Theor Appl Genet (1997) 95:490–496

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Introgression of resistance to root-knot nematodes from wild Central American *Solanum* species into *S. tuberosum* ssp. *tuberosum*

Received: 25 November 1996 / Accepted: 14 February 1997

Abstract Crossing experiments were conducted to introduce resistance to the root-knot nematodes, *Meloidogyne chitwoodi* and *M. fallax*, from various polyploid Central American *Solanum* spp. into the cultivated potato, *S. tuberosum* ssp. *tuberosum*. The most effort was put into producing tetraploid hybrids through inter-EBN (Endosperm Balance Number) crosses. From the crosses of tetraploid *S. tuberosum* (4 EBN) with tetraploid *S. stoloniferum* and *S. fendleri* (both 2 EBN), few seeds were derived that led to viable plants. In vitro culture of immature seeds also yielded several hybrid plants. From crosses of diploid *S. tuberosum* (2 EBN) with hexaploid *S. hougasii* (4 EBN) four hybrids were obtained through in vitro culture. Backcrosses were made with selected hybrids and a variable number of seeds was produced depending on the hybrid genotype. The successful introgression of resistance into backcross populations is shown. A scheme is presented for the introgression of traits at a tetraploid level from allotetraploid *Solanum* species into autotetraploid *S. tuberosum* through sexual crosses. The relevance of EBN for potato breeding is discussed.

Key words EBN · In vitro seed culture · Potato breeding

Introduction

Wild *Solanum* species are potential sources of valuable traits for potato breeding such as resistance to various diseases and pests. However, not all *Solanum* species can be readily crossed with the cultivated potato, *S. tuberosum* ssp. *tuberosum*, due to the presence of pre- and/or postzygotic barriers, differences in ploidy levels and/or structural genomic differences (Ross 1986; Hawkes 1990; Hermsen 1994).

With regard to the postzygotic barriers, Johnston et al. (1980) proposed the Endosperm Balance Number (EBN) hypothesis to explain the results of interspecific crosses between *Solanum* species. Each species with a certain ploidy level has been assigned a hypothetical value ranging from 1 to 4 (e.g. listed by Hawkes and Jackson 1992; Hanneman 1994), and only interspecific combinations with an equal EBN will have a normal endosperm development and give rise to viable seeds and hybrid plants. The hypothesis bases the success of intra- and interspecific crosses on a female: male EBN ratio in the endosperm of 2:1 (Johnston et al. 1980), and the EBN seems to be genetically controlled by two or three unlinked loci (Ehlenfeldt and Hanneman 1988; Camadro and Masuelli 1995).

In order to circumvent incompatible reactions resulting from different EBN values, breeders have applied several alternative strategies. In unreduced gametes, the so-called 2n gametes, the actual EBN number equals the somatic number and this can abolish the EBN difference in certain interspecific crosses. With the use of 2n gametes F_1 hybrids or first backcrosses have been obtained from *S. stoloniferum* and *S. acaule* with *S. tuberosum* (Von Wangenheim 1955; Adiwilaga and Brown 1991). Also, colchicine treatment of the lower EBN parent to double chromosome numbers has a similar effect and has made these interspecific (back-) crosses possible (Swaminathan 1951; Watanabe et al. 1992). Somatic hybridisation is another approach by which to obtain interspecific genotypes as a first step of introgression. This technique has been applied for the establishment of hybrids of *S. tuberosum* with *S. brevidens* (Barsby et al. 1984), *S. circaeifolium* (Mattheij et al. 1992), *S. bulbocastanum* (Austin et al. 1993),

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Communicated by G. Wenzel

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S. commersonii (Cardi et al. 1993) and *S. pinnatisectum* (Ward et al. 1994).

However, these alternatives can have certain disadvantages in the prolonged process of introgression. In the case of somatic hybrids, aneuploid chromosome numbers are common (Ramulu et al. 1989), and recombination is not automatically enhanced. The use of 2n gametes in crosses between tetraploid species or the treatment of triploid genotypes with colchicine will lead to hexaploid genotypes, which are suitable for continued introgression. However, in these genotypes homoeologous pairing and recombination between chromosomes is not likely to occur due to preferential pairing of homologous chromosomes and disomic behaviour of the polyploid genome (Brown 1988; Watanabe et al. 1992). On the other hand, true tetraploid hybrids from sexual crosses should enhance homoeologous pairing and recombination and therefore facilitate the introgression of desired traits (Iwanaga et al. 1991; Watanabe et al. 1992, 1994; Bamberg et al. 1994), but these interspecific hybrids have been difficult to find due to the EBN differences between the wild *Solanum* species and *S. tuberosum*. Still, several successful inter-EBN combinations leading to viable hybrids have been reported (reviewed and discussed by Hermsen 1994).

