·226 $\pm$ 0·001	
			Jut	2	0·241 $\pm$ 0·001	
			ObWa	2	0·231 $\pm$ 0·003	
			Vos	9	0·225 $\pm$ 0·008	0·229 $\pm$ 0·010
D. $\times$ issleri	AC	$2x$	BaFo	7	0·287 $\pm$ 0·012	
			BlFo	1	0·286	
			BoFo	1	0·281	
			ObWa	3	0·285 $\pm$ 0·007	
			Vos	2	0·284 $\pm$ 0·005	
			ST	4	0·280 $\pm$ 0·005	0·284 $\pm$ 0·009
D. $\times$ oellgaardii	AT	$2x$	BaFo	6	0·279 $\pm$ 0·009	
			BlFo	2	0·268 $\pm$ 0·010	
			HeOd	1	0·263	
			Jut	1	0·283	
			ObWa	3	0·281 $\pm$ 0·007	
			Vos	6	0·267 $\pm$ 0·005	
			ST	1	0·278	0·273 $\pm$ 0·009
D. $\times$ zeilleri	CT	$2x$	BaFo	6	0·246 $\pm$ 0·007	
			BoFo	1	0·230	
			HeOd	2	0·234 $\pm$ 0·001	
			Jut	1	0·243	
			ObWa	3	0·250 $\pm$ 0·002	
			Vos	5	0·231 $\pm$ 0·006	0·238 $\pm$ 0·009
D. hybrid	AAC	$3x$	ObWa	1	0·429 $\pm$ 0·003	0·429 $\pm$ 0·003
D. hybrid	AAT	$3x$	ObWa	1	0·416 $\pm$ 0·004	0·416 $\pm$ 0·004
D. hybrid	ACC	$3x$	BaFo	1	0·402 $\pm$ 0·003	0·402 $\pm$ 0·003

For abbreviations used, see Table 1; letters used for genome composition: A, *D. alpinum*; C, *D. complanatum*; T, *D. tristachyum*.

species, ranging from 5·52 pg in *D. $\times$ zeilleri* to 6·63 pg in *D. $\times$ issleri* (Table 3). The values are exactly intermediate between those of their assumed parents and agree closely with those calculated from their putative parents by adding the corresponding 1C values for a single genome (Table 5). The concordance between the measured and expected values convincingly supports their hybrid origin, the assumed parentage (as inferred from morphological characters) as well as their homoploid status. Similarly to the species, the geographical variation (within and between regions) is small and statistically not significant ($P < 0.01$). Homoploid hybrids as confirmed by flow cytometry occur in all nine geographical regions studied (Fig. 1). In the Vosges Mountains, the Bavarian Forest, the Oberpfälzer Wald and the Bohemian Forest the occurrence of all three hybrids was confirmed, while in the remaining five areas the existence of two hybrids was established. *Diphasiastrum $\times$ zeilleri* appears to be the most common hybrid taxon and was shown to occur in seven out of nine regions.

Just like in the species, our estimates exceed the values extracted from appendix 2 in Aagaard *et al.* (2009*b*) by about 1 pg (Table 4).

Diploid backcrossing

It has been proposed that in *Diphasiastrum*, backcrosses between hybrids and their parent species occur at the diploid level (Aagaard *et al.*, 2009*a*). Exemplified for *D. $\times$ issleri*, backcrossing would require this primary hybrid to produce germinable haploid meiospores (with 23 chromosomes) containing chromosomes from both parents, *D. alpinum* and *D. complanatum*. If we assume the assortment of homologues in meiosis to be random (which is likely) and to follow a Gaussian distribution, 99 % of the spores produced contain 6–17 chromosomes of each parent. During fertilization, such a mixed chromosome set would be added to a single *D. alpinum* genome (23 chromosomes), resulting in a sporophyte with the majority (63–87 %) of its chromosomes

