A CONTRIBUTION TO THE PHYLOGENY OF *DRYOPTERIS REMOTA* BY GENOTYPING OF A FRAGMENT OF THE NUCLEAR *PgiC* GENE

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ABSTRACT

Since its discovery by A. Braun in 1834, there has been speculation on the origins of *Dryopteris remota*. The present contribution suggests that none of the previous assumptions are correct. *Dryopteris remota* is triploid and of hybrid derivation. Its parental species are the sexual allotetraploid *D. carthusiana* and an as yet unidentified sexual diploid species, most likely identical with the unknown parent of the apomictic diploid *D. affinis*. These assertions are based on comparison of traits, including habit, apomixis, colour of insertions of pinnae of the species under examination, and fragment length variation of the nuclear *PgiC* gene.

INTRODUCTION

The phylogeny and taxonomic status of *Dryopteris remota* (A.Br.) Druce is probably one of the most controversially discussed of any Central European fern species. When Alexander Braun discovered the plant near Baden-Baden in 1834 (Baden-Württemberg/ S-Germany), he thought the new taxon to be a variation of *D. villarii* (Bellardii) Woynar ex Schinz & Tell. As such it was listed in 1843 by J. C. Döll in "Rheinische Flora". In his book "Betrachtungen über die Erscheinung der Verjüngung in der Natur" of 1851, Braun revised his view in a footnote and described the taxon as a hybrid of *D. filix-mas* (L.) Schott and *D. carthusiana* (Vill.) H.P.Fuchs or *D. dilatata* (Hoffm.) A.Gray. He nonetheless attributed species rank to this taxon by calling it "*Aspidium remotum*", i.e. in current nomenclature *D. remota*. Since then a substantial number of renowned fern experts have speculated on its origin and taxonomic classification. In 1973 Benl and Eschelmüller presented an exhaustive description of this continuing dispute among scientists.

Further confusion had arisen following the discovery of a fern hybrid near Lake Windermere in north-west England in the middle of the 19th century. The morphology of this hybrid resembled very much that of *D. remota,* but it had abortive spores. In 1859 A. Braun found a single plant of *D. remota* among a large population of *D. filix-mas* near Aachen (Nordrhein-Westfalen/ Germany), which led him to the conclusion that *D. remota* must be a variation of *D. filix-mas*. He did not realize that this plant was not *D. remota* but actually the same hybrid as that from Lake Windermere. He transplanted this hybrid to the Botanical Garden of Berlin and for approximatively 15 years he sent incorrectly labelled exsiccata from there to herbaria and to individual scientists before this mistake was discovered (Benl & Eschelmüller,

1973). Further examination of a frond sample in the herbarium at Bonn revealed that Braun's finding was the second record of the tetraploid hybrid *D. carthusiana* × *D. filix-mas,* as discovered earlier at Lake Windermere (Krause et al., 2001), and which has now been typified as *D.* × *brathaica* Fraser- Jenk. & Reichst. (1977). It is now possible to distinguish the two taxa through cytology (Manton, 1950), morphology (Fraser-Jenkins & Reichstein, 1984; Krause et al., 2001; Freigang et al., 2013), chemotaxonomy (Widén et al., 1976) and molecular genetics (Freigang et al., 2013).

Dryopteris remota is today recognised as a triploid species of hybrid derivation, but the dispute concerning its origin continues. From fundamental works by W. Döpp (1932; 1933; 1935; 1939; 1955) and I. Manton (1932; 1938; 1939; 1950), Döpp considered *D. remota* to be the hybrid of *D. dilatata* × *D. affinis* (Lowe) Fraser-Jenk. (Döpp, 1955). Reichstein, Fraser-Jenkins and others are of the opinion that the diploid apomictic *D. affinis* must be one of the parents due to the characteristics of *D. remota* (Fraser-Jenkins & Reichstein, 1984). In consequence the second parent would have to be a diploid sexual taxon that contributes the characteristics known from the *D. carthusiana* group to the genotype of *D. remota. Dryopteris expansa* (C.B.Presl) Fraser-Jenk. & Jermy was proposed (Widén et al., 1971, Fraser-Jenkins & Reichstein, 1984), even though chemo-taxonomic results did not match well (Widén et al., 1970; 1971; 1996). Furthermore, in an on-line discussion of the British Fern Group, Fraser-Jenkins (30 December 2008) reiterated doubts as to *D. expansa* being involved: "Although I agree that *D. expansa* is the most likely second ancestor of *D. remota*, it ought to have a ? along that line as it has never been proved in any way and the possibility of some other, even perhaps Asiatic species being involved still remains."