Our interest concerns primarily the transfer of root-knot nematode resistance into the potato gene pool. High levels of resistance to *Meloidogyne chitwoodi* and *M. fallax* have been identified in the Central American *Solanum* species *S. fendleri*, *S. stoloniferum* and *S. hougasii* (Janssen et al. 1996). With the tetraploid 2 EBN species *S. stoloniferum* and *S. fendleri*, successful interspecific hybridisations with *S. tuberosum* have been reported, mostly as a result of 2n gametes and ploidy manipulations (Swaminathan 1951; Van Soest 1985; Adiwilaga and Brown 1991). However, on some rare occasions tetraploid hybrids of *S. stoloniferum* with *S. tuberosum* have been obtained (Von Wangenheim 1955), also in combination with embryo rescue pollination (Singsit and Hanneman 1991). The hexaploid 4 EBN species *S. hougasii* is sexually crossable with tetraploid *S. tuberosum*, but with diploid *S. tuberosum* a tetraploid level would be obtained giving better opportunities for the introgression of genes (Ross 1986; Watanabe et al. 1992).

This report study describes attempts to cross root-knot nematode-resistant *S. fendleri*, *S. stoloniferum* and *S. hougasii* genotypes with cultivated potato, *S. tuberosum*, following normal crossing procedures and, for some combinations, using in vitro culture of immature seeds. The first steps of the introgression of resistance to *Meloidogyne* spp. from these wild *Solanum* spp. through intra- and inter-EBN crosses is described as well as our efforts to retain a tetraploid level.

Materials and methods

Plant material

Genotypes were selected from trials for resistance to *Meloidogyne* spp. (Janssen et al. 1996). The characteristics of the genotypes used are presented in Table 1. The wild *Solanum* genotypes have moderate-to-high levels of resistance to *M. chitwoodi* and *M. fallax*, whereas all of the *S. tuberosum* genotypes used are susceptible (Janssen et al. 1997a). All genotypes were good pollinators on the basis of pollen production and pollen stainability with lactophenol-acid fuchsine. The wild *Solanum* genotypes were crossed with diploid and tetraploid *S. tuberosum* genotypes in a glasshouse from April to July 1994. Flowers were emasculated 1 or 2 days before anthesis and pollinated in the morning once the flowers were open. Berries were harvested 6 weeks after inoculation. In the spring and summer of the following year selected hybrid genotypes were backcrossed with *S. tuberosum* genotypes mostly differing from those used in the original cross.

In vitro seed culture

For some cross combinations, in vitro culture of immature seeds was applied to obtain hybrid plants. The procedure is based on dissecting berries and plating whole immature seeds in contrast to embryo culture, where excised embryos are cultured. Young developing berries were harvested approximately 20 days after pollination, surface-sterilised by soaking for 10 s in 70% ethyl alcohol and for 10 min in 1% NaOCl solution and then rinsed twice for 10 min in sterile water. Young seeds were carefully excised from the berries and placed in a sterile petri dish with HLH 50 medium (Neal and Topoleski 1983). Dishes were first put in the dark for 14 days and then in the light in a growth chamber (23° ± 1°C). Germinating seeds were transferred to sterile tubes containing MS 30 medium (Murashige and Skoog 1962). Rooted plants were transplanted into soil in the glasshouse and further treated as other genotypes.