TABLE 3. *DNA 2C values (pg) for the diploid* Diphasiastrum *taxa studied*

Taxon	Accession number	Region	Locality	2C ± s.d. mean for locality	2C ± s.d. mean for region	2C ± s.d. overall mean
D. alpinum	H 09/75	BaFo	W Finsterau	7·50 ± 0·04		
	H 09/65	BaFo	Hirschenstein	7·49 ± 0·07		
	H 09/73	BaFo	NNW Finsterau	7·45 ± 0·03	7·49 ± 0·05	
	H 08/01	Har	Gr. Sonnenberg	7·55 ± 0·05	7·55 ± 0·05	7·52 ± 0·06
D. complanatum	H 09/76	BaFo	W Finsterau	5·73 ± 0·06	5·73 ± 0·06	
	H 09/69	BoFo	Strázny	5·74 ± 0·01	5·74 ± 0·01	
	H 09/43 and H 09/88	ObWa	Voithenberg	5·73 ± 0·07	5·73 ± 0·07	
	H 08/02	Har	Gr. Sonnenberg	5·78 ± 0·03	5·73 ± 0·07	5·76 ± 0·05
D. tristachyum	H 09/79	BaFo	W Finsterau	5·26 ± 0·05		
	H 09/85	BaFo	E Spiegelau	5·23 ± 0·07	5·25 ± 0·06	
	H 09/48 and H 09/91	ObWa	Voithenberg	5·29 ± 0·06	5·29 ± 0·06	5·26 ± 0·06
D. × *issleri*	H 09/77	BaFo	W Finsterau	6·68 ± 0·05		
	H 09/66	BaFo	Hirschenstein	6·59 ± 0·07		
	H 09/67	BaFo	Haidmühle	6·59 ± 0·05		
	H 09/74	BaFo	NNW Finsterau	6·67 ± 0·09		
	H 09/81	BaFo	Rindel-Berg	6·52 ± 0·06	6·61 ± 0·07	
	H 09/70	BoFo	Strázny	6·61 ± 0·06	6·61 ± 0·06	
	H 09/45 and H 09/89	ObWa	Voithenberg	6·61 ± 0·04	6·61 ± 0·04	
	H 09/59	BlFo	Feldberg	6·73 ± 0·08	6·73 ± 0·08	
	H 09/60	Vos	Tanneck, type locality	6·62 ± 0·01		
	H 09/61	Vos	Tanneck	6·62 ± 0·02	6·62 ± 0·02	
	H 08/03	Har	Gr. Sonnenberg	6·65 ± 0·05	6·65 ± 0·05	6·64 ± 0·07
D. × *oellgaardii*	H 09/78	BaFo	W Finsterau	6·45 ± 0·06		
	H 09/83	BaFo	E Spiegelau	6·36 ± 0·10	6·40 ± 0·09	
	H 09/71	BoFo	Strázny	6·34 ± 0·08	6·34 ± 0·08	
	H 09/46 and H 09/90	ObWa	Voithenberg	6·40 ± 0·06	6·40 ± 0·06	6·39 ± 0·08
D. × *zeilleri*	H 09/80	BaFo	W Finsterau	5·51 ± 0·03		
	H 09/84	BaFo	E Spiegelau	5·46 ± 0·04	5·50 ± 0·05	
	H 09/49 and H 09/92	ObWa	Voithenberg	5·52 ± 0·07	5·52 ± 0·07	
	H 09/52	HeOd	Beerfelden 1	5·55 ± 0·04		
	H 09/53	HeOd	Beerfelden 2	5·56 ± 0·02	5·55 ± 0·03	
	H 08/04	Har	Gr. Sonnenberg	5·56 ± 0·04	5·56 ± 0·04	5·53 ± 0·05

For abbreviations used, see Table 1.

being derived from *D. alpinum* and only a minor fraction (13–37 %) from *D. complanatum*.

Backcrosses exhibiting such more or less intermediate genotypes should not only be morphologically striking but also detectable by flow cytometry due to their intermediate DNA contents. Our 2C DNA values, however, are virtually invariable within each species and hybrid ($P < 0.01$), and the differences in genome sizes between all taxa are highly significant ($P < 0.0001$).

