

A bicontinental origin of polyploid Australian/New Zealand *Lepidium* species (Brassicaceae)? Evidence from genomic *in situ* hybridization

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• **Background and Aims** Incongruence between chloroplast and nuclear DNA phylogenies, and single additive nucleotide positions in internal transcribed spacer (ITS) sequences of polyploid Australian/New Zealand (NZ) *Lepidium* species have been used to suggest a bicontinental hybrid origin. This pattern was explained by two trans-oceanic dispersals of *Lepidium* species from California and Africa and subsequent hybridization followed by homogenization of the ribosomal DNA sequence either to the Californian (C-clade) or to the African ITS-type (A-clade) in two different ITS-lineages of Australian/NZ *Lepidium* polyploids.

• **Methods** Genomic *in situ* hybridization (GISH) was used to unravel the genomic origin of polyploid Australian/NZ *Lepidium* species. Fluorescence *in situ* hybridization (FISH) with ribosomal DNA (rDNA) probes was applied to test the purported ITS evolution, and to facilitate chromosome counting in high-numbered polyploids.

• **Key Results** In Australian/NZ A-clade *Lepidium* polyploids, GISH identified African and Australian/NZ C-clade species as putative ancestral genomes. Neither the African nor the Californian genome were detected in Australian/NZ C-clade species and the Californian genome was not detected in Australian/NZ A-clade species. Five of the eight polyploid species (from 7x to 11x) displayed a diploid-like set of rDNA loci. Even the undecaploid species *Lepidium muelleriferdinandi* ($2n = 11x = 88$) showed only one pair of each rDNA repeat. In A-clade allopolyploids, *in situ* rDNA localization combined with GISH corroborated the presence of the African ITS-type.

• **Conclusions** The nuclear genomes of African and Australian/NZ C-clade species were detected by GISH in allopolyploid Australian/NZ *Lepidium* species of the A-clade, supporting their hybrid origin. The presumed hybrid origin of Australian/NZ C-clade taxa could not be confirmed. Hence, it is assumed that Californian ancestral taxa experienced rapid radiation in Australia/NZ into extant C-clade polyploid taxa followed by hybridization with African species. As a result, A-clade allopolyploid *Lepidium* species share the Californian chloroplast type and the African ITS-type with the C-clade Australian/NZ polyploid and African diploid species, respectively.

Key words: *Lepidium*, Brassicaceae, FISH, GISH, hybridization, polyploidy, long-distance dispersal, ITS, rDNA, Australia, New Zealand.

INTRODUCTION

Lepidium is one of the largest genera in the Brassicaceae, consisting of approximately 230 species distributed worldwide (Al-Shehbaz *et al.*, 2006). This genus is characterized by reduced floral organs, an autogamous mating system and mucilaginous seeds (Al-Shehbaz, 1986). Recent molecular phylogenetic studies using the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS), non-coding chloroplast DNA (cpDNA) and single-copy nuclear DNA sequences (an intron of the PISTILLATA gene, PI), respectively, clarified only some relationships within the genus (Bowman *et al.*, 1999; Mummenhoff *et al.*, 2001; Lee *et al.*, 2002). All molecular phylogenies support three main infrageneric lineages, corresponding to (1) section *Monoploca sensu stricto* (*s.s.*) (Australia), (2) section *Lepia* with *Cardaria* included (Eurasia) and (3) *Lepidium s.s.* (referred to hereafter as *Lepidium*) representing by far the bulk of *Lepidium* species on every continent. The fossil data, easily dispersible mucilaginous seeds, common autogamous breeding systems and low

levels of sequence divergence between *Lepidium* species from different continents or islands suggest a rapid radiation of *Lepidium* by long-distance dispersal in the Pliocene/Pleistocene (Mummenhoff *et al.*, 2001). The large number of polyploid species (Warwick and Al-Shehbaz, 2006) suggests reticulate evolution in *Lepidium* and this was indicated also in the analysis of PISTILLATA intron sequences, in which many polyploid taxa harbour two or more phylogenetically distinct sequences (Lee *et al.*, 2002). Chromosome numbers have been reported for about 70 *Lepidium* species and subspecies (Warwick and Al-Shehbaz, 2006), of which all except five have $x = 8$ chromosomes (Al-Shehbaz 1986). Vaarama (1951) speculated that species with $2n = 24$ (e.g. *L. sativum*) are hexaploids based on $x = 4$, but this interpretation was not supported by any further research. More than half of *Lepidium* species studied are polyploids, and chromosome numbers vary widely ($2n = 16, 24, 28, 32, 40, 48, 64$ and 80 ; Warwick and Al-Shehbaz, 2006).