Table 1 Some characteristics of *Solanum* species and the number of genotypes used

Solanum species	Accession[a]	Ploidy	EBN	Abbreviation	Number of genotypes	Resistance[b]
S. stoloniferum	7229	4x	2	sto	4	Resistant
S. fendleri	8083, 23568	4x	2	fen	4	Resistant
S. hougasii	55203	6x	4	hou	4	Resistant
S. tuberosum	–	2x	2	tbr2	5	Susceptible
S. tuberosum	–	4x	4	tbr4	5	Susceptible

[a] BGRC accessions from the Dutch-German potato gene bank, Wageningen, The Netherlands

[b] Resistance to the root-knot nematodes *Meloidogyne chitwoodi* and *M. fallax*

Resistance tests

The level of resistance to the root-knot nematodes *M. chitwoodi* and *M. fallax* was investigated for parent genotypes and putative hybrid and backcross genotypes in various glasshouse experiments. Inheritance studies had previously revealed the presence of single dominant resistance genes in *S. fendleri* and *S. hougasii*, whereas the resistance of *S. stoloniferum* seemed to be based on several additive genes (Janssen et al. 1997b). The genotypes of *S. fendleri* and *S. hougasii* used appeared to be homozygous for the resistance. Genotypes were tested in four replications per nematode population as described by Janssen et al. (1997a). In this study , genotypes were determined to resistant if the mean number of egg masses on roots was less than 5% compared to the susceptible control, potato cv 'Nicola', and susceptible if the roots contained more.

Results

The results of the interspecific crosses are summarised in Table 2. The intra-EBN crosses were partially successful, but large differences were observed between reciprocal crosses indicating unilateral incompatibility. The inter-EBN crosses often resulted in seedless berries, but some crosses produced a few plump seeds in mature berries which led to viable true hybrid plants. The hybrid status was confirmed on the basis of morphological features and with the identification of parent-specific random amplified polymorphic DNA (RAPD) bands in the band pattern of the hybrid (Xu et al. 1993). There were indications that the number of seeds per cross and the success of a cross differed between the *S. tuberosum* genotypes used. In general, the best results were obtained with cv Bildtstar. The use of the higher EBN parent as female seemed to give better results than the reciprocal cross, as indicated by Hermsen (1994). However, the numbers of seeds were too low and climatological conditions too variable to analyse these observations statistically.

Plants were derived from different inter-EBN crosses with immature seed culture also (Table 3). Many developing seeds could be extracted from the berries of inter-EBN crosses, and the percentages of germinated seeds varied from 0 to 3.5%. For comparison, seed culture was also applied to one intra-EBN combination, from which 13% germinating seeds were obtained.

Ploidy analyses revealed two hybrids which deviated from the expected ploidy level (Table 4). The hybrids M94-126-1 and -2 were obtained from crosses of *S. fendleri* with tetraploid *S. tuberosum* and were hexaploid, probably as a result of the 2n eggs of *S. fendleri* which would have resolved the inter-EBN difference. The resistance tests showed that most hybrids were resistant to both *M. chitwoodi* and *M. fallax*. Only two of the hybrids with *S. stoloniferum* were susceptible.

Table 2 Berry set and production of mature plump seeds of reciprocal crosses of wild *Solanum* species with *S. tuberosum*

Cross	EBN	Ploidy	Number of pollinations	Number of berries[a]	Number of mature seeds	Number of seeds/berry
sto × tbr2	2 × 2	4x × 2x	95	32 (1)	200	6.5
Reciprocal	2 × 2	2x × 4x	39	7	0	0
sto × tbr4	2 × 4	4x × 4x	322	160 (8)	0	0
Reciprocal	4 × 2	4x × 4x	76	36 (5)	30	1.0
fen × tbr2	2 × 2	4x × 2x	108	23	13	0.6
Reciprocal	2 × 2	2x × 4x	47	15	620	41.3
fen × tbr4	2 × 4	4x × 4x	199	37 (8)	4	0.1
Reciprocal	4 × 2	4x × 4x	116	4	1	0.3
hou × tbr2	4 × 2	6x × 2x	121	21 (3)	1	0.1
Reciprocal	2 × 4	2x × 6x	65	2	0	0
hou × tbr4	4 × 4	6x × 4x	50	27	25	0.9
Reciprocal	4 × 4	4x × 6x	49	0	–	–

[a] Number of berries used for in vitro seed culture (see Table 3)

Table 3 Germination and plant formation from immature seeds with in vitro culture

From cross	EBN	Number of berries used	Number of immature seeds	Number of plants obtained	% germination
sto × tbr2	2 × 2	1	68	9	13.2
sto × tbr4	2 × 4	8	329	1	0.3
tbr4 × sto	4 × 2	5	327	5	1.5
fen × tbr4	2 × 4	8	276	0	0
hou × tbr2	4 × 2	3	113	4	3.5

