Ancestral Chromosomal Blocks Are Triplicated in Brassiceae Species with Varying Chromosome Number and Genome Size[1]

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The paleopolyploid character of genomes of the economically important genus *Brassica* and closely related species (tribe Brassiceae) is still fairly controversial. Here, we report on the comparative painting analysis of block F of the crucifer Ancestral Karyotype (AK; $n = 8$), consisting of 24 conserved genomic blocks, in 10 species traditionally treated as members of the tribe Brassiceae. Three homeologous copies of block F were identified per haploid chromosome complement in Brassiceae species with $2n = 14$, 18, 20, 32, and 36. In high-polyploid ($n \geq 30$) species *Crambe maritima* ($2n = 60$), *Crambe cordifolia* ($2n = 120$), and *Vella pseudocytisus* ($2n = 68$), six, 12, and six copies of the analyzed block have been revealed, respectively. Homeologous regions resembled the ancestral structure of block F within the AK or were altered by inversions and/or translocations. In two species of the subtribe Zillineae, two of the three homeologous regions were combined via a reciprocal translocation onto one chromosome. Altogether, these findings provide compelling evidence of an ancient hexaploidization event and corresponding whole-genome triplication shared by the tribe Brassiceae. No direct relationship between chromosome number and genome size variation (1.2–2.5 pg/2C) has been found in Brassiceae species with $2n = 14$ to 36. Only two homeologous copies of block F suggest a whole-genome duplication but not the triplication event in *Orychophragmus violaceus* ($2n = 24$), and confirm a phylogenetic position of this species outside the tribe Brassiceae. Chromosome duplication detected in *Orychophragmus* as well as chromosome rearrangements shared by Zillineae species demonstrate the usefulness of comparative cytogenetics for elucidation of phylogenetic relationships.

Approximately 240 cruciferous species of the *Brassicaceae* family classified into approximately 52 genera and sharing distinct morphological characters with the genus *Brassica* are taxonomically treated as the tribe Brassiceae (Schulz, 1936; Hedge, 1976; Gomez-Campo, 1980; Warwick and Sauder, 2005; Al-Shehbaz et al., 2006). The Brassiceae represent a natural monophyletic group comprising several phylogenetic lineages (Lysak et al., 2005; Al-Shehbaz et al., 2006; Bailey et al., 2006). Taxonomic treatment of the tribe chiefly based on morphological and anatomical characters traditionally involved the recognition of seven (Schulz, 1936; Hedge, 1976) or six subtribes (Gomez-Campo, 1980). However, in a recent study (Warwick and Sauder, 2005), only four subtribes (Cakilinae, Savignyinae, Vellinae, and Zillinae) were shown to be natural phylogenetic lineages identified with four ITS- and ITS/*trn*L-based clades. In contrast, there was little support for the recognition of the traditionally circumscribed subtribes Brassicinae, Moricandiinae, and Raphaninae, as these were shown to be polyphyletic (Warwick and Sauder, 2005). The placement of genera *Calepina* Adanson, *Conringia* Heist. ex Fabr., and *Orychophragmus* Bunge into the tribe is currently the subject of intense debate, as these genera have repeatedly been placed within or excluded from the Brassiceae (Schulz, 1936; Gomez-Campo, 1980; Anderson and Warwick, 1999; Lysak et al., 2005; Warwick and Sauder, 2005).

The Brassiceae are distributed throughout the Mediterranean, Irano-Turanian, and Saharo-Sindian regions with only a few representatives occurring in eastern and southern Africa (Hedge, 1976; Gomez-Campo, 1980; Appel and Al-Shehbaz, 2003; Warwick and Sauder, 2005). The highest genetic and, consequently, taxonomic diversity of the group is confined to the southwestern Mediterranean, particularly to southeastern Spain and northwestern Africa: 47 out of 52 Brassiceae genera occur here, with 23 species being endemic to this region (Hedge, 1976). In contrast, only three genera exhibit their maximum diversity in the eastern Mediterranean, and five are distributed across the Irano-Turanian region (Gomez-Campo, 1980). In the Saharo-Sindian region, mainly including desert regions of the Sahara, Arabia, and Sindh (Pakistan), 11 out of 19 endemic cruciferous genera belong to

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Brassiceae (Hedge, 1976). Subtribes Savignyinae and Zillinae are exclusively confined to the Saharo-Sindian region (*Schouwia* DC.), and to a lesser extent (*Physorhynchus* Hook.) to the Irano-Turanian region (Hedge, 1976; Gomez-Campo, 1980; Appel and Al-Shehbaz, 2003).

Recently, chromosome colinearity shared between the Brassiceae allopolyploid *Brassica napus* ($n = 19$; subtribe Brassicinae) and Arabidopsis [*Arabidopsis thaliana* (L.) Heynh.; $n = 5$; tribe Camelineae] was analyzed by extensive comparative genetic mapping (Parkin et al., 2005). Arabidopsis and *Brassica* genomes were shown to share a minimum of 21 conserved syntenic blocks, which have been duplicated and reshuffled to build up the allopolyploid *B. napus* genome. Comparative linkage maps between Arabidopsis and two $n = 8$ Camelineae species [*Arabidopsis lyrata* (L.) O'Kane & Al-Shehbaz: Kuittinen et al., 2004; *Capsella rubella* Reut.: Boivin et al., 2004] along with the reconstruction of karyotype evolution in Arabidopsis and its four close relatives ($n = 6$–8) by comparative chromosome painting (CCP) paved the way for establishing a putative $n = 8$ Ancestral Karyotype (AK; Lysak et al., 2006). Schranz et al. (2006) integrated this AK karyotype with the concept of 21 syntenic blocks in *Brassica* (Parkin et al., 2005) to produce an ancestral karyotype with 24 conserved genomic blocks (A–X). These 24 blocks can be identified as single copies among the AK, Arabidopsis, and its close relatives; however, within the *Brassica rapa* (A genome) component of *B. napus*, they occur mostly in triplicate (Schranz et al., 2006).

