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Chromosomal collinearity and karyotype evolution in crucifers (Brassicaceae)

Ph.D. Thesis

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Abstract

The Brassicaceae (Crucifereae) comprises 49 tribes, 321 genera and 3660 species, and belongs to the largest plant families. Whole-genome sequencing of the model plant *Arabidopsis thaliana* fuelled the interest of scientists in the mustard family as well as rapid development of comparative phylogenomics and cytogenomics, including the invent of chromosome painting in *A. thaliana* and comparative chromosome painting (CCP) in other Brassicaceae species. The Brassicaceae is the only plant family in which large-scale CCP is feasible. CCP provides unique insights into the karyotype and genome evolution in plants by comparing chromosome collinearity, identification of chromosome rearrangements, construction of comparative cytogenetic maps, and reconstruction of ancestral karyotype structures.

This PhD thesis deals with the karyotype evolution in the Brassicaceae family uncovered by comparative chromosome painting. The introductory part is divided into four chapters. The first chapter introduces chromosomes, karyotypes, karyotypic variation, and the role of chromosome rearrangements and polyploidy in the karyotype and genome evolution. In the methodical second part, principles of chromosome painting are described. The core third chapter focuses on comparative cytogenomics in the Brassicaceae, and summerizes the current knowledge on karyotype and genome evolution in this family. The final chapter provides comparison of karyotype evolution in the Brassicaceae with evolutionary trends in other plant families. Eight publications document author's contribution in the following research areas: 1) Optimization and application of the large-scale CCP in the Brassicaceae. 2) Identification of the mechanisms responsible for chromosome number reduction. 3) Evaluation of the role of polyploidy in karyotype and genome evolution. 4) Re-examination of the role of antient chromosome rearrangements in plant speciation.

Abstrakt

Čeleď brukvovitých (Brassicaceae, Crucifereae) patří k největším rostlinným čeledím; zahrnuje 49 tribů, 321 rodů a 3660 druhů. Zájem vědců o tuto čeleď vzrostl především díky ustanovení huseníčku rolního (*Arabidopsis thaliana*) modelovým druhem a sekvenování jeho genomu. To odstartovalo mimo jiné bouřlivý rozvoj srovnávací fylogenomiky a cytogenomiky, včetně úspěšného zavedení metody malování chromosomů (chromosome painting, CP) huseníčku a jejího rozšíření na další zástupce čeledi brukvovitých (srovnávací malování chromosomů, comparative chromosome painting, CCP). Metoda CCP umožňuje studium chromosomové kolinearity, rozpoznání přestaveb a rekonstrukci struktury karyotypů. Brukvovité jsou jedinou rostlinnou čeledí, u níž je v tomto rozsahu metoda CCP použitelná. Srovnávací cytogenetické mapy brukvovitých rostlin tak představují unikátní typ dat o evoluci rostlinných karyotypů a genomů.

Předkládaná disertační práce se věnuje evoluci karyotypu v čeledi brukvovitých studované pomocí metody srovnávacího malování chromosomů. Úvod disertační práce je rozdělen na čtyři části. První kapitola představuje chromosomy, karyotypy, jejich variabilitu a evoluci ovlivněnou chromosomovými přestavbami a polyploidií. Druhá metodická část pojednává o principech malování chromosomů. Stěžejní třetí kapitola disertační práce shrnuje naši současnou znalost o evoluci karyotypu a genomu v čeledi Brassicaceae. Poslední kapitola poskytuje srovnání s ostatními rostlinnými čeleděmi. Osm přiložených publikací, které významně přispěly k poznání studované problematiky, dokumentuje vědecké výsledky autora disertační práce v těchto základních oblastech: 1) Optimalizace a použití metody CCP u různých brukvovitých druhů. 2) Určení mechanismu zodpovědného za snižování chromosomového počtu. 3) Studium role polyploidie v evoluci karyotypu a genomu. 4) Přezkoumání vlivu chromosomových přestaveb na rostlinnou speciaci.