In Australia and New Zealand (NZ), *Lepidium* is represented by 19 and seven native species, respectively. Incongruence between the chloroplast and nuclear DNA phylogenetic

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TABLE 1. Composition of the ITS sequences of Californian, Australian/New Zealand and African *Lepidium* species

	ITS1	ITS2
	11111111112233344	67880013446775936933
	83478965680241795402	
Californian C-clade species		
<i>L. nitidum</i> , <i>L. oxycarpum</i> , <i>L. dictyotum</i>	CATTAGCCCGTCATCGG	-TA
Australian/NZ C-clade polyploids		
<i>L. aschersonii</i>	CATTATCCCGTCATCGG	-TW
<i>L. muelleriferdinandi</i>	CATTATCCCGTCATCGG	-TA
Australian/NZ A-clade polyploids		
<i>L. hyssopifolium</i> (KM 1670)	YWYKRSSYSWACYTA	-TCT
<i>L. hyssopifolium</i> (KM 1669)	TTCGCGTGAACCTA	-TCT
<i>L. pseudohyssopifolium</i>	TTCGCGTGAACCYA	-TCT
African A-clade species		
<i>L. africanum</i> , <i>L. transvaalense</i>	TTCGCGTGAACCTA	-TCT

This data matrix contains only those positions of a complete alignment (not shown) that distinguish Californian from African *Lepidium* taxa. Californian and African species are extant members of two different lineages suggested to have been involved in the hybridogenous origin of Australian/NZ *Lepidium* species. R = A and G; W = A and T; Y = C and T; K = G and T; S = G and C. Site numbers are those of the complete alignment. For details see Mummenhoff *et al.* (2004).

analyses indicated a hybridogenous genomic constitution of Australian/NZ *Lepidium* species: all 18 studied species shared a Californian cpDNA-type, while 11 Australian/NZ species appeared to harbour a Californian ITS-type (C-clade) and a group of the remaining seven species shared an African ITS-type (A-clade) (Mummenhoff *et al.*, 2004; Table 1, Fig. 1). This pattern was explained by two trans-oceanic dispersals of ancestral taxa from California and Africa to Australia/NZ and subsequent hybridization followed by sequence homogenization of the rDNA either to the Californian or to the African ITS-type in the two different lineages, with single additive nucleotide positions remaining, representing the ancestral parental input (Table 1, Fig. 1). However, Mummenhoff *et al.* (2004) did not assess the ploidy level of Australian/NZ species and the conclusion of a bicontinental hybridogenous genomic constitution was based only on two markers representing nuclear (ITS) and chloroplast genomes (three non-coding cpDNA regions).

The present study aimed to (1) assess ploidy level and chromosome number variation among Australian/NZ *Lepidium* species, (2) test the purported allopolyploid bicontinental origin of Australian/NZ species of the A- and C-clades using genomic *in situ* hybridization (GISH) and (3) gain insight into the physical organization of rDNA loci in the presumed parental species and their Australian/NZ hybrid derivatives.

MATERIALS AND METHODS

Plant material

Collection data of selected species of *Lepidium* L. used in the current study are given in Table 2. The presumed parental and hybrid species were used exemplarily, based on results of previous studies (Mummenhoff *et al.*, 2001, 2004, 2009).

Chromosome preparations

Plants were grown from seeds in plastic Petri dishes on sieved potting soil in a phytotron with long day illumination (16 h light at 20 °C, 8 h dark at 15 °C). Young inflorescences were fixed in ethanol/acetic acid (3:1, v/v) fixative for 24 h at 4 °C. Fixative was replaced by 70 % ethanol and the material stored at -20 °C until further use. Chromosome spreads were prepared as described by Lysak *et al.* (2006). Slides were examined under phase contrast for the presence of suitable mitotic metaphase spreads. Selected slides were post-fixed in 4 % formaldehyde in 2× sodium saline citrate (SSC, 5 min), washed in 2× SSC (twice for 5 min), and dehydrated in an ethanol series (70, 90 and 100 %, 2 min each). The preparations were stained with 2 µg mL⁻¹ 4,6-diamino-2-phenylindole (DAPI) in Vectashield antifade (Vector Laboratories, Burlington, Ontario, Canada) and screened for the quality of metaphase and the presence of cytoplasm under an epifluorescence microscope (BX-61, Olympus, Tokyo, Japan). If appropriate, the slides were treated with pepsin (0.1 g L⁻¹) in 0.01 M HCl at 39 °C for 15–45 min, and post-fixed and dehydrated as described above.

DNA probes

The *Arabidopsis thaliana* BAC clone T15P10 (AF167571) bearing 45S rRNA gene repeats was used for *in situ* localization of 45S rDNA, and *A. thaliana* clone pCT 4.2 (M65137), corresponding to a 500-bp 5S rRNA repeat, was used for localization of 5S rDNA loci. For GISH, total genomic DNA (gDNA) was extracted from healthy young leaves according to Dellaporta *et al.* (1983) followed by RNase treatment (50 µg mL⁻¹). Extracted gDNA was checked for protein, starch or RNA contamination using a Beckmann photospectrometer and run on a 1 % (w/v) agarose gel in 1× Tris-acetate-EDTA (TAE) buffer. DNA probes were labelled either by biotin- or by digoxigenin-dUTP using the Nick Translation Mix (Roche, Mannheim, Germany) according to the manufacturer's instructions.

In situ hybridization

Labelled 5S and 45S rDNA probes were precipitated and the pellet was resuspended in 20 µL hybridization mixture containing 50 % formamide and 10 % dextrane sulfate in 2× SSC. The probe was applied to the slide, covered with a cover slip and denatured on a hot plate at 80 °C for 2 min. Slides were hybridized at 37 °C for approx. 12 h. To prevent drying, cover slips were framed by rubber cement. Labelled gDNA probes were prepared and denatured as described above. Hybridization time for GISH was between 12 and 48 h.