Early comparative linkage mapping among three *Brassica* species (*Brassica nigra*, *Brassica oleracea*, and *B. rapa*; Lagercrantz and Lydiate, 1996), and between Arabidopsis and these three *Brassica* species (Lagercrantz, 1998), respectively, revealed that the "diploid" *Brassica* genomes were triplicated probably by an ancient hexaploidization event. These findings were corroborated and extended by in-depth genetic mapping studies confirming the ancient triplication of ancestral genomic blocks in *B. napus* (Parkin et al., 2005; see above) and white mustard (*Sinapis alba*; Nelson and Lydiate, 2006). Recently, the ancestral whole-genome triplication event shared by all Brassiceae taxa has been supported by comparative cytogenetic data. Lysak et al. (2005) analyzed the structure of chromosome regions homeologous to the ancestral block U by chromosome painting using a bacterial artificial chromosome (BAC) contig (continuous set of BAC clones) of Arabidopsis chromosome At4 in 21 crucifer species. In Brassiceae species from subtribes Brassicinae, Cakilinae, Moricandiinae, Raphaninae, and Vellinae ($n = 7$–19), CCP identified three and six homeologous copies of block U. The absence of the genome triplication in *Calepina* and *Conringia* species ($n = 7, 8$) suggested the placement of these genera outside the tribe Brassiceae (Lysak et al., 2005). Similar to the previous study, three different ancestral blocks (J, M, and N) were found to be triplicated in the *B. oleracea* ($n = 9$) complement upon CCP analysis (Ziolkowski et al., 2006). Hence, cytogenetic data corroborate the results of genetic mapping studies and provide strong evidence for the occurrence of an ancient whole-genome triplication event defining the tribe Brassiceae as a natural monophyletic lineage (Lysak et al., 2005; Lysak and Lexer, 2006).

This study aims to provide new insights into the origin and evolution of the ancient whole-genome triplication in the tribe Brassiceae. Here, we report on CCP analysis of two further ancestral blocks (blocks F and L) of AK (Schranz et al., 2006) in 10 previously unsampled Brassiceae species that vary considerably in chromosome number ($n = 7$–60; Table II), including those purported to have a polyploid origin ($n \geq 14$; Warwick and Al-Shehbaz, 2006). The second aim is to analyze how the ancient triplication event is reflected by present-day ploidy level and genome size variation among the analyzed taxa.

RESULTS

We have analyzed an approximately 11.5-Mb chromosome segment of the upper arm of Arabidopsis chromosome At3 by CCP in 10 Brassiceae species (Table I; Fig. 1). This BAC contig corresponds to ancestral crucifer blocks F and L as defined by Schranz et al. (2006) (Fig. 2A). To corroborate our findings, the At4-b BAC contig (approximately 8.7 Mb; Lysak et al., 2005) was used in some species. This contig comprises a large part of the bottom arm of Arabidopsis chromosome At4 and corresponds to the ancestral block U (Schranz et al., 2006; Fig. 3A).

Analysis of proximal chromosome regions within pachytene spreads has frequently been hampered by the presence of pericentromeric heterochromatin that impairs hybridization of painting probes to the

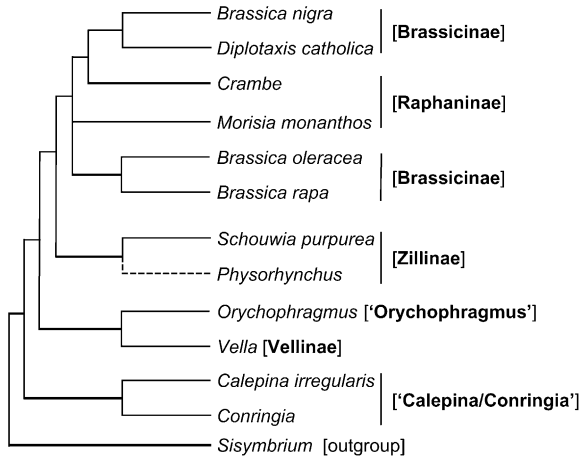

Figure 1. Schematic relationships of species analyzed and/or discussed in this article. The phylogenetic reconstruction based on analysis of combined ITS/*trn*L sequence data sets was adapted from Warwick and Sauder (2005). The position of *Physorhynchus* was inferred based on restriction site analysis of cpDNA by Warwick and Black (1994); the bootstrap value for the *Crambe* clade was adopted from Francisco-Ortega et al. (1999). Subtribe/clade assignments are given in brackets. Bootstrap support >70% is indicated by thickened lines.