To shed light on the ongoing discussions, the nuclear *PgiC*-marker of many species of the genus *Dryopteris*, including *D. remota*, has been examined.

MATERIAL AND METHODS

Material

The samples used in the investigation are listed in Table 1. Herbarium vouchers, currently in the collectors' private herbaria will be deposited in JE.

Methods

Samples were analyzed at the nuclear *PgiC* gene (cytosolic 6-Phosphoglucose-isomerase), individual fragments were generated and their lengths compared. The intron fragment between exons 15 and 16 proved especially suitable for comparison as it shows a species specific length for a number of species of the genus *Dryopteris*, especially in the *D. carthusiana* group (Juslén et al., 2011, Freigang et al., 2013). For this fragment Ishikawa et al., 2002 constructed an 'EPIC'- primer pair (Exon Primed Intron Crossing), which offers almost general applicability. Due to a high frequency of poor PCR yields or complete PCR failures, the method described in Freigang et al. (2013) was partly replaced by the PCR-direct method of ThermoScientific (Phire Plant Direct PCR Kit, F-130WH). This method worked without DNA isolation and PCR yields were often higher and, according to the producer instructions the workflow consisted of only two steps.

1. A disc of 0.3-0.5 mm diameter was punched out from fresh or silica gel-dried leaf material and put into PCR-solution. 2. PCR was carried out according to the producer instructions. The uncleaned amplicon was genotyped in a sequencer (ABI 3500, Applied Biosystems or MegaBace Instrument, Healthcare). Employment of different

sequencers made transformation of results necessary (marked with an asterisk). The analysis of the generated data was first made with Fragment Profiler software (Healthcare), then later with GeneMapper software (Applied Biosystems).Sometimes the sequencing device produced a double peak in consecutive basepairs (bp). This could be an artefact or may be the result of PCR-conditions.

The relative height of signals within an electropherogram depended firstly on the number of sets of chromosomes causing the signal and, secondly, on the relative PCR yield of the 15/16-fragment of the existing alleles.

RESULTS

The genotypes of samples 1 to 24 are summarised in Table 2. Signals of electropherograms which were not separated are noted in the same table-field. Electropherograms of a subset of samples are shown in Figures 1-3 (underlined in Tables 1 and 2).

DISCUSSION

In the case of apomictic reproduction in ferns the sporophyte grows directly from a gametophyte with the unreduced number of chromosomes, and without gametes being involved. The sporophyte produces genetically uniform diplospores through a restitution nucleus; in the case of apomicts of *Dryopteris*, restitution occurs in the last mitotic cell division before the production of spore mother cells (Manton, 1950), resulting in the production of eight, rather than the usual 16 spore mother cells. Sporogenesis then results in the production of 32 (instead of the usual 64) spores, each with the same number of chromosomes as the sporophyte.

Fischer (1909; 1919) detected apomixis in *D. remota* and Manton demonstrated that it is a triploid taxon, with three different (non-homologous) chromosome sets (i.e. a heterotriploid fern) as in the occasional production of a spore mother cell of the '16 cell type', the chromosomes are unpaired (non-homologous), with 123 univalents (3x). The heterotriploid character is also shown by the electropherogram of *PgiC* 15/16 fragments of the species (Figure 1), where three distinct peaks are observed.The electropherogram shows the following: The genome of *D. remota* contains at least three chromosome sets possessing fragments of different length and therefore derived from different alleles. This supports the results of cytology that the species is heterotriploid. The two shorter fragments of 488 bp and 500 bp correspond to the results seen in *D. carthusiana* (Figure 2), an allotetraploid species; no other taxon of the European ferns of the genus *Dryopteris* examined up to now shows this pattern.