Table 4 Ploidy level and presence of root-knot nematode resistance in selected interspecific hybrid genotypes

Genotype	Cross		Ploidy		Resistance?
	Parents	EBN	Parents	F_1/hybrid	
M94-133-1	tbr4 × sto	4 × 2	4x, 4x	4x	Resistant
M94-133-3	tbr4 × sto	4 × 2	4x, 4x	4x	Susceptible
M94-134-1	sto × tbr2	2 × 2	4x, 2x	3x	Susceptible
M94-124-1	tbr2 × fen	2 × 2	2x, 4x	3x	Resistant
M94-125-1	tbr4 × fen	4 × 2	4x, 4x	4x	Resistant
M94-126-1	fen × tbr4	2 × 4	4x, 4x	6x	Resistant
M94-126-2	fen × tbr4	2 × 4	4x, 4x	6x	Resistant
M94-127-1	fen × tbr2	2 × 2	4x, 2x	3x	Resistant
M94-144-1	fen × tbr4	2 × 4	4x, 4x	4x	Resistant
M94-109-1	hou × tbr4	4 × 4	6x, 4x	5x	Resistant
M94-110-2	hou × tbr4	4 × 4	6x, 4x	5x	Resistant
M94-111-3[b]	hou × tbr2	4 × 2	6x, 2x	4x	Resistant
M94-111-4[b]	hou × tbr2	4 × 2	6x, 2x	4x	Resistant

[a] Analysed with a flow cytometer

[b] Obtained via in vitro seed culture

Table 5 Berry set and production of mature plump seeds in backcrosses of interspecific hybrid genotypes with tetraploid *S. tuberosum*

Original wild species used	Hybrid used as parent	Ploidy of backcross	Number of pollinations	Number of berries	Number of seeds	Number of seeds/berry
S. stoloniferum	M94-133-1	4x × 4x	5	4	4	1.0
	reciprocal	4x × 4x	12	0	–	–
S. fendleri	M94-125-1	4x × 4x	38	9	63	7.0
	reciprocal	4x × 4x	9	0	–	–
S. fendleri	M94-126-1	6x × 4x	47	16	720	45.0
	reciprocal	4x × 6x	7	7	750	107.1
S. fendleri	M94-126-2	6x × 4x	26	11	500	45.5
	reciprocal	4x × 6x	10	9	450	50.0
S. fendleri	M94-144-1	4x × 4x	40	20	65	3.3
	reciprocal	4x × 4x	30	1	16	16.0
S. hougasii	M94-109-1	5x × 4x	35	6	46	7.7
	reciprocal	4x × 5x	4	0	–	–
S. hougasii	M94-110-2	5x × 4x	56	13	74	5.7
	reciprocal	4x × 5x	16	0	–	–
S. hougasii	M94-111-3	4x × 4x	33	19	9	0.5
	reciprocal	4x × 4x	40	0	–	–
S. hougasii	M94-111-4	4x × 4x	65	11	9	0.8
	reciprocal	4x × 4x	43	0	–	–

Backcrosses were made reciprocally between the hybrids and tetraploid *S. tuberosum*. The most seeds were obtained from backcrosses with the hexaploid *S. fendleri*/*S. tuberosum* hybrids (Table 5). The backcrosses of the pentaploid hybrids from *S. hougasii* were successful only with the hybrid as female parent. Of the backcrosses with the inter-EBN hybrid genotypes, the crosses with the tetraploid hybrids from *S. fendleri* gave the most seeds, but a few seeds were also produced in the backcrosses of the tetraploid hybrids from *S. stoloniferum* and *S. hougasii*. Again, the best results were obtained using the hybrid as female. In the case of the tetraploid and pentaploid hybrids, the pollen stained poorly after treatment with lactophenol-acid fuchsine, indicating low male fertility.

From each backcross population 3–15 genotypes were tested for resistance to the root-knot nematodes. Most populations segregated for resistance with the exception of populations M95-206 and M95-207, of which all 9 and 10 genotypes tested, respectively, appeared to be resistant (Table 6). This is in line with the expectation that the F_1 hybrids M94-126-1 and -2 arose from a 2n gamete of *S. fendleri*. In that case one complete genome would still remain in all pentaploid genotypes, leading to resistance.