Table I. *Subtribal classification, basic chromosome numbers (x), and the origin of species analyzed in this study*

Species	Subtribe[a]	Generic Basic No. (x)[b]	Origin/Source
B. oleracea	Brassicinae	7, 8, 9, 10, 11	Rapid-cycling *B. oleracea*
B. rapa			Rapid-cycling *B. rapa*
D. catholica (L.) DC.		7, 8, 9, 10, 11, 13	Portugal: Serpa; Botanic Garden Bordeaux, #210
C. cordifolia Stev.	Raphaninae	15	#2003–2035; living collection of RBG, Kew
C. maritima			#1976–4072; living collection of RBG, Kew
M. monanthos (Viv.) Asch.		7	#1992–2229; living collection of RBG, Kew
O. violaceus (L.) O.E. Schulz	Not assigned	12	Chiltern seeds, Bortree Stile, Ulverston, UK, #928B
P. chamaerapistrum (Boiss.) Boiss.	Zillinae[c]	14, 16	Oman: Muscat, Wadi Ghul; 23°11′ N, 57°8′ E (Millenium Seed Bank, RBG, Kew; #0096366)
S. purpurea (Forssk.) Schweinf.	Zillinae	18	Somalia: 5 km SW Mogadishu to Merka, 2 km from the airport; 2°5′ N, 45°20′ E (Millenium Seed Bank, RBG, Kew; #0050016)
V. pseudocytisus	Vellinae	17	#1975–5209; living collection of RBG, Kew

[a]Warwick and Sauder (2005). [b]Anderson and Warwick (1999); Warwick and Al-Shehbaz (2006). [c]Warwick and Black (1994).

homeologous chromosomal targets and/or impedes tracing of individual chromosomes at proximal regions. In numerous cases we were unable to identify unambiguously the L block hybridization signal at proximal regions, although it yielded a strong signal on Arabidopsis chromosome At3 (Fig. 2A). When tracing of this block was impossible, only block F (approximately 9.5 Mb) was analyzed in pachytene spreads.

Three Copies of the F Block Were Found in *Morisia*, Two *Brassica* Species, and *Diplotaxis*

In *Morisia monanthos* ($2n = 14$), *B. oleracea* ($2n = 18$), *B. rapa* ($2n = 20$), and *Diplotaxis catholica* ($2n = 18$), CCP with the F (+L) block revealed three homeologous copies (Fig. 2, B–D). In *M. monanthos* (Fig. 2B), two homeologous copies of block F are arranged as in AK but differ as to their position on the respective chromosome. The first and the second homeologous segments are positioned distally and at the middle part of homeologs M1 and M2, respectively. The M3 homeolog harbors the analyzed segment at an interstitial position with inverted orientation. In contrast, we could localize only one of the expected three copies of block L (homeolog M4).

In the two *Brassica* species, the homeology patterns corresponding to block F were very similar (Fig. 2, C and D). Two homeologs (O1/R1 and O2/R2) again resembled the AK structure, although minor rearrangements within the labeled chromosome regions cannot be ruled out. The third homeolog (O3/R3) showed the same structure but at an interstitial position. Interestingly, the two *Brassica* species differed as to their heterochromatin patterns visualized after 4′,6-diamidino-2-phenylindole (DAPI) staining. Distinct interstitial and terminal heterochromatin knobs can be identified on pachytene chromosomes of *B. rapa* (Fig. 2D), but are lacking or less pronounced on chromosomes of *B. oleracea* (Fig. 2C). In particular, *B. rapa* homeologs R1 and R2 are characterized by a terminal heterochromatin that was not found on homeologs of other Brassiceae species.

In *D. catholica*, regions homeologous to block F displayed the AK structure and distal position on three different chromosomes (data not shown).

Two of the Three F + L Homeologous Copies Are Fused in *Physorhynchus* and *Schouwia*

In two species traditionally classified as members of the subtribe Zillinae (Fig. 1; Table I), *Physorhynchus chamaerapistrum* ($2n = 32$) and *Schouwia purpurea* ($2n = 36$), the F + L block labeled two different chromosomes. One homeolog (P1 or S1) shows the ancestral structure, while the second chromosome (P2 or S2) revealed a much longer painting signal than observed in any other species analyzed (Fig. 2, E and F). Homeologs P2 and S2 most likely represent a fusion chromosome combining two homeologous regions. In *P. chamaerapistrum*, two other homeologs were identified: P3 with a part of block F and P4 bearing a signal corresponding to block L (Fig. 2E); the latter two signals were not observed in *S. purpurea* (Fig. 2F).

Orychophragmus Revealed a Chromosome Duplication Rather Than a Triplication

Within the chromosome complement of *Orychophragmus violaceus* ($2n = 24$), the F + L block was found as two copies (Fig. 2G). One copy (homeolog O1) resembles the arrangement found in the AK, whereas the second (O2) harbors only the distal part of block F. The missing proximal part of the O2 homeolog is most likely represented by the red signal on homeolog O3. Homeologous regions corresponding to block L were observed close to the pericentromeric heterochromatin of two other chromosomes (O4 and O5). No third copy of the F + L block was found in *Orychophragmus*.

Six and Twelve F + L Homeologous Copies Occurred in Species of Higher Ploidy Level

Three highly polyploid ($n \geq 30$) Brassiceae species were analyzed using CCP with the F + L block as a