Comparison with *D. cristata* (L.) A.Gray (sexual allotetraploid) and *D. intermedia* (Mühl. ex Willd.) A.Gray (sexual diploid) reveals that the peak at 488 bp differs from that of *D. intermedia* (500 bp), and is generated by the chromosome set derived from the common parental taxon of *D. cristata* and *D. carthusiana*, referred to as *D. "semicristata"* (Sc) by Walker (1955), a diploid species as yet unknown. The signal at 500 bp in *D. remota* is identical with that of *D. intermedia* (I). The chromosome sets of *D. carthusiana* can be symbolized by ScScII. The assumption that the parental species of *D. carthusiana* are *D. intermedia* and *D. "semicristata"* is supported (Table 2) and confirms the result of Sessa et al. (2012). The results are also in agreement with chloroplast sequence data by Juslén et al. (2011), which showed that *D. carthusiana* and *D. remota* share a maternally inherited set of chromosomes, referred to as *"semicristata"*. Table 2 shows that the 15/16-fragment length of *D. expansa* is the same

Table 1: Genotyped samples (Nomenclature according Fraser-Jenkins & Reichstein, 1984 and Fraser-Jenkins, 2007). RF: numbers according to rasters (see Niklfeld, 1971). Abbreviations of the names of collectors: GZ (G. Zenner/ Kirn), HWB (H. W. Bennert/ Ennepetal), JF (J. Freigang/ Bergatreute), SJ (S. Jeßen/ Chemnitz), WB (W. Bujnoch, Trier).

Sample no. & species	Origin	Collector/ Determination/ Date	Internal Data
1. *Dryopteris aemula*	Portugal/ Azores/ São Miguel/ N-side and ridge at Pico da Vara, 1100 m MSL	GZ 28.5.2013 Dr 8	MPb1458, DE807, RUN32/2H
2. *D. affinis*	Portugal/ Madeira/ N Funchal/ c. 600 m S of restaurant Ribeiro Frio at the roadside EN 103	JF/GZ 25.8.2011 JFV1086	MPb1724, RUN45/7A
3. *D. affinis*	Germany/ RP/ Palatinate/ NE Wilgartswiesen, rock at Wiligartsburg, ca. 230 m MSL, RF 6713/32	WB/GZ 6.8.1996	DE032, RUN21/11B
4. D. carthusiana	Germany/ NRW/ Märkisches Land, SW Hagen/ near Hasper dam, RF 4603	HWB 27.05.2013	MPb1442, DE791, RUN32/1B
5. *D. carthusiana*	Germany/ NRW/ niederrheinisches Tiefland/ E Venlo/ Nettetal/ Poelvenn–Kuhlen/ RF 4603/23	N.Neikes 05.07.2009	MPb1482, DE812, RUN32/3E
6. *D. carthusiana*	Germany/ RP/ middle Mosel/ W Trier/ Sirzenicher Bachtal, RF 6205/23	WB 17.7.2005	DE083, RUN22/10B
7. *D. carthusiana*	Germany/ BW/ Oberschwaben/ NE Ravensburg/ SE Bergatreute/ near Kiliansweiher, RF 8124/32	JF 22.10.2006 JF 2006	DE385, RUN20/12A
8. *D. cristata*	Germany/ RP/ Eifel/ SSE Daun/ Schalkenmehrener Maar, RF 5807/13	WB 28.8.1984 GWBT2 spore cultured	DE024 RUN20/G11
9. *D. dilatata*	France/ Haut–Rhin/ Vosges/ S Col de la Schlucht/ Sentier des Roches/ NE "les Trois Four", 1050 m MSL, RF 7908/31	GZ/P.Holveck 27.8.2014 Dr 4	MPb1665, RUN40/11D
10. *D. expansa*	Germany/ BY/ upper Allgäu/ West–Grünten/ N Burgberg/ N „Weinhalde", northern slope, 780–795 m MSL, RF 8427/41	GZ 19.7.2004 Dr 20	MPb1229, DE695, GWBT156 RUN28/11E
11. *D. expansa*	Switzerland/ Wallis/ S Leukerbad/ Dala Valley/ lower Lochwald, 1260 m MSL, RF 9611/23	GZ 18.7.2000 Dr 1	MPb1230 DE696, GWBT182 RUN28/11F