Following hybridization, stringent washing was carried out in 50 % formamide in 2× SSC (v/v) at 42 °C three times for 5 min. Detection of hybridization signals was performed according to Lysak *et al.* (2006). The biotin-labelled probes were detected by avidin–Texas Red (Vector Laboratories), and signals amplified by biotinylated goat anti-avidin (Vector Laboratories) and avidin–Texas Red. Digoxigenin-labelled probes were detected by mouse anti-digoxigenin (Roche) and goat anti-mouse–Alexa Fluor 488 antibodies

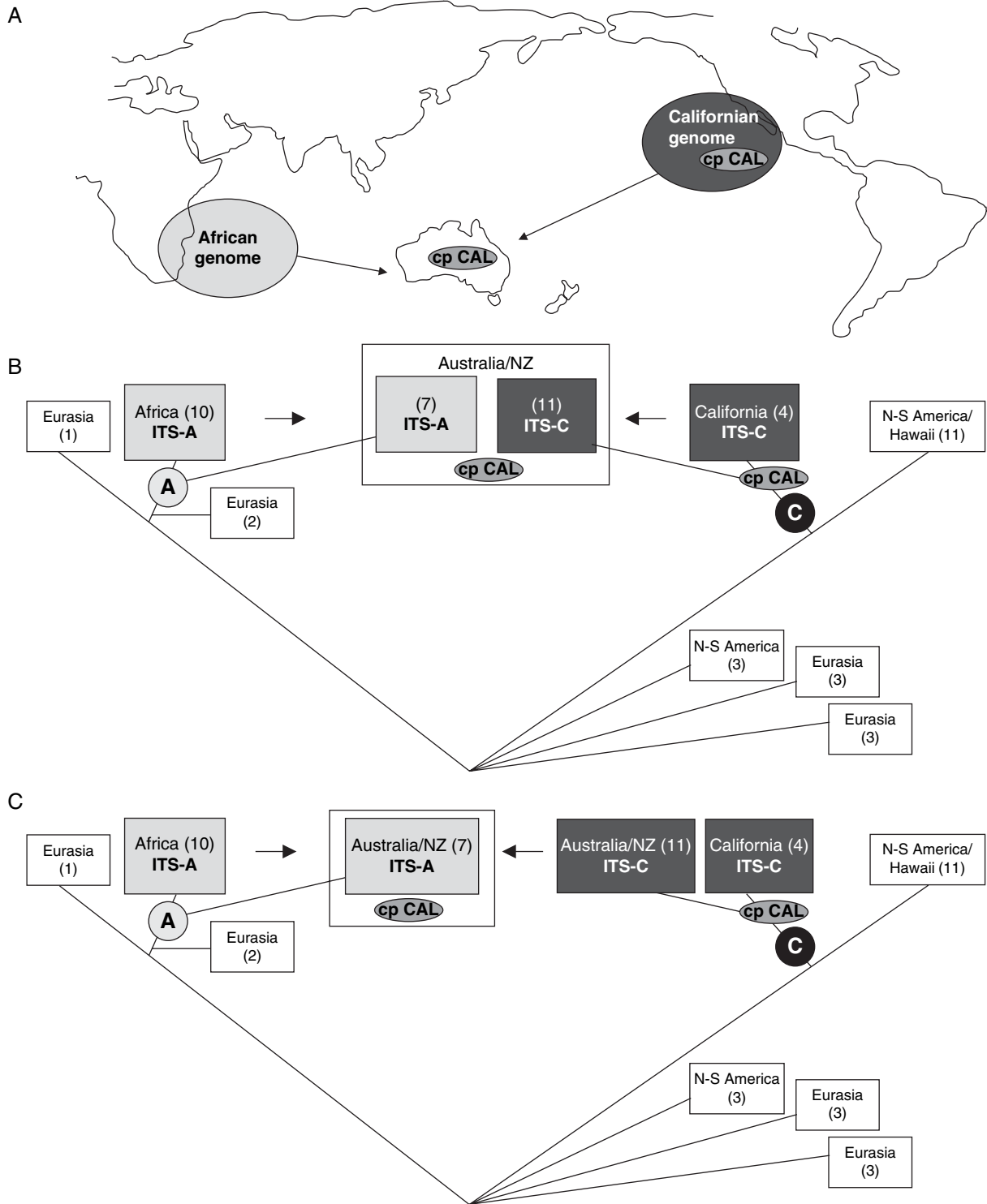


FIG. 1. Organismal and geographical context of the genome evolution of polyploid Australian/NZ A- and C-clade *Lepidium* species. (A) Geographical context of the evolution of polyploid Australian/NZ *Lepidium* species further outlined in (B) and (C). (B) Evolution of polyploid Australian/NZ A- and C-clade *Lepidium* species as inferred from ITS- and cpDNA sequence data (Mummenhoff *et al.*, 2004). All Australian/NZ species harbour a Californian chloroplast type (cp CAL), while bidirectional concerted evolution of ribosomal DNA (ITS) subsequent to hybridization between African and Californian parental species resulted in the presence of either of the parental ITS-types, i.e. African (ITS-A) and Californian (ITS-C), in the two lineages (Australian/NZ A- and C-clade species, respectively). Arrows indicate hybridization. Numbers in boxes represent number of analysed species of given geographical origin. Encircled letters A and C refer to A- and C-clade species, respectively. See text for more details. (C) Evolution of polyploid Australian/NZ A- and C-clade *Lepidium* species as inferred from ITS and cpDNA sequence data (Mummenhoff *et al.*, 2004) and current GISH experiments. In this alternative scenario polyploid Australian/NZ C-clade species are the outcome of rapid radiation of an ancient Californian ancestor in Australia/New Zealand. Australian/NZ A-clade species originated from hybridization between African and Australian/NZ C-clade species, still harbouring the ancestral Californian chloroplast type. In this scenario concerted evolution operated unidirectionally towards the African ITS-type in Australian/NZ A-clade hybrids. Arrows indicate hybridization. Numbers in boxes represent number of analysed species of given geographical origin. Encircled letters A and C refer to A- and C-clade species, respectively. See text for more details.

TABLE 2. Origin, chromosome number, ploidy level and number of rDNA loci for *Lepidium* species studied

Species	Accession no.	Origin/Collector	Chromosome no.	No. of 45S rDNA loci	No. of 5S rDNA loci