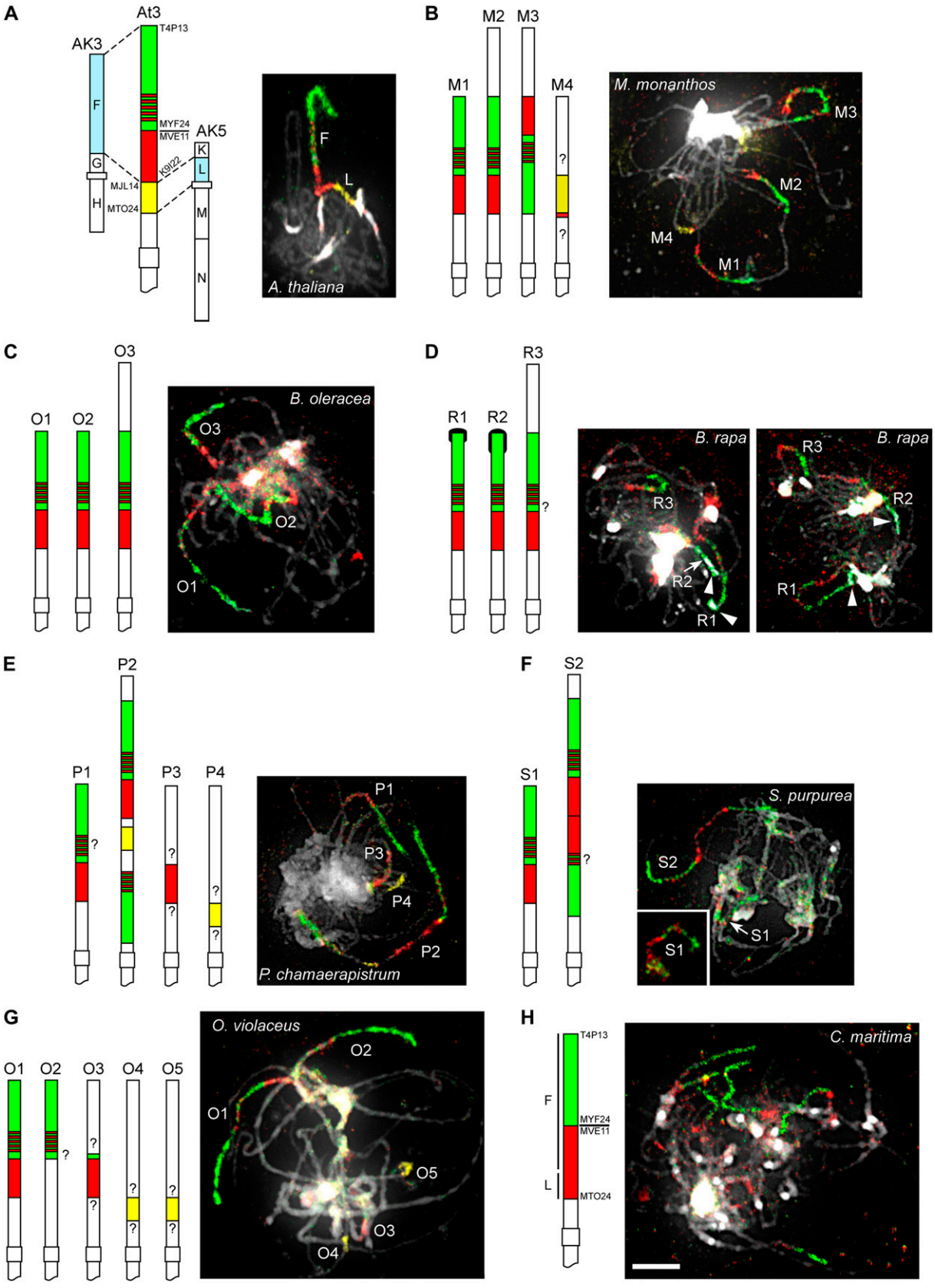

Figure 2. CCP of ancestral blocks F and L. A, Labeling scheme of Arabidopsis BAC contigs corresponding to blocks F and L on chromosomes AK3 and AK5 of AK (Schranz et al., 2006), and their fluorescence in situ localization on chromosome At3 of

painting probe. Analysis of painting signals within pachytene spreads of these plants was hampered (1) by high bivalent numbers causing frequent intermingling of painted homeologs, (2) by formation of multivalents involving also painted homeologs, and (3) by prominent heterochromatin clusters particularly in proximal chromosome regions. In *Vella pseudocytisus* ($2n = 68$), six copies of block F were found in most pachytene spreads. Although the internal structure of all six copies could not always be analyzed, most if not all homeologs showed the AK structure (data not shown). Similarly, in *Crambe maritima* ($2n = 60$) six chromosome regions homeologous to F block were found. The labeling scheme used here does not allow us to make conclusions as to the internal structure of the homeologs; however, all six copies apparently exhibit the AK-like structure (Fig. 2H). Interpretation of CCP results in *Crambe cordifolia* ($2n =$ approximately 120) was difficult due to the high chromosome number and the multivalent formation. However, in several spreads 10 to 12 copies of the F block were identified (data not shown).

CCP of the Ancestral Block U Confirms the Chromosome Duplication in *Orychophragmus*

To corroborate the absence of a third copy of the F + L block in *O. violaceus*, block U has been used as an additional CCP probe (Fig. 3A). The ancestral block U is identical with the At4-b BAC painting probe used by Lysak et al. (2005). Block U was also found in only two copies, both showing arrangement as in the AK having a distal position on two chromosomes (Fig. 3B). In *Physorhynchus* and *Schouwia*, both of which showed the fusion of two F + L homeologous copies, CCP with U block labeled three homeologs with similar signal patterns in both species. Two chromosomes (P1 and P2, or S1 and S2) resemble the AK structure, whereas the corresponding region on homeologs P3 and S3 is partly inverted (Fig. 3, C and D).