A rough estimate for Central Europe, based on data from field observations, revision of herbarium specimens and regional literature, reveals that the 15–17 populations of each hybrid included in this study (see Table 1) represent 7 % of the approx. 230 known populations in *D.* × *zeilleri*, 15 % in *D.* × *issleri* with about 110 colonies, and 71 % in *D.* × *oellgaardii* with 21 records. Considering the high crossability of species and the fact that agglomerations with 4–6 taxa co-occurring are not rare, backcrosses would be expected to be not uncommon and should be detectable in our sampling.

Aagaard *et al.* (2009*b*) presented evidence that reciprocal crosses occur repeatedly in *D.* × *issleri* and × *zeilleri*. In such reciprocal crosses no DNA content differences would be expected.

Genetic variability was observed in neighbouring or inter-mixed clones in two of the diploid hybrids (*D.* × *issleri* and × *zeilleri*) by means of isoenzyme gel electrophoresis (see Horn, 1997), which also suggests repeated hybridization events.

Molecular studies are in preparation by one of the authors (M. Schnittler) to (*a*) prove the status of the hybrid taxa by using species-specific uniparental inherited markers and (*b*) develop markers that allow genotyping of individuals to test the occurrence of diploid backcrossing.

Nuclear DNA C values and possible parentage of three putative triploid hybrids

Considerably higher 2C DNA amounts than in the diploids were found in three accessions, with values ranging from 9·48 to 10·30 pg (Table 5). These exceed those of *D. alpinum* (which has the largest genome size of the diploids) by 2 to almost 3 pg, providing strong evidence that these plants are polyploids. Figure 2 shows the various genome sizes to be expected for the triploid and tetraploid genotype combinations based on the three diploid species. They range from 7·89 pg (in an autotriploid *D. tristachyum*, TTT) to 15·04 pg (in an auto-tetraploid *D. alpinum*, AAAA). As expected, the tetraploid cytotypes occupy the upper range, starting with 10·52 pg for an autotetraploid *D. tristachyum* (TTTT). This value is 0·22 pg higher than the largest 2C DNA amount measured

for the new hybrids, allowing the conclusion that they represent triploids and not tetraploids. The closest concordances between measured and expected values were found assuming the following genome compositions: ACC for the accession 09/82 from the Bavarian Forest, and AAC for the accession 09/86 and AAT for the accession 09/87a, both from the

TABLE 4. *DNA 2C values (pg) for several north temperate* Diphasiastrum *species and hybrids*

Taxon	Wang et al. (2005)	Aagaard et al. (2009c)	Aagaard et al. (2009b)	This study
D. alpinum		6·40	6·45 (6·78) [6·97]	7·52
D. complanatum		4·82	4·81 (5·12) [5·37]	5·76
D. digitatum	5·45			
D. madeirense		5·63		
D. tristachyum		4·45	4·28 (4·70)	5·26
D. × issleri			5·61	6·64
D. × oellgaardii			5·21	6·39
D. × zeilleri			4·53	5·53

Data are from Wang *et al.* (2005), Aagaard *et al.* (2009*b*, *c*) and from this study; values in parentheses are mentioned by Aagaard *et al.* (2009*b*) under results and were obtained using Feulgen densitometry and those in square brackets were obtained by flow cytometric measurements using fresh material.

Aagaard *et al.* (2009*b*, appendix 2) presented a comprehensive list of Feulgen DNA image densitometry data on numerous *Diphasiastrum* accessions without, however, naming the taxa. The only information given is that of the origin of the chloroplast DNA ('maternal lineage'). As a certain maternal chloroplast haplotype occurs in the corresponding species, but may also be present in the two hybrids derived from it, each accession could belong to three different taxa. For example, the chloroplast haplotype of *D. alpinum* is contained in *D. alpinum* itself, but also occurs in *D. × issleri* and *D. × oellgaardii*, provided *D. alpinum* was the female parent. We have attempted to identify the taxa of the accessions analysed by Aagaard *et al.* (2009*b*, appendix 2) by first selecting the three possible candidates on the basis of the maternal lineage and then comparing the reported 2C content with the values given by Aagaard *et al.* (2009*c*) for the three species. Taxa with intermediate values were interpreted as the corresponding hybrids. Some data sets could not be assigned due to incompatibility of data.