<i>L. africanum</i> (Burm.f.) DC.*	KM 1702	Australia, Melbourne, Royal Park, South of Park Street/Scarlett, N.	$2n = 2x = 16$	2	2
	KM 1793	South Africa, Eastern Cape/Clark, V. R.	$2n = 2x = 16$	2	2
	KM 1794	South Africa, Eastern Cape/Clark, V. R.	$2n = 2x = 16$	2	2
	KM 1792	South Africa, Eastern Cape/Clark, V. R.	$2n = 2x = 16$	2	2
<i>L. transvaalense</i> Marais	KM 1711	USA, California, Tucker Herbarium at UC Davis/Bowman, J.	$2n = 7x = \text{approx. } 56$	2	2
<i>L. nitidum</i> Nutt.	KM 1713	USA, California, Central Valley, Los Banos/Bowman, J.	$2n = 7x = \text{approx. } 56$	2	2
<i>L. oxycarpum</i> Torr. & Gray	KM 1714	USA, California, Carrizo plain/Bowman, J.	$2n = 7x = \text{approx. } 56$	2	4
<i>L. dictyotum</i> Gray	KM 1669	Australia, New South Wales, Bathurst/Scarlett, N.	$2n = 9x = 72$	2	4
<i>L. hyssopifolium</i> Desv.	KM 1670	Australia, Victoria, Belfast Lough, Port Fairy end of the Lough/Scarlett, N.	$2n = 9x = 72$	2	4
<i>L. pseudohyssopifolium</i> Hewson	KM 1666	Australia, Victoria, Melbourne, Fairfield, Banks of the Yarra River/Parsons, R. F.	$2n = 9x = 72$	2	2
<i>L. ginninderrense</i> Scarlett	KM 1673	Australia, Australian Capital Territory, Belconnen Naval Station, floodplain of Ginninderra Creek/Scarlett, N.	$2n = 14x = 112$	4	2
<i>L. muelleriferdinandi</i> Thell.	KM 1710	Australia, Northern Territory, Alice Springs Desert Park, outside herbarium/Albrecht, D. E.	$2n = 11x = 88$	2	2
<i>L. aschersonii</i> Thell.	KM 1668	Australia, Victoria, Lake Beac/Scarlett, N.	$2n = 7x = 56$	2	2

* *L. africanum* is native to South Africa, and an introduced weed in Australia.

(Molecular Probes, New Haven, CT, USA). Chromosomes were counterstained with DAPI ($2 \mu\text{g mL}^{-1}$) in Vectashield. Fluorescence signals were analysed with an Olympus BX-61 epifluorescence microscope and AxioCam CCD camera (Carl Zeiss, Jena, Germany). Individual images were merged and processed by using Photoshop CS software (Adobe Systems).

RESULTS

Chromosome counts

In the analysed *Lepidium* species, chromosome numbers varied from $2n = 16$ to $2n = 112$ (see Table 2 for all chromosome counts). The two African species (*L. africanum*, *L. transvaalense*) have a diploid chromosome number ($2n = 2x = 16$), whereas all Australian/NZ and Californian species are polyploid (Fig. 2). Four different ploidy levels ($7x$, $9x$, $11x$ and $14x$) were discerned in Australian/NZ species. In the three Californian species, we could not obtain suitable chromosome spreads to determine exact chromosome numbers. Observed chromosome numbers were tentatively interpreted as heptaploid ($2n = 7x = 56$).