Genome Size Variation Does Not Coincide with Chromosome Number Variation in Brassiceae Species

Results for genome size and base-pair composition (AT/GC) are shown in Table II. The genome size variation ranged from 1.2 pg/2C in *D. catholica* ($2n = 18$) to 9.5 pg/2C in *C. cordifolia* ($2n =$ approximately 120). A general correlation between DNA content and chromosome number was found (Pearson parametric correlation $r = 0.96$ at $P < 0.01$). However, there is no direct relationship between chromosome number and genome size variation among species sharing the whole-genome triplication (Table II). Although species with the lowest chromosome numbers (*M. monanthos*, $2n = 14$; *D. catholica*, $2n = 18$) possess the smallest genomes, a comparable genome size was found in *S. purpurea* ($2n = 36$). Genome sizes in highly ($n \geq 30$) polyploid *Vella* and *Crambe* species corroborate our findings based on CCP data. In *V. pseudocytisus* ($2n = 68$) and *C. maritima* ($2n = 60$), the 2C values roughly equal double the values for Brassiceae species with the triplicated genome. Similarly, 2C value of the octoploid *C. cordifolia* ($2n =$ approximately 120) is approximately 2-fold greater than the value for the tetraploid *C. maritima* ($2n = 60$). Thus, genome size data correspond to the presence of six or 12 homeologous copies of ancestral blocks in these species. CCP analyses showed that the *O. violaceus* genome ($2n = 24$) is duplicated and has not undergone the Brassiceae-specific triplication event. This finding is not supported by the 2C value of *O. violaceus*, which is larger than genome sizes of Brassiceae species with $2n = 14$ to 36 (Table II). The variation in GC content among the analyzed taxa was very low, ranging from 34.2% in *D. catholica* to 36.7% in *S. purpurea* and *C. cordifolia* (Table II). No significant correlation between base composition and genome size was found (tested nonparametrically by Spearman's rho).

DISCUSSION

This study has primarily focused on chromosomal in situ localization of ancestral blocks F and L via CCP using Arabidopsis BAC contigs in several species assigned to the tribe Brassiceae. Three homeologous copies of block F found in Brassiceae species studied herein are in agreement with genetic mapping data (Parkin et al., 2005, and refs. therein) and provide further evidence for the ancient triplication of the Brassiceae genomes as previously shown for ancestral block U in a number of Brassiceae taxa (Lysak et al., 2005) and for blocks J, M, and N in *B. oleracea* (Ziolkowski et al., 2006).

Figure 2. (*Continued.*)
Arabidopsis. The BAC subcontigs were visualized by Alexa 488 (green), Texas Red (red), and Cy3 (yellow) fluorescence, respectively. B, CCP in *M. monanthos* ($2n = 14$); C and D, CCP in *B. oleracea* ($2n = 18$; C) and *B. rapa* ($2n = 20$; D). Black spheres on homeologs R1 and R2 indicate heterochromatin arrays and knobs on these chromosomes (arrowheads). E and F, CCP in *P. chamaerapistrum* ($2n = 32$; E) and *S. purpurea* ($2n = 36$; F) revealing the fusion chromosomes P2 and S2, respectively. G, Two homeologous copies of block F (+L) in *O. violaceus* ($2n = 24$). H, Six homeologous copies of block F (+L) in *C. maritima* ($2n = 60$). Note that the naming of *Brassica* chromosomes (O1-3, R1-3) used here does not correspond to the linkage groups in Parkin et al. (2005). Question marks within schematic chromosomes refer to the unknown orientation of a particular chromosome bearing the painting signal; question marks beside diagrams indicate that the presence or structure of a particular chromosome region is uncertain. Scattered/dot-like red and green hybridization signals at pericentromeric chromosomal regions correspond to cross-hybridizing repetitive elements present within the used BAC contigs. Scale = 5 μm.

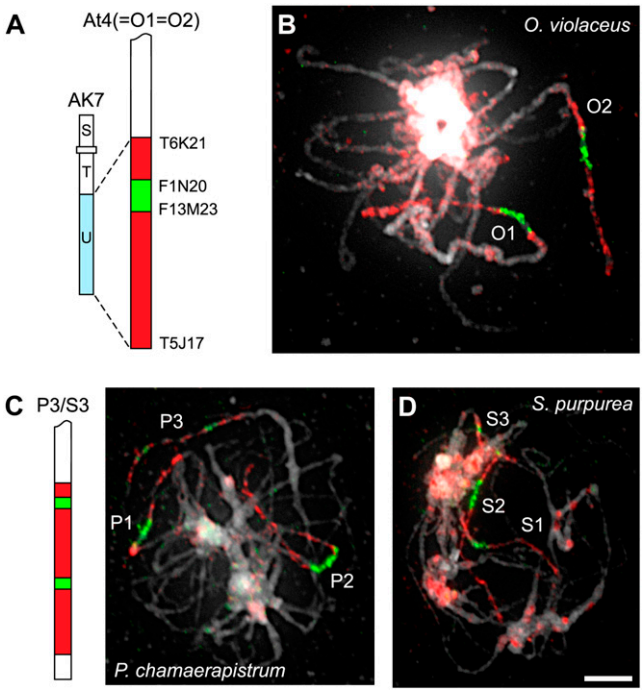

Figure 3. CCP of ancestral block U. A, Labeling scheme of Arabidopsis BAC contigs corresponding to block U on chromosome AK7 of AK (Schranz et al., 2006). B, Two homeologs (O1, O2) identified in the genome of *O. violaceus* ($2n = 24$). C and D, Three homeologs (P1–P3 or S1–S3) of block U revealed in the genomes of *P. chamaerapistrum* ($2n = 32$; C) and *S. purpurea* ($2n = 36$; D). The structure of the third homeolog (P3 or S3) as compared to the structure of block U is altered by a paracentric inversion (see C). Red signals, particularly at pericentromeric heterochromatin regions, correspond to cross-hybridization of repetitive elements present within the used BAC contigs. Scale = 5 μm.