FIG. 1. Map of Central Europe showing the distribution of *Diphasiastrum* taxa identified; a, *D. alpinum*; c, *D. complanatum*; i, *D. × issleri*; o, *D. × oellgaardii*; t, *D. tristachyum*; z, *D. × zeilleri*. Code numbers for the regions: [1]Denmark, [2]Harz Mountains, [3]Hessischer Odenwald, [4]Vosges Mountains, [5]Black Forest, [6]Bavarian Forest, [7]Oberpfälzer Wald, [8]Bohemian Forest, [9]South Tyrol.

TABLE 5. *DNA 2C values for the species and hybrids of* Diphasiastrum *studied*

Taxon and genome formula	Genome formula	Values measured (pg ± s.d.)	Values predicted (pg)	Difference measured – predicted (pg)
Species				
D. alpinum	AA	7·52 ± 0·06		
D. complanatum	CC	5·76 ± 0·05		
D. tristachyum	TT	5·26 ± 0·06		
Diploid hybrids				
D. × issleri	AC	6·64 ± 0·07	6·64	0·00
D. × oellgaardii	AT	6·39 ± 0·08	6·39	0·00
D. × zeilleri	CT	5·53 ± 0·05	5·51	0·02
Triploid hybrids				
D. alpinum × D. × issleri	AAC	10·30 ± 0·06	10·40	−0·10
D. alpinum × D. × oellgaardii	AAT	9·97 ± 0·07	10·15	−0·18
D. complanatum × D. × issleri	ACC	9·48 ± 0·12	9·52	−0·04

For the hybrids, a comparison is made between the values measured and those predicted on the basis of the parental genome sizes; the calculation for the triploids was made in accordance with the assumed origin (e.g. by summing the size of an unreduced genome of a hybrid and one half of the parental species' genome; see text); the assumed hybrid origin is indicated by genome formulae.

Oberpfälzer Wald (Table 5). For unknown reasons, the matches are not as close as in the case of the diploid hybrids, a phenomenon observed in diploid and triploid *Equisetum* hybrids as well (Bennert *et al.*, 2005). In all three *Diphasiastrum* hybrids the predicted values are slightly higher than those measured. Genome downsizing may be involved (see Leitch and Bennett, 2004), but, to our knowledge, has not been reported for lycophytes or ferns so far.

The morphology of the triploid hybrids corroborates our interpretation (Fig. 3). The plant of accession 09/82 is generally intermediate between *D. complanatum* and *D. × issleri*, but displays a strong influence of *D. complanatum*, such as the distinctly flattened upright axes and the spreading lateral leaves, which narrow abruptly toward the slender apices and reduced lanceolate ventral leaves. In accession 09/86, considered to be an intermediate between *D. alpinum* and
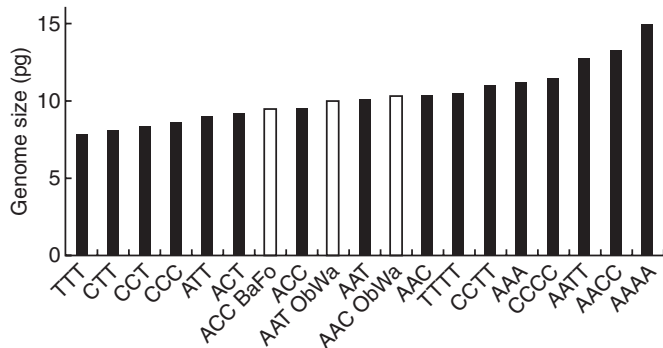

FIG. 2. Bar chart displaying genome sizes (DNA values in pg) to be expected for the ten triploid (seven allotriploid and three autotriploid) and six tetraploid (three allotetraploid and three autotetraploid) genotype combinations (black columns) as calculated from the values measured for the six diploid taxa. The DNA contents of the three triploids found in nature are shown by white columns; for abbreviations used for regions, see Table 1; for letters used for genome composition, see Table 2.