Mitotic chromosomes of *Lepidium* species are small (approx. $2\text{--}5 \mu\text{m}$) with the bulk of heterochromatin concentrated within pericentromeres, and often fuzzy euchromatic chromosome ends (Figs 2 and 3). In the two African species, chromosomes are larger and possess a less distinct eu-/heterochromatin profile than chromosomes in the other two geographical groups. The presumably different chromosome structure of African species is particularly apparent upon GISH in the A-clade allopolyploids, whereby chromosomes of African origin show a more even labelling pattern and appear to be larger than the remaining chromosomes (Fig. 3).

Localization of rDNA loci

Most *Lepidium* species exhibit one pair of 5S and 45S rDNA loci (Table 2 and Fig. 2). This pattern was also observed in high-numbered polyploid species such as *L. nitidum*, *L. oxycarpum*, *L. pseudohyssopifolium*, *L. muelleriferdinandi* and *L. aschersonii*. Two species (*L. dictyotum* and *L. hyssopifolium*) possess two pairs of 5S rDNA, and one species (*L. ginninderrense*) possesses two pairs of 45S rDNA loci (Table 2 and Fig. 2). In *L. oxycarpum*, 5S and 45S loci were found to be partly co-localized (Fig. 2C). 5S rDNA loci are positioned on chromosomes interstitially, whereas 45S rDNA loci showed terminal localization (Fig. 2).

GISH in polyploid Australian/NZ *Lepidium* species

With the aim to discern the origin of Australian/NZ *Lepidium* polyploids, labelled gDNA of putative African and Californian parental species was hybridized to mitotic chromosomes of Australian/NZ polyploid A- and C-clade taxa. In two A-clade species (*L. hyssopifolium*, *L. pseudohyssopifolium*), gDNA of presumed African parents (*L. africanum*, *L. transvaalense*) labelled 16 of the 72 chromosomes, whereas no chromosomes were identified using gDNA of presumed Californian parental species (*L. dictyotum*, *L. oxycarpum*) (data not shown). No chromosome-specific hybridization signals were observed in

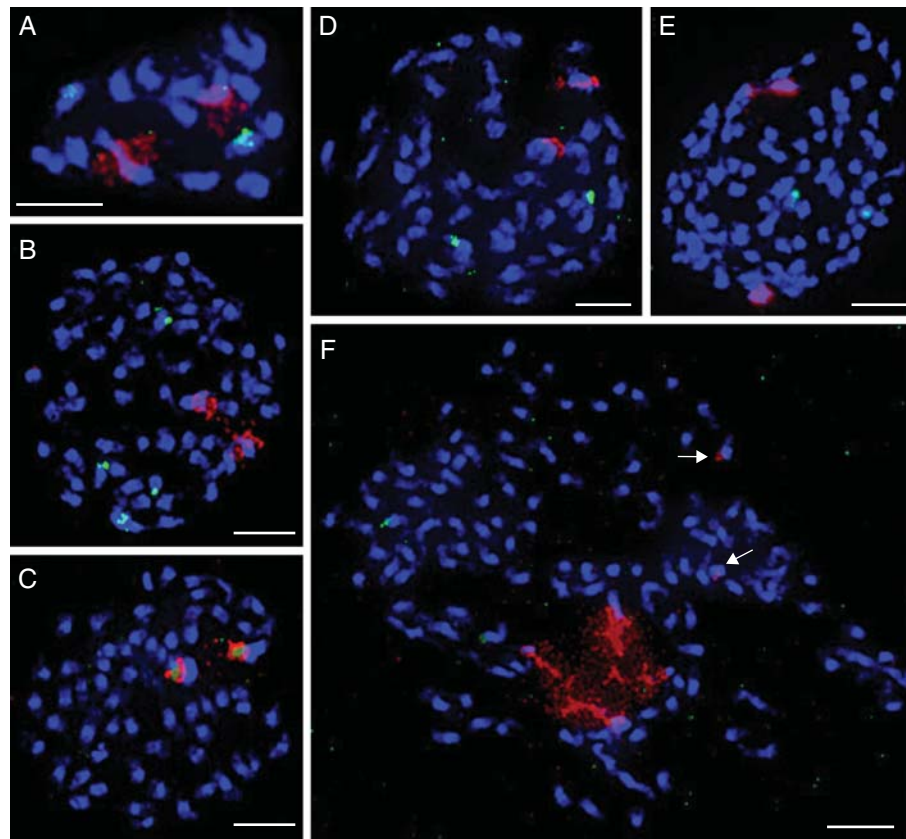


FIG. 2. DAPI-stained mitotic chromosome spreads from flower bud tissue of *Lepidium* species. Chromosomes were hybridized with 5S (green) and 45S (red) rDNA probes and counterstained with DAPI. (A) *L. africanum* ($2n = 2x = 16$), (B) *L. dictyotum* ($2n = 7x = 56$), (C) *L. oxycarpum* ($2n = 7x = 56$), (D) *L. aschersonii* ($2n = 7x = 56$), (E) *L. muelleriferdinandi* ($2n = 11x = 88$), (F) *L. ginninderrense* ($2n = 14x = 112$). In (F) one pair of major 45S rDNA loci is decondensed around the nucleolus; two minor 45S rDNA loci are indicated by arrows. Scale bars = 5 μm .