Block F is located distally on the top arm of ancestral chromosome AK3, and retained the same structure and position on the top arm of Arabidopsis chromosome At3 (Fig. 2A; Lysak et al., 2006, Schranz et al., 2006). We have demonstrated that homeologous chromosome regions identified in all Brassiceae species analyzed retained the conserved structure and position of block F as within AK in at least one of the homeologs. Block L is located on chromosome AK5 in AK, whereas it has an adjacent position to block F on the top arm of At3 (Fig. 2A). With the exception of *P. chamaerapistrum* (Fig. 2E), block F was found separated from block L in Brassiceae species. Thus, our data suggest that the Brassiceae species share the ancestral pattern for blocks F and L (i.e. AK3: block F; AK5: block L). CCP data are in good agreement with the location of blocks F and L on different chromosomes in the genetic map of *B. napus* (Parkin et al., 2005). Although in most cases more than one homeologous copy of block L was observed, in situ localization of this block was hampered by the presence of pericentromeric heterochromatin that impairs hybridization of painting probes to the homeologous chromosomal targets, and, thus, its exact copy number remains largely unresolved.

The ancestral pattern of blocks F and L has frequently been altered by chromosome rearrangements. In *Morisia* (Fig. 2B), the position of the block F at the middle part of homeolog M2 most likely results from a reciprocal translocation. The pattern of the M3 homeolog can be explained by a paracentric inversion in combination with a reciprocal translocation. In *Orychophragmus* (Fig. 2G), the missing proximal subcontig of homeolog O2 is represented by the red signal on another chromosome (O3), possibly as result of a reciprocal translocation.

The two species belonging to the subtribe Zillineae (Fig. 1; Warwick and Black, 1994; Warwick and Sauder, 2005), *P. chamaerapistrum* ($2n = 32$) and *S. purpurea* ($2n = 36$), revealed three homeologous copies of block F on only two chromosomes (Fig. 2, E and F). The internal structure of the homeologs P2 and S2 indicates a fusion of two F + L blocks by a complex rearrangement including partial deletion(s). Although the fusion chromosomes P2 and S2 exhibit comparable structure at the distal and proximal regions in both species, the structure of the central part differs. The fusion has occurred most likely via a reciprocal translocation between the two F + L homeologous copies accompanied by one or more inversions. Due to these (and later) chromosomal alterations, parts of the participating homeologous segments were lost, giving the fusion chromosomes their present-day structure. Possibly, the combination of two copies on one chromosome preceded the divergence of both species. Inversions were shown to be the most frequent chromosome rearrangements involving block U in the Brassiceae species studied previously (Lysak et al., 2005). Similarly, an apparently identical paracentric inversion splitting the green subcontig of block U into two parts has been identified on homeologs P3 and S3 in *Physorhynchus* and *Schouwia*, respectively (Fig. 3, C and D). Shared chromosome rearrangements underline the close relationship of the two taxa and support a monophyletic status for the Zillineae (Fig. 1; Warwick and Black, 1994; Warwick and Sauder, 2005).

Unexpectedly, one complete and one truncated copy of block F were identified in *Orychophragmus* (Fig. 2G). This finding indicated that *O. violaceus* has not undergone the whole-genome triplication event shared by other Brassiceae species. To further test this, we analyzed ancestral block U located on ancestral chromosome AK7 and the bottom arm of Arabidopsis chromosome At4 (Schranz et al., 2006). Two copies of block U found in the *O. violaceus* complement (Fig. 3B) corroborated conclusions based on the localization of block F. The genus *Orychophragmus* comprises two species (*O. violaceus* and *O. limprichtianus*) endemic to China and Korea (Al-Shehbaz and Yang, 2000). *O. violaceus* has a wider distribution area and is also cultivated as an ornamental plant in China as well as outside eastern Asia. The genus shows an unusual geographic distribution as well as atypical morphological characters compared to the remainder of Brassiceae species (for review, see Anderson and Warwick,

Table II. *Chromosome number, ploidy levels, 2C values, and base composition of species analyzed in this study*

Species	2*n*	Ploidy Level Based on x	(Paleo)ploidy Level Based on CCP Data	2C DNA	±SD	AT[a]	GC[a]
				pg		%	%
M. monanthos	14	2	6	1.578	0.014	65.34	34.66
D. catholica	18	2	6	1.206	0.007	65.77	34.23
O. violaceus	24	2	4	2.944	0.009	63.47	36.53
P. chamaerapistrum	32	2	6	2.481	0.006	63.63	36.37
S. purpurea	36	2	6	1.781	0.014	63.29	36.71
V. pseudocytisus	68	4	12	3.796	0.021	65.60	34.40
C. maritima	60	4	12	4.837	0.009	65.01	34.99
C. cordifolia	Approx. 120	8	24	9.468	0.181	63.33	36.67
Variation				7.85-fold		2.48%	
Used standards							
Arabidopsis (Columbia)	10	2	–	0.320[b]	–	65.30[c]	34.70[c]
Lycopersicon esculentum	24	2	–	1.972[b]	–	66.75	33.25

[a]Based on DAPI/PI dye factor, binding length = 4 (compare with Barow and Meister, 2002), and Arabidopsis AT/GC content (Arabidopsis Genome Initiative, 2000). [b]Based on Arabidopsis C value of 157 Mb (Bennett et al., 2003). [c]Based on known Arabidopsis sequence data (Arabidopsis Genome Initiative, 2000).