D. × issleri (AAC), the *D. complanatum*-like characters are less distinct, and the influence of *D. alpinum* is more pronounced as becomes manifest by longer ventral and dorsal leaves (Fig. 3). Accession 09/87 (intermediate between *D. alpinum* and *D. × oellgaardii*, AAT) is not reminiscent of *D. complanatum*, but shares characters with *D. × oellgaardii*, such as the length and shape of the ventral leaves; the shoots, however, are terete to triangular, approaching those of *D. alpinum*. Additional morphological details (growth form and habit of plants, number and insertion of strobili), giving further evidence for the assumed origin of the three triploids, will be published separately.

In total, seven allotriploid genome combinations are possible (Figs 2 and 4): six with a genome pair derived from the same parental species combined with a third chromosome set from another species (like AAC), and a unique combination composed of one genome each from all three diploid species (ACT). As shown in Fig. 4, there are three different routes by which this hybrid could be achieved. While in *Diphasiastrum* this three-parent hybrid is unknown, such a hybrid was detected in the genus *Equisetum* (Bennert *et al.*, 2005; Lubienski *et al.*, 2010).

Out of the seven possible triploids, three apparently exist in nature and, together with the diploid hybrids, deliver an impressive example of reticulate evolution in *Diphasiastrum*.

How could triploids have formed in nature?

When studying spore morphology, in all three diploid hybrids, besides well-formed spores and aborted spores, diplospores were discovered, which are recognizable by their larger size and more globose shape; details of this study will be published separately. The occurrence of diplospores was reported earlier for *D. × oellgaardii* by Øllgaard and Tind (1993). These unreduced diplospores, sometimes called 'giant' or 'basketball' spores (Wagner *et al.*, 1986; Øllgaard and Tind, 1993), result
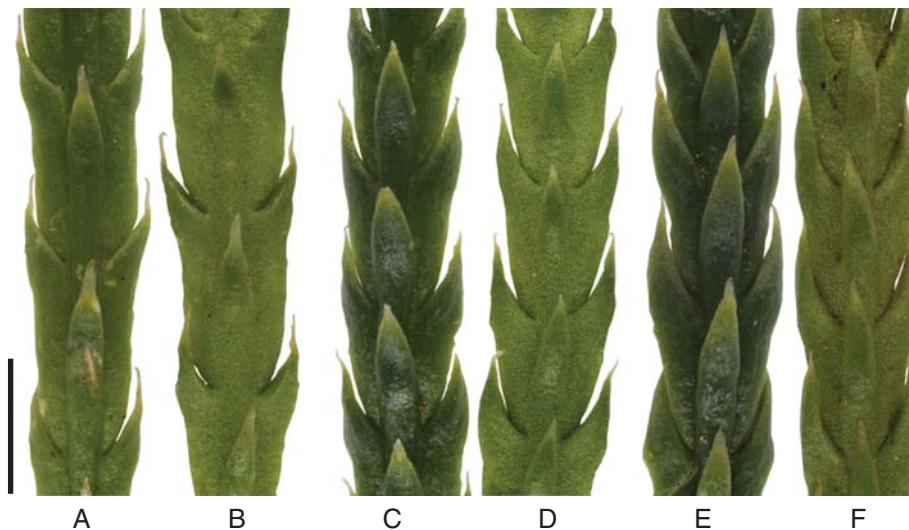