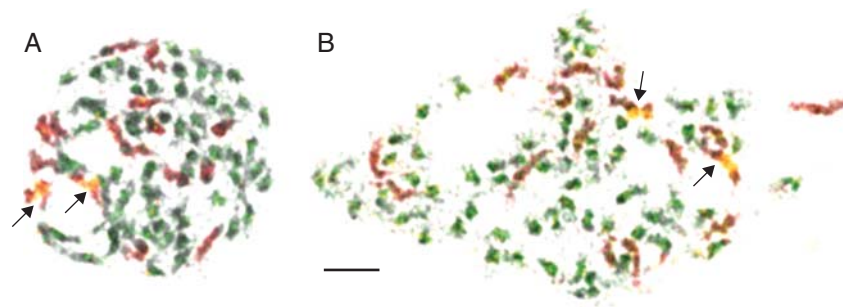


FIG. 3. GISH in two nonaploid ($2n = 9x = 72$) Australian/NZ *Lepidium* A-clade species. Chromosomes of *L. hyssopifolium* (A) and *L. pseudohyssopifolium* (B) hybridized with gDNA of *L. africanum* (16 red chromosomes) and gDNA of an Australian/NZ C-clade species, i.e. *L. muelleriferdinandi* (56 green chromosomes). Arrows mark 45S rDNA-bearing chromosomes (yellow/red). Scale bar = 5 μm .

C-clade species (*L. aschersonii*, *L. muelleriferdinandi*) using the same probes as used in the A-clade species (data not shown).

In follow-up GISH experiments, 56 of 72 chromosomes in the Australian/NZ A-clade species *L. hyssopifolium* and *L. pseudohyssopifolium* were detected using gDNA of two Australian/NZ C-clade species, i.e. *L. muelleriferdinandi* (Fig. 3) and *L. aschersonii* (data not shown). In the two A-clade allopolyploids ($2n = 72$), 16 chromosomes correspond to *L. africanum* and 56 chromosomes were labelled by gDNA of *L. aschersonii*/*L. muelleriferdinandi* (Fig. 3). This experiment also showed that 45S rDNA in *L. hyssopifolium*

and *L. pseudohyssopifolium* has probably been contributed by the African parental genome(s).

DISCUSSION

rDNA patterns in polyploid Lepidium species

The present study followed three principal goals as far as *in situ* localization of rDNA repeats is concerned. First, we were interested to see if the three geographical species groups (Africa, Australia/NZ and California) are characterized

by specific rDNA patterns. Secondly, the number of rDNA loci in Australian/NZ polyploids was compared with the number of the same loci in the presumed progenitor species, and finally, the origin of 45S rDNA loci in Australian/NZ polyploid species was inferred from GISH analysis and compared with conclusions based on ITS sequence data (Mummenhoff *et al.*, 2004).

From the limited data set (only ten of 230 *Lepidium* species were analysed) no apparent geographical pattern of rDNA variation emerges. In all but one polyploid species analysed, only a diploid-like number of 45S rDNA loci were found, with two pairs of 45S identified in the tetradecaploid *L. ginninderrense* ($2n = 112$). This finding is somewhat surprising given the high ploidy levels (from $7x$ to $14x$) of the analysed taxa. Regardless of prevailing modes of polyploid evolution (auto- vs. allopolyploidy) in the Californian and Australian/NZ groups, respectively, the present data indicate that there is a strong tendency toward diploidization of rDNA loci in both geographical species groups. Although less likely, preferential loss of rDNA-bearing chromosomes in odd-numbered (7 , 9 and $11x$) *Lepidium* polyploids cannot be ruled out.

Ali *et al.* (2005) analysed three different crucifer species with the same polyploid chromosome number ($2n = 48$). In two species, the number of 45S was high (approx. 14 in *Camelina microcarpa*, and $10 + 6$ minor loci in *Olimarabidopsis cabulica*), whereas only one pair has been recorded in *Aethionema schistosum* (Ali *et al.*, 2005). It could be speculated that this limited comparison corroborates the generally accepted correlation between the number of 45S rDNA loci and the age of polyploid species as the genus *Aethionema* is sister to and older than the rest of Brassicaceae (Al-Shehbaz *et al.*, 2006). However, the *Aethionema* polyploid species may be of a similar age to two Camelinae species analysed. Hence, it appears to be difficult to establish a direct link between the age of a phylogenetic split and the diploidization rate in crucifer taxa.

With each polyploidization event the number of rDNA loci increases and some rDNA sequences become redundant. These ITS sequences can be changed by bidirectional interlocus concerted evolution, resulting in complete homogenization to either of the parental types or to mosaic-like sequences with additive nucleotides from both parents (Wendel, 2000). In the long term, genome diploidization events as discovered for ancient allopolyploid *Nicotiana* species of the section *Repandae* (4.5 Myr old) can occur, resulting in the loss of redundant rDNA loci and a diploid-like number of rDNA loci, which can be even lower than the number of 45S loci found in the progenitor species (Clarkson *et al.*, 2005). However, more recently formed *Nicotiana* allopolyploids (e.g. *N. tabacum*, 0.2 Myr old) display the sum of the rDNA loci of their progenitors (Clarkson *et al.*, 2005). As a maximum estimated age for the origin of polyploid Australian/NZ *Lepidium* species is approx. 1.3 Ma (Mummenhoff *et al.*, 2004), this or a shorter period of time has been sufficient for (nearly) complete diploidization of rDNA loci (Table 2).