1999; Warwick and Sauder, 2005). However, the shared presence of isozyme duplications (Anderson and Warwick, 1999) and interspecific crossing data with various *Brassica* species (for review, see Warwick and Sauder, 2005) led to the inclusion of the genus in the tribe Brassiceae. Later, the inclusion of *Orychophragmus* was supported by a phylogenetic study based on nuclear (ITS) and chloroplast sequence data (Warwick and Sauder, 2005). In the above studies, however, *O. violaceus* formed a sister group to the core Brassiceae (i.e. formed a clade with the Vellinae based on ITS data; Fig. 1) or, based on cpDNA data associated with the *Calepina/Conringia* clade, a group that does not share the whole-genome triplication (Lysak et al., 2005). Based on the CCP data acquired here, we argue that *O. violaceus* is a tetraploid taxon sharing the same ancestor with Brassiceae but not the tribe-specific triplication event. It is tempting to speculate that the tetraploid *Orychophragmus*, in particular, *O. violaceus* ($2n = 4x = 24$), represents a bridge between the diploid *Calepina/Conringia* lineage and the hexaploid Brassiceae (Fig. 1). This would imply an ancestral Brassiceae genome having $n = 6$ chromosomes and the primary hexaploid ancestor with 36 chromosomes ($2n = 6x = 36$). However, the atypical geographic distribution outside the tribal diversity center in the southwestern Mediterranean and outside Saharo-Sindian/Irano-Turanian regions makes *Orychophragmus* a less likely candidate for a surviving ancestral genome. An in-depth multigenic sequence analysis is needed to identify the closest relatives of the Brassiceae, as well as to elucidate the phylogenetic relationship between *Orychophragmus* and the *Calepina/Conringia* clade.

Chromosome duplications detected in *Orychophragmus* as well as chromosome rearrangements shared by *Physorhynchus* and *Schouwia* clearly demonstrate the usefulness of CCP for detection of rare genomic changes and thus for elucidation of phylogenetic relationships (Lysak and Lexer, 2006). The long-lasting controversy over the inclusion of *Orychophragmus* in the tribe Brassiceae appears to be resolved by our CCP data.

The chromosome number across the tribe Brassiceae ranges from $n = 6$ to 75 (Anderson and Warwick, 1999). Within several genera species differ as to their basic chromosome numbers (x; e.g. *Brassica*, *Diplotaxis*; Table I). Some genera (e.g. *Vella*, *Schouwia*) apparently comprise only polyploid species (Warwick and Al-Shehbaz, 2006). Without information on the genome evolution of the tribe, basic chromosome number(s) and corresponding ploidy levels have been traditionally inferred as half of the lowest somatic chromosome count ($2n$) in a given genus (see Tables I and II). This situation has changed with the advent of CCP revealing the ancient as well as recent ploidy history of the Brassiceae species. A previous (Lysak et al., 2005) and this study showed that species ranging in haploid chromosome number from $n = 7$ (*Diplotaxis*, *Morisia*) to $n = 18$ (*Schouwia*) are in fact ancient hexapolyploids, sharing the whole-genome triplication. These data suggest that the traditional concept to estimate basic numbers (x) and ploidy levels is not valid. Although the high level of polyploidy hampers tracing of homeologous chromosomes and chromosome segments in meiotic chromosome spreads, six copies of block F were identified in *C. maritima* ($2n = 60$) and *V. pseudocytisus* ($2n = 68$), indicating that *C. maritima* and *V. pseudocytisus* are neopolyploids similar to the allopolyploid *Brassica* species or *Erucastrum gallicum* (Lysak et al., 2005). Consequently, 12 homeologous chromosome segments were expected in the genome of *C. cordifolia* with approximately 60 chromosome pairs. Indeed, approximately 12 copies were countable in some cells. From these findings we predict that quasi-diploid *Crambe* species ($2n = 30$) possess three copies of ancestral blocks as other Brassiceae species.

The ancient whole-genome triplication was found in Brassiceae species with $2n = 14, 16, 18, 20, 22, 28, 30,$

32, and 36. The lowest chromosome number reported for the Brassiceae is $2n = 14$ in *Diplotaxis erucoides* and *Morisia*. Both species possess three homeologous copies of the analyzed ancestral chromosomal blocks (Lysak et al., 2005; this study). The whole-genome triplication found in $n = 7$ taxa implies that the first paleohexaploid ancestor of Brassiceae likely had a higher chromosome number than 7 because of the ancestral genome triplication. An ancestral genome with $n = 6$ or 7 would yield a $2n = 36$ or 42 complement of the paleohexaploid ancestor. While *S. purpurea* represents a Brassiceae species with $2n = 36$, taxa with $2n = 42$ chromosomes were not yet identified within the tribe (Anderson and Warwick, 1999; Warwick and Al-Shehbaz, 2006). According to the current knowledge of *Brassicaceae* genome evolution, a hexaploid genome with $2n = 6x = 48$ based on an $n = 8$ AK (Lysak et al., 2006; Schranz et al., 2006) might be considered as most parsimonious. However, a Brassiceae species with $2n = 48$ has not been reported in the Brassiceae (Anderson and Warwick, 1999; Warwick and Al-Shehbaz, 2006). Furthermore, the scenario of independent and recurrent chromosome number reduction from the ancestral $n = 24$ genome toward the lowest known chromosome number ($n = 7$) via the inversion-reciprocal translocation cycle (Lysak et al., 2006; Schubert, 2007) accompanied by genome downsizing is not supported by genome size data (Table II).