12. *D. intermedia*	USA/ Vermont/ Rutland/ Leicester Hollow Trail/ NNE Brandon	HWB 15.8.2001 SJ-3476	DE368 RUN25/A11
13. *D. oreades*	France/ Hérault/ Haut Languedoc,/W Bédarieux/ N Colombières-s-Orb/ near "La Fage", c. 760 m MSL	GZ/WB 23.7.1994 Dr 1	DE067, GZ-58, RUN18/11C
14. *D. oreades*	Italy/ Toscana/ Appennino Tosco Emiliano/ NW Pistoia/ W Abetone/ W M Gomito/Val di Luce, c. 1480 m MSL	SJ/J.Müller 12.8. 1992 SJ-2226	DE288, RUN19/11B
15. *D. pallida* subsp. *pallida*	Italy/ Sicily/ Mount Etna above Zafferana	JF 1.1.2007 JFV542	MPb1562 DE861 RUN37/B12
16. *D. remota*	Germany/BW/ Northern–Schwarzwald/ SSE Bad Herrenalb/ valley of Rothenbach, 440 m MSL, RF 7216/21	GZ/WB 1.8.1990 spore cultured	FZ-90/7, GWBT 32, DE26, RUN19/12G
17. *D. remota*	Germany/ BY/ W Berchtesgaden/ Ramsau/ SW of Schwarzbachwacht, c. 900 m MSL, RF 8343/31	GZ/JF 5.8.2013 Dr 2,	MPb1516, DE 824, RUN35/11B
18. *D. remota*	Schwitzerland/ Berner Oberland/ Innertkirchen/ Urbachtal in Vordertalwald, RF 9315/12	SJ 21.7.1992 SJ-2270	MPb1235, DE 637, RUN28/ 11C
19. *D. remota*	Austria/ Salzburg/ Pinzgau/ E Rauris/ valley of Geißbach, c. 1060 m MSL, RF 8744/31	GZ 6.8.2014 Dr 10	MPb1650, RUN44/11H
20. *D. remota*	Romania/ S-Karpaten/ N- Făgăraş/ NW des Rifugio "Negoiu"/ SSE Porumbacu de Sus/ Şerbota Tal c. 1000 m MSL	SJ 5.8.1985 SJ-2560-2	MPb1236, DE638, RUN28/ 11D
21. *D. remota*	Germany/ BY/ West-Allgäu/ NW Scheidegg/ forest N "Lötz", c. 700 m MSL, RF 8425/11	JF 4.6.2011 JF-V1008	MPb1250, DE710, RUN28/12D
22. *D. remota*	North-Italy/ Prov. Varese /E Luino/ W Ponte Tresa/ Tresa-Valley near Biviglione, 250 m MSL, RF 0018/21	GZ/JF/SJ 15.6.2014 Dr 33	MPb1580, RUN40/9D
23. *D. remota*	Northeast-Turkey/ Rize/ E Trabzon, S Ardeşen, Cat - Çamlihemşin, c. 400m MSL	SJ-3026, TR-4011, CRFJ 4037, 27.08.1973	MPb1738, RUN47/A1
24. *D. villarii*	Bulgaria / North-Pirin/ E des Kameniski vrah/ NW des Vihren/ W Bansko, Bela-reka-Valley, 2150 m MSL	SJ 24.7.1981 SJ-335	MPb1452 DE801, RUN32/2C

Table 2: Genotyping results for the length of the 15/16 *PgiC* fragment of samples 1 to 24 in base pairs (bp). The sample numbers in Table 2 correspond with those in Table 1. The measurements in Table 2 indicate that the two shorter fragments of *D. remota* correspond with those of *D. carthusiana* and the third fragment of *D. remota* with that of *D. affinis* and *D. oreades*.