Within the set of the 72 chromosomes of Australian/NZ A-clade species (*L. hyssopifolium*, *L. pseudohyssopifolium*) combined GISH and rDNA fluorescence *in situ* hybridization (FISH) localization detected two 45S rDNA loci on two

chromosomes belonging to the 16 chromosomes of African origin. The 56 remaining chromosomes displayed no 45S rDNA loci (Fig. 3). This is in agreement with the ITS phylogenetic data showing that all Australian/NZ A-clade species harbour the African ITS-type (Mummenhoff *et al.*, 2004; Fig. 1C, Table 1). For these species, physical loss of C-clade rDNA loci must be assumed subsequent to hybridization. Furthermore, the African ITS-type in polyploid Australian/NZ A-clade species can be explained by unidirectional concerted evolution to the African ITS-type with single additive nucleotides remaining (Table 1, *L. hyssopifolium* KM 1670).

Previous hypothesis on the origin of polyploid Australian/NZ Lepidium species

The rDNA ITS regions are the most widely used nuclear-encoded phylogenetic markers (Álvarez and Wendel, 2003). Their high evolutionary rate permits discrimination of closely related putative parental species and the identification of additive patterns in hypothesized allopolyploids (Marhold and Lihová, 2006). Although concerted evolution can erase nucleotide additivity, bidirectional, nearly complete homogenization or intergenomic recombination may result in a mosaic-like structure of ITS sequences, which has been described in the literature (Marhold and Lihová, 2006). In the Brassicaceae, such analyses unravelled hybridization events in *Thlaspi* (Mummenhoff *et al.*, 1997), *Cardamine* (Franzke and Mummenhoff, 1999; Lihová *et al.*, 2006) and *Boechera* (Koch *et al.*, 2003). Support for the bicontinental hybrid origin of polyploid Australian/NZ *Lepidium* species was provided by bidirectional, nearly complete concerted evolution of the ITS to either of the two presumed parental ITS-types (African and Californian) with single additive (parental) nucleotides remaining (Mummenhoff *et al.*, 2004; Table 1). It appears that all Australian/NZ polyploids share a Californian cpDNA-type (Mummenhoff *et al.*, 2004), but this cpDNA evidence was detected only in 60% of the maximally parsimonious trees (Mummenhoff *et al.*, 2004). Furthermore, this scenario of a bicontinental hybridogenous genomic constitution was based on only two markers representing the nuclear (ITS) and chloroplast genome (Mummenhoff *et al.*, 2004). Thus, GISH experiments were performed to gain deeper insight into the origin and evolution of polyploid Australian/NZ *Lepidium* species.

Origin of Australian/NZ C-clade species: neither African nor Californian progenitors detected by GISH

In previous studies based on sequence analysis of nuclear ITS and non-coding cpDNA (Mummenhoff *et al.*, 2004), a bicontinental hybrid origin of polyploid Australian/NZ *Lepidium* species has been suggested. In GISH experiments, gDNA probes of presumed African (*L. africanum*, *L. transvaalense*) and Californian parental species (*L. dictyotum*, *L. oxycarpum*) did not label specific chromosomes in the Australian C-clade species. This result is in contrast to the ITS and cpDNA analysis (Mummenhoff *et al.*, 2004). The evidence of a bicontinental hybridogenous genomic constitution of C-clade species is based on weak cpDNA evidence (see above), ITS sequence homology of

Australian/NZ species with Californian taxa, and single additive nucleotide positions representing both parental ITS-types. If this additivity in single ITS nucleotide positions is not random and indicates an African input, present-day African species used for GISH experiments do not represent the original African progenitors. This means that the genome of the original African species contributing to the ITS additivity in the polyploid Australian/NZ C-clade species is too distinct from the genome of extant African taxa used in the present study (*L. africanum*, *L. transvaalense*), and thus gDNA probes of *L. africanum*/*L. transvaalense* could not be used to detect the original African parental genome in C-clade species.

Ancestral Californian species (migrating to Australia) may have transmitted the Californian ITS-type to the allopolyploid Australian/NZ C-clade species via hybridization with an unknown taxon, potentially of African origin. At the same time the ancestral Californian taxa might have evolved into the present-day polyploid Californian species, probably by several rounds of hybridization and polyploidization events, accompanied by the dynamic evolution of genome-specific dispersed repeats. As a consequence, the extant Californian species differ significantly in genome constitution from their presumed polyploid Australian/NZ descendants, and thus their gDNAs do not reveal the ancestral Californian genome in the polyploid Australian/NZ C-clade species. This can be analogous to the decreased efficiency of GISH and its failure in approx. 1- and 5-Myr-old *Nicotiana* allopolyploids, respectively (Lim *et al.*, 2007).

Australian/NZ C-clade species: simply descendants of Californian ancestors?