Although on chromosome number of the first paleohexaploid ancestor of the Brassiceae can only be speculated, any evolutionary scenario must include the process of chromosome number reduction resulting in present-day chromosome number variation. The inversion-reciprocal translocation cycle, leading to loss of one centromere and potential loss of a terminal nucleolus organizing region, seems to be the most feasible mechanism of chromosome number reduction in the *Brassicaceae* (Lysak et al., 2006; Schranz et al., 2006; Schubert, 2007). The evolutionary loss of centromeric and nucleolus organizing regions should be reflected by genome downsizing and potential changes in base (AT/GC) composition, providing that this process is not fully counterbalanced by retroelement amplification. Although some correlation between DNA content and chromosome number variation has been found, species with varying chromosome numbers ($2n = 14, 18, 36$) showed comparable genome size as well as AT/GC composition (Table II). Hence, the present data indicate that chromosome number reduction accompanied by losses of large genomic tracts is not directly reflected by genome downsizing, and has been counteracted by genome expansion most likely through retroelement amplification.

To date, Arabidopsis BAC contigs corresponding entirely or partially to seven ancestral blocks (B, F, J, L, M, N, and U) on five chromosomes (AK1, 3, 4, 5, and 7) of AK ($n = 8$; Schranz et al., 2006), i.e. approximately 28.5 Mb or 18% of the Arabidopsis genome (157 Mb), have been analyzed by CCP (Lysak et al., 2005; Ziolkowski et al., 2006; this article). Except for a short (approximately 1.5 Mb) segment of block B (located on AK2; Ziolkowski et al., 2006), all ancestral blocks were found to be triplicated in genomes of 24 Brassiceae species (Lysak et al., 2005; Ziolkowski et al., 2006; this study). Almost 10 years since Lagercrantz (1998) for the first time postulated a whole-genome triplication for *Brassica* species, compelling genetic and cytogenetic evidence supporting an ancestral triploidization at the origin of the tribe Brassiceae is at hand.

MATERIALS AND METHODS

Plant Material

Plants grown from seeds and plants from the living collection at Royal Botanic Gardens (RBG), Kew were used as experimental material. The origins of investigated species are given in Table I. Entire inflorescences were fixed in ethanol:chloroform:acetic acid (6:3:1) and stored in 70% ethanol at −20°C until the use. Fresh leaves were used for genome size estimation by flow cytometry.

Painting Probes

Two BAC contigs were used as painting probes: (1) Arabidopsis (*Arabidopsis thaliana*) BAC clones of the top arm of chromosome At3 corresponding to the ancestral blocks F and L (Schranz et al., 2006) labeled according to two labeling schemes (Fig. 2, A and H), and (2) BAC clones of the bottom arm of chromosome At4 corresponding to the ancestral block U (Schranz et al., 2006) and the At4-b segment in Lysak et al. (2005) (Fig. 3A).

Multicolor CCP

Spreading of meiotic chromosomes at pachytene stage from individual flower buds and anthers has been carried out as described previously (Lysak et al., 2006). The slides were treated with pepsin (100 μg/mL in 0.01 M HCl) prior to CCP. DNA from individual BAC clones was isolated by a standard alkaline extraction and labeled by biotin-, digoxigenin-, and Cy3-dUTP, respectively, via nick translation. Labeled clones were mixed, precipitated, and dissolved in equal volumes of 100% formamide and 20% dextran sulfate in 4× SSC buffer. Probes and chromosomes were denatured together on slides at 80°C for 2 min, hybridized at 37°C for approximately 60 h, and subsequently washed in 20% formamide in 2× SSC at 42°C. Biotin-labeled BAC clones were detected using avidin~Texas Red and anti-avidin~biotin (both Vector Laboratories). The digoxigenin-labeled probes were detected by mouse anti-digoxigenin (Jackson ImmunoResearch) and by goat anti-mouse~Alexa 488 (Molecular Probes). Slides were analyzed using a Zeiss Axioplan 2 fluorescence microscope equipped with a Spot CCD camera. Captured images were merged and processed using Adobe Photoshop CS2.

Genome Size Estimation

DNA content and base composition was measured by flow cytometry in the flow cytometry laboratory at the Institute of Botany and Zoology, Masaryk University, Brno, Czech Republic. Two cytometers and two fluorochromes were used: Cy Flow (Partec) cytometer with propidium iodide (PI) for absolute DNA content estimation, and PA-1 (Partec) with DAPI for the AT proportion of the relative DNA content (needed for calculation of AT and GC content). A two-step procedure (Otto, 1990) was used for sample preparation. A piece of leaf was chopped using a razor blade together with internal standard in a glass petri dish containing 1 mL of Otto I buffer (0.1 M citric acid, 0.5% Tween 20). An additional 1 mL of Otto I buffer was added and the crude suspension of nuclei was filtered through a 50-μm nylon mesh. The suspension was divided into the two sample tubes to which 1 mL of Otto II buffer (0.4 M $Na_2HPO_4.12H_2O$) supplemented either with DAPI (2 μg/mL) or PI plus RNase (both 50 μg/mL) was added. All samples were measured on both cytometers simultaneously with *Lycopersicon esculentum* cv 'Stupické polní tyčkové ranné' as a primary internal standard. All measurements were repeated three times and results were averaged.

The AT and GC content was calculated from comparison of the parallel measurements with PI and AT-specific DAPI. For the calculation we used the

"Dye Factor," the proportion between the sample/standard fluorescence ratio with AT-specific DAPI, and the sample/standard fluorescence ratio with base-unspecific PI (equation 6 in Barow and Meister, 2002). The exact values of GC content were calculated according to Barow and Meister (2002) (equations 7 and 8) using binding length 4 and the mathematical approximation, the regula falsi method (compare with Barow and Meister, 2002; Meister and Barow, 2007), calculated in an automated Excel sheet (http://www.sci.muni.cz/botany/systemgr/Data/Festuca/ATGCFlow.xls).

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