Species	**Fragment length in base pairs (bp)**					
1. *Dryopteris aemula*				525		
2*. *D. affinis* subsp. *affinis*					530	
3. *D. affinis* subsp. *affinis*					530	
4. *D. carthusiana*	487	499				
5. *D. carthusiana*	486	499 500				
6. *D. carthusiana*	486 487	498 499				
7. *D. carthusiana*	487	500				
8. *D. cristata*	487			521		
9. *D. dilatata*		499	517			
10. *D. expansa*		499 500				
11. *D. expansa*		499 500				
12. *D. intermedia*		500				
13. *D. oreades*					529 530	
14. D. oreades					530	
15. *D. pallida* subsp. *pallida*			516			
16. *D. remota*	487 488	499 500			530	
17. *D. remota*	487	500			531	
18. *D. remota*	487	499			530	
19*. *D. remota*	489	501			530	
20. *D. remota*	487	500			530	
21. *D. remota*	488	500			530	
22. *D. remota*	489	501			531	
23*. *D. remota*	489	501			531	
24. *D. villarii*						532 533

* Employment of different sequencers made transformation of results necessary.

as that of *D. intermedia.* As mentioned in the introduction, the involvement of *D. expansa* in the parentage of *D. remota* has been the subject of much discussion. But this scenario would entail that the second parent of *D. remota* must be a diploid apomictic taxon of the *D. affinis* agg. with signals at 488 bp and 530 bp. Despite study of many taxa of the *D. affinis* agg., this combination has never been found. The same applies to the combination of signals at 500 and 530 bp.

During the first half of the last century *D. remota* was thought to be a hybrid of *D. carthusiana* × *D. filix-mas*, and Döpp endeavoured to backcross *D. remota* with the supposed parental species. He was successful in hybridizing *D. remota* only with *D. carthusiana* (Döpp, 1935). This provides some support for the conclusion that *D. carthusiana* is one parent of *D. remota*.

Already H. Fischer pointed to the fact that such a hybrid should also emerge in nature and he mentioned that the pharmacist H.K. Woynar (Graz) allegedly found intermediate forms in mixed stands of the two species at different sites in Tirol. Since 2011 putative hybrids of *D. remota* × *D. carthusiana* have been found at various locations in the wild (Freigang et al., in prep.).

The remaining question relates to the unknown identity of the third signal in the electropherogram of *D. remota*, at 530 bp. The chromosome set causing this third signal at 530 bp would have to contribute two qualities to *D. remota* that are characteristic of it and cannot be derived from the genetic material of *D. carthusiana* - the obligatory apomixis and the blue-black colour at the insertion of the pinnae (Fraser-Jenkins & Reichstein, 1984). Both features also exist in the *D. affinis* agg., but not in that of *D. filix-mas*. Examination of the diploid taxon *D. affinis* (Figure 3) shows a single peak at 530 bp.

According to current opinion *D. affinis* is a diploid apomict fern that shows non-homology of the two chromosome sets in 16-celled sporangia (Fraser-Jenkins, 2007) and it is presumed to have been derived from a cross between the sexual diploid *D. oreades* Fomin (OO) and a sexual diploid taxon, not yet identified but referred to as *D.* "*semiaffinis"* (SaSa) (Krause, 1998). The *PgiC* 15/16 fragments of both taxa correspond to the third peak in *D. remota.* As *D. remota* is triploid and its genome

Figure 1. Electropherogram of *Dryopteris remota* (Schwarzwald, Smp.No. 16)

Figure 2. Electropherogram of *Dryopteris carthusiana* (Bergatreute, Smp. No. 7)

Figure 3. Electropherogram of *Dryopteris affinis* (Wilgartswiesen, Smp. No. 3)

contains two sets of chromosomes of allotetraploid *D. carthusiana*, *D. affinis* as the second parent is out of question, because as an apomictic taxon it would contribute both its chromosome sets to *D. remota*, resulting in a tetraploid taxon. This implies that the third peak (530 bp) in *D. remota* must be derived from a diploid sexual taxon with the 530 bp peak, and the only possible candidate from the results presented here are *D. oreades* or the missing diploid taxon *D. "semiaffinis"*.