Negative results using gDNA of Californian and African species in C-clade Australian/NZ polyploids suggest long-distance dispersal of ancestral Californian species to Australia followed by radiation into extant C-clade polyploid species without hybridization with African species being involved. All Australian/NZ C-clade species appear to harbour a Californian ITS- and cpDNA-type (Mummenhoff *et al.*, 2004) and only one additive nucleotide position each (representing African and Californian ancestral nucleotides, respectively) in the ITS sequences of two C-clade species studied (*L. aschersonii*, *L. muelleriferdinandi*). This single African nucleotide in these two C-clade species could also be explained by random mutations or could represent an input by hybridization with an unknown species. The remaining 32 chromosomes of the genome of present-day *L. muelleriferdinandi* ($2n = 88$) not detected in Australian A-clade polyploids ($2n = 72$) could indeed indicate some sort of hybridization/polyploidization with unknown taxa in the evolution of *L. muelleriferdinandi* subsequent to the hybridization of an ancestor of *L. muelleriferdinandi* (presumably with $2n = 56$) with African species ($2n = 16$). GISH signals of an African genome input could not be detected, suggesting that Australian/NZ C-clade species are not the outcome of a bicontinental allopolyploidization scenario as originally described (Mummenhoff *et al.*, 2004). Calibration of molecular trees yielded ages of approx. 0.7–1.3 and 0.3–0.55 Ma for the Australian/NZ species of the C- and

A-clades, respectively (Mummenhoff *et al.*, 2004). These age differences might indicate that the radiation of Californian ancestors into Australian/NZ C-clade species predates the later arrival of African ancestors and the origin of A-clade allopolyploids.

Origin of Australian/NZ A-clade species: allopolyploid hybrids between African and Australian/NZ C-clade species

GISH experiments clearly detected 16 chromosomes of African origin (*L. africanum*, *L. transvaalense*) in the two polyploid Australian/NZ A-clade species analysed (*L. hyssopifolium*, *L. pseudohyssopifolium*). The remaining 56 chromosomes in the A-clade allopolyploid genomes were not labelled by any of the Californian presumed parental gDNAs used. Thus, the presumed direct Californian genomic input into the Australian/NZ A-clade *Lepidium* species could not be confirmed by GISH.

One might thus speculate that Australian/NZ C-clade taxa have contributed the Californian cpDNA-type to the Australian/NZ A-clade taxa by hybridization with African species in Australia. To test this scenario, GISH experiments were conducted using gDNAs of Australian/NZ C-clade species (*L. aschersonii* $2n = 56$, *L. muelleriferdinandi* $2n = 88$) as probes onto the Australian/NZ A-clade polyploid species *L. hyssopifolium* and *L. pseudohyssopifolium* ($2n = 72$). Of the 72 chromosomes, 56 were labelled by gDNA of Australian/NZ C-clade species and, as outlined above, the remaining 16 chromosomes were labelled by gDNA of *L. africanum*/*L. transvaalense*. The remaining 32 chromosomes of the genome of present-day *L. muelleriferdinandi* ($2n = 88$) not detected in Australian A-clade polyploids ($2n = 72$) could indicate some sort of hybridization/polyploidization with unknown taxa in the evolution of *L. muelleriferdinandi* subsequent to the hybridization of an ancestor of *L. muelleriferdinandi* (presumably with $2n = 56$) with African species ($2n = 16$).

This general scenario is in agreement with the cpDNA phylogeny in which the Australian/NZ A-clade hybrids harbour a Californian chloroplast type (Mummenhoff *et al.*, 2004). There is some additional evidence for this alternative scenario: one accession of an Australian/NZ A-clade polyploid (*L. hyssopifolium*, KM 1670) shows additivity in almost every diagnostic nucleotide position that differs between the C-clade and A-clade species group (Table 1). Regardless, all GISH and rDNA FISH experiments using two different accessions of *L. hyssopifolium* (KM 1670, KM 1669) show the same results. As ITS homogenization can occur very rapidly, as also observed in experiments with recent and synthetic allopolyploids where 45S rDNA homogenization was apparent within a few generations (Franzke and Mummenhoff, 1999; Skalická *et al.*, 2003; Kovarik *et al.*, 2005; Shcherban *et al.*, 2008), it is possible that additivity in almost all diagnostic nucleotide position in *L. hyssopifolium* (KM 1670) indicates recent and/or recurrent hybridization between African and Australian/NZ C-clade species in Australia.

Thus, the main conclusions from GISH analysis are as follows. The nuclear genomes of African and Australian/NZ C-clade species were detected in allopolyploid Australian/NZ *Lepidium* A-clade species. The presumed hybrid origin of

Australian/NZ C-clade taxa with African and Californian parents involved (Mummenhoff *et al.*, 2004) could not be confirmed. This does not mean that hybridization processes did not play a role in the evolution of polyploid Australian C-clade species, for example *L. muelleriferdinandi* with $2n = 88$ chromosomes. Hence, it is assumed that ancestral Californian taxa subsequent to their dispersal to Australia experienced a rapid radiation in Australia and New Zealand into extant C-clade taxa that hybridized with African species. As a result, A-clade allopolyploid *Lepidium* species in Australia/NZ share the Californian chloroplast type and the African ITS-type with polyploid Australian/NZ C-clade and diploid African species, respectively.

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