Table 6 Ploidy level and presence of root-knot nematode resistance in genotypes of various backcross populations

Original wild species used	Population	Cross	Ploidy[a]		Resistance?
		Parents	Parents	BC hybrid	
S. stoloniferum	M95-214	M94-133-1 × tbr4	4x, 4x	4x	Segregating
S. fendleri	M95-206	M94-126-1 × tbr4	6x, 4x	5x	All resistant
S. fendleri	M95-207	M94-126-2 × tbr4	6x, 4x	5x	All resistant
S. fendleri	M95-208	M94-125-1 × tbr4	4x, 4x	4x	Segregating
S. fendleri	M95-209	M94-144-1 × tbr4	4x, 4x	4x	Segregating
S. fendleri	M95-211	tbr4 × M94-144-1	4x, 4x	4x	Segregating
S. hougasii	M95-201	M94-110-2 × tbr4	5x, 4x	4x to 5x	Segregating
S. hougasii	M95-202	M94-109-1 × tbr4	5x, 4x	4x to 5x	Segregating
S. hougasii	M95-203	M94-111-4 × tbr4	4x, 4x	4x	Segregating

[a] Analysed with a flow cytometer

Discussion

The transfer of resistance to root-knot nematodes from *S. fendleri*, *S. stoloniferum* and *S. hougasii* into BC_1 genotypes through sexual crosses has been described. The most attention was paid to the production of true tetraploid hybrids to enhance recombination and promote rapid introgression (Iwanaga et al. 1991; Watanabe et al. 1992, 1994; Bamberg et al. 1994). With regard to the efficiency of introgression, a schematic representation is given in Fig. 1 of possible routes by which to introgress traits from allotetraploid species; in this example *S. fendleri* into *S. tuberosum*. Similar schemes of alternative routes A and C have been described earlier by Van Soest (1985), Adiwilaga and Brown (1991) and Watanabe et al. (1992). Route B, however, has distinct advantages. The possibility of selecting true tetraploid hybrids and backcrosses (route B) enables a rapid introgression of traits, like resistance, without any dependence on 2n gametes or special treatments to double the genome to continue introgression. Moreover, besides an expected lower percentage of the wild genome in the F_1 and BC_1 than along the alternative routes, the desired tetraploid level is automatically reached regardless of the negative selection of wild characteristics.

Whether recombination is truly enhanced depends on the pairing ability of the homoeologous chromosomes and the formation of chiasmata leading to crossing-overs. A reduced homoeologous pairing of chromosomes in wide crosses can be expected due to structural chromosome differences, but this will depend on the relatedness, i.e. genome constitutions, of species (Matsubayashi 1991; Hermsen 1994). In general, recombination seems to occur in wide crosses between *Solanum* species. Cytological observations of tetraploid hybrids of hexaploid *S. demissum* with diploid