Unfortunately the length of *PgiC* 15/16 fragments of *D. oreades* and *D. "semiaffinis"* cannot be distinguished with the method used here. Of these two, *D. "semiaffinis"* is the more likely parent; *D. oreades* does not exhibit blue-black insertions, nor apomixis. We are left to infer that both diploid *D. affinis* and triploid *D. remota* have inherited these characteristics from *D. "semiaffinis"*, and hence the phylogeny of *D. remota* could be analogous to that of *D. affinis,* as shown in Table 3.

All the evidence suggests that "*semiaffinis*" induces apomixis in its hybrids and species of hybrid derivation, in contrast to hybrids involving *D. oreades*. This is the case in all heterodiploid as well as heterotriploid taxa of the *D. affinis* complex, which are all fertile but to varying degrees. In hybrids of taxa of the *D. affinis* complex with *D. filix-mas*, apomixis is also expressed, despite that fact the "*semiaffinis*" chromosome set is only one of four or five sets in the nucleus. The hybrid of *D. carthusiana* and *D. "semiaffinis"* (Table 3) has three non-homologous sets of chromosomes and would be expected to be sterile, in contrast to *D. remota* which is fertile and can produce viable offspring in the wild. This case of *D. remota*, gives further support to the hypothesis that

Table 3. Diagram of the hypothetical genesis of *Dryopteris remota* and *D. affinis*
Abbreviations of chromosome sets: O = *oreades*, Sa = "*semiaffinis*", I = *intermedia*, Sc = *"semicristata"*

Parental Taxa	*D. oreades* homodiploid, sexual	*D. "semiaffinis"* homodiploid, sexual	*D. carthusiana* allotetraploid, sexual
Chromosome sets of a somatic cell	OO	SaSa	IScISc
Chromosome set(s) of a gamete	O	Sa	ISc
Hybridisation			
Primary hybrid		OSa heterodiploid, sterile	SaISc heterotriploid, sterile
Development of apomixis			
Progeny		*D. affinis*	*D. remota*
chromosome sets of a somatic cell		OSa heterodiploid, apomictic fertile	SaISc heterotriploid, apomictic fertile

the set of chromosomes from "*semiaffinis*" has the ability to transfer the qualities of dark insertion of pinnae, and apomixis coupled with fertility.

The distribution of *D. remota* lies between the Pyrenees and the Caucasus and thus falls within the distribution of *D. carthusiana* and *D. affinis* (Fraser-Jenkins & Reichstein, 1984). The genetic variability of *D. remota* was examined by the isozyme-method (Schneller & Holderegger, 1994) and also by RAPDs (Schneller et al., 1998). The first method did not reveal any genetic variability within or between populations, the second only little. The authors suggested that the species developed relatively recently and only once, presumably in Caucasus or the Mediterranean region, and spread over the now populated area by 'long-distance spore dispersal'. Since spores of apomicts produce sporophytes without the need for sexual fertilization, then an individual spore has the potential to give rise to a new individual or eventually even a new population.

During the past 180 years of discussion about the origin of *D. remota* other taxa have been proposed as possible parents, for example *D.villarii* (Braun in Döll, 1843), *D. dilatata* (Döpp, 1935; 1955), *D. aemula* (Aiton) O.Kuntze (Widén et al., 1971) or *D.pallida* subsp. *pallida* (Bory) Fomin and subsp. *raddeana* Fraser-Jenk. (Peroni et al., 1991). The results in Table 2 rule out almost all of these possibilities, with the exception of *D. pallida* subsp. *raddeana*, as samples of this taxon were not included in the study.

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