FIG. 3. Photographs of shoots of the three putative triploid *Diphasiastrum* hybrids: (A, B) dorsal and ventral view of the hybrid interpreted as *D. complanatum × D. × issleri* (ACC), (C, D) dorsal and ventral view of the hybrid interpreted as *D. alpinum × D. × issleri* (AAC), (E, F) dorsal and ventral view of the hybrid interpreted as *D. alpinum × D. × oellgaardii* (AAT). Scale bar = 2·5 mm.
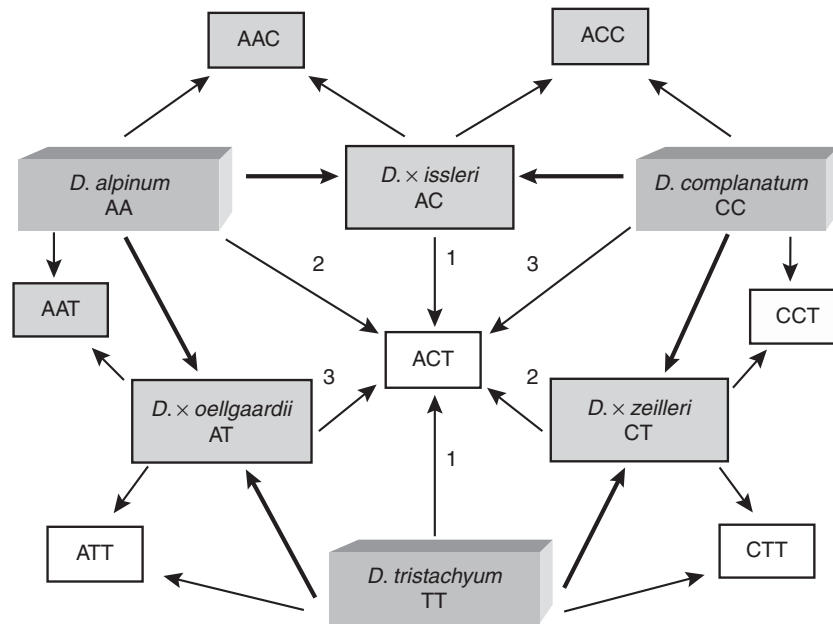
F<small>IG</small>. 4. Hybridization scheme showing the origin of the three known diploid and all seven possible triploid hybrids within *Diphasiastrum* in Central Europe; diploid species and hybrids as well as the three new triploids are shown in the grey boxes; for the triploid genotype ACT, all three possible origins are indicated.

from incomplete meiotic divisions and are believed to be capable of germinating and producing diploid gametophytes.

Tetraploids are unknown in European *Diphasiastrum* species, and our screening also failed to prove the existence of tetraploids in the regions studied. Thus, the common route of yielding triploids, i.e. from a cross between a tetraploid and a diploid species or cytotype, obviously is unlikely. We propose that the triploids arose instead as crosses between diploid gametophytes (stemming from a hybrid's diplospore) and a haploid gametophyte (derived from a normal meiospore of the second parent).

For the formation of the three putative triploid hybrids (ACC, AAC and AAT), only two diplospore-producing diploid hybrids would be required, namely *D.* × *issleri* (AC) and *D.* × *oellgaardii* (AT). For establishment of the genotypes AAC and ACC, a diploid *D.* × *issleri* gametophyte would backcross with either parent (*D. alpinum* or *D. complanatum*), while to obtain AAT a diploid gametophyte of *D.* × *oellgaardii* is required, which would backcross with *D. alpinum*. At the locality in the Bavarian Forest where the triploid ACC is growing, all six diploid taxa are present, and the same applies for the stand of the triploid AAT in the Oberpfälzer Wald, while the colony of AAC is separated by about 750 m from the main *Diphasiastrum* assemblage also containing the maximum number of six diploid taxa. So far, the range of the triploid hybrids is confined to southeastern Germany (Fig. 1). This is one centre of distribution of the diploid hybrids (*D.* × *issleri* and *D.* × *oellgaardii*) that are considered as parents (Bennert, 1999).

The significance of diplospores for the formation of triploids has been shown, e.g. for *Athyrium* (Schneller and Rasbach, 1984; Rasbach *et al.*, 1991) and *Equisetum*, where several triploid taxa exist (Bennert *et al.*, 2005), and, as in European *Diphasiastrum*, no tetraploids are known.

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