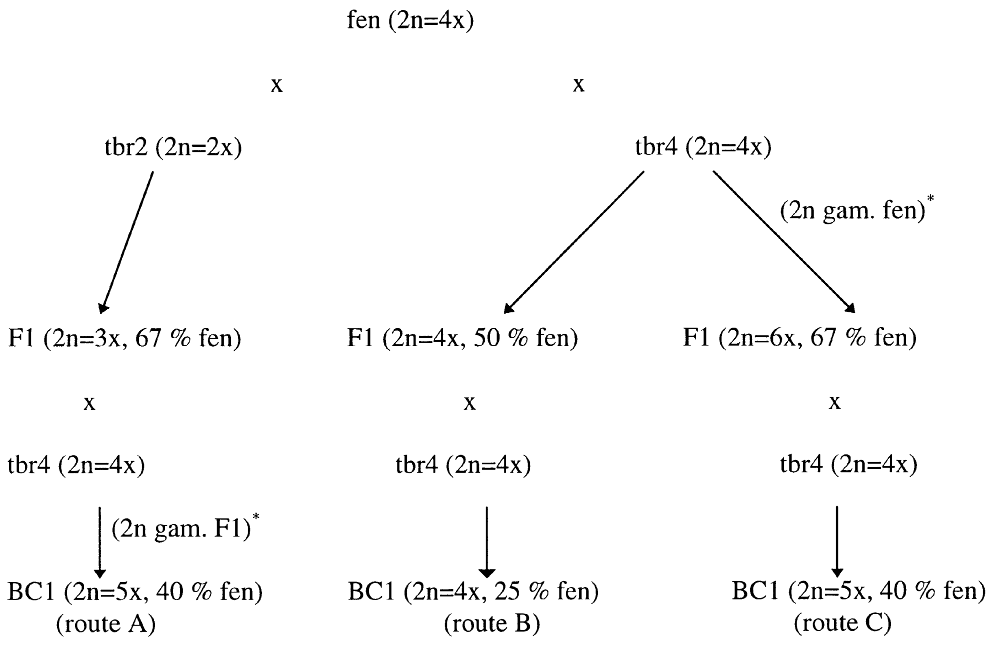

Fig. 1 Efficiency of the introgression of traits from *Solanum fendleri* into *S. tuberosum* via different routes. *: Instead of 2n gametes, the doubling of the parent genotype through colchicine treatment would give a similar effect

S. tuberosum or *S. verrucosum* showed regular bivalent chromosome pairing between the homoeologous genomes (Ramanna and Hermsen 1979), whereas in tetraploid hybrids of *S. acaule* with *S. tuberosum* a high frequency of bivalents but also several univalents were observed (Watanabe et al. 1994). Also, sexually obtained diploid hybrids of *S. brevidens* with *S. tuberosum* showed some meiotic aberrations, but chromosome associations suggested the likely occurrence of recombination between the genomes of nontuberous and tuber-bearing species (Watanabe et al. 1995). It is expected that the chiasmata frequency will be lower in these wide interspecific crosses, but the advantage of at least some recombination at an early stage of introgression remains worthwhile.

Most successful inter-EBN crosses appear to have been the result of embryo rescue or rescue pollinations or a combination of these techniques (Iwanaga et al. 1991; Singsit and Hanneman 1991; Watanabe et al. 1992, 1995). In the crossing experiments reported here, true tetraploid hybrids were obtained from crosses of *S. tuberosum* with *S. stoloniferum* and *S. fendleri* following normal crossing procedures. Tetraploid hybrids of *S. tuberosum* with *S. hougasii* were obtained after in vitro seed culture. One seed was derived from a mature berry but failed to germinate. Nevertheless, this might indicate that more crosses possibly would have yielded hybrid plants without having to resort immature seed culture. Such seed culture is a simplification of the embryo-culture technique, which has already proven to be powerful in overcoming crossing barriers (Hermsen 1994). Using embryo rescue, Eijlander and Stiekema (1994) obtained hybrids between the hexaploid weed black nightshade, *Solanum nigrum*, and potato cv 'Desiree', and sexual crosses between genera might even be possible (Watanabe et al. 1995). In vitro seed culture is to be preferred in interspecific hybridisations, which are expected to be little successful but not impossible through sexual crosses, such as the cross combinations described in this study.

The EBN hypothesis is based on the (lack of) endosperm development in developing seeds (Johnston et al. (1980). However, there are more factors involved which determine the actual success of crosses, and it is not an absolute rule. Since in some inter-EBN combinations one or two seeds per berry can be found, an arbitrary criterion of ten plump seeds per fruit has been used to determine the success of a cross and to assign an EBN value (Johnston and Hanneman 1980; Hanneman 1994). Without violating the theory on which the EBN hypothesis is based, it can be stated that EBN is not a major limiting factor for potato breeding, since a few hybrids are sufficient as a first step in gene transfer.

The introgression of resistance to root-knot nematodes from *S. fendleri*, *S. stoloniferum* and *S. hougasii* has advanced to resistant BC_1 genotypes for further backcrosses. At this stage other agronomically important characteristics also need to be selected for at the cost of the majority of the traits from the wild species. The introgression strategy using the tetraploid hybrids (Fig. 1, route B) is simple and efficient and should provide valuable breeding materials for a new generation of potato cultivars. In combination with marker-assisted selection, these hybrids should even make a more efficient selection towards the wild *Solanum* genome possible.

Acknowledgements The authors wish to thank J. H. W. Bergervoet and H. A. Verhoeven for their cooperation with the ploidy analyses and L. T. Colon, J. G. Th Hermsen and E. J. Jacobsen for critical reading of the manuscript.

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