Content

1 Chromosomes and karyotypes

Motto: "*I am so inconspicuous that no one can see me with the naked eye. For millenia no one knew of my existence until microscopes were developed allowing an object to be magnified as many as 1000 times. But it was not so easy. The organs and tissues, of plants and animals, had to be separated into that unit of life that is the cell. Subsequently, only by breaking the cell, could I be found, well encased in its bosom, in company with the other minute molecular structures. Lockes in this prison, with thick membranes and walls surrounding me everywhere, I looked pale, transparent and hardly distinguishable from my surroundings. Ladies, of the upper classes, had been colouring their cheeks and lips with a dye called carmine, extracted from the body of a scale-insect, which lived on the cactuses of tropical America. When a drop of carmine was placed over the disrupted cell I easily became coloured a bright red and could be well recognized.*" (Lima-de-Faria 2008)

1.1 The chromosome discovery

Heinrich von Waldeyer (1888) introduced the term **chromosome** (from the Greek *chroma* for colored and *soma* for body) to designate filaments in the cell nucleus involved in cell division (mitosis) previously described by Walther Flemming (1878). During the first decade of the twentieth century, the Mendel's laws of heredity in which each individual presents two factors for each trait, with one factor coming from each parent, was rediscovered. In 1902, Walter Sutton (1902) observed chromosomes during meiosis to be organized in pairs, and gametes receiving only one of the two homologous chromosomes. This observation supported the idea that Mendel's factors responsible for heredity are localized on chromosomes. Seven years later, Wilhelm Johannsen (1909) introduced the term gene (from the Greek *genno* for give birth) instead of Mendel's factors to describe units of heredity. Thomas Morgan (1911) by his work on the *Drosophila* sex chromosome X proved Sutton's theory (Sutton 1902) that chromosomes are carriers of genes and thus established the chromosomal theory of inheritance. In 1931, Harriet Creighton and Barbara McClintock (1931) demonstrated by an elegantly simple experiment in maize, that exchanges between genes are accompanied by exchange of cytologically visible chromosome parts, indicating that genes are physically aligned along the chromosome. Today it is a well-known fact that DNA is the hereditary material and that the vast majority of the DNA of an organism is housed in chromosomes.

1.2 Karyotype evolution

The chromosomal constitution of each organism is reflected by its **karyotype**. It is characterized by a species-specific number of chromosomes of particular size and shape. Karyotypes may change via primary and secondary chromosome rearrangements (chapter 1.3 and 1.4), as well as ploidy mutations (chapter 1.5 and 1.6). Because karyotypes are dynamic structures, the reconstruction of ancestral karyotypes based on well described extant karyotypes is necessary for the understanding of trends in karyotype evolution (Schubert and Lysak 2011).

The development of complementary techniques for studying chromosomes made possible to reconstruct and compare karyotypes between species, genera and even families to the extent not feasible before. The study of chromosome collinearity, and the identification of chromosome rearrangements became feasible with the invent of diverse chromosome banding techniques (reviewed by Bickmore 2001) and, more recently, fluorescence *in situ* hybridization methods like comparative chromosome

painting in mammals (reviewed by Ferguson-Smith and Trifonov 2007) and plants (e.g., Brassicaceae and grasses; Mandáková and Lysak 2008, Mandáková et al. 2010a, b, 2012, Idziak et al. 2011). Alternatively, karyotype and genome evolution can be studied by comparative genetic mapping (i.e., Devos and Gale 1997, Duran et al. 2009, Gale and Devos 1998, Paterson et al. 2000, Salse et al. 2008) and comparative analyses of whole-genome sequences (e.g., Cannon et al. 2009, Ming et al. 2008, Salse 2012, Schmutz et al. 2010).

1.3 Variation in the chromosome structure

Structural chromosome alterations are the result of primarily or secondary rearrangements. Primary rearrangements are caused by illegitimate recombination during double-strand break repair, either via direct joining of ends between different double-strand breaks, or through recombination with ectopic homologous sequences. Primary rearrangements have breakpoints preferentially within heterochromatic regions enriched with repetitive sequences (reviewed by Bzymek and Lovett 2001, Lönnig and Saedler 2002, Schubert and Lysak 2011).

Primary chromosome rearrangements are insertion, deletion, duplication, peri- and paracentric inversion, and intra- or interchromosomal reciprocal translocation.

Deletion is the loss of genetic material. Two breaks can produce an interstitial deletion. In principle a single break can cause a terminal deletion but, because of the need for telomeres, it is likely that terminal deletions in fact include two breaks, one close to the telomere. Deletions are tolerated only in polyploids or when dispensable sequences are involved (Schubert and Lysak 2011).

Duplication results in the origin of an additional copy that is free from selective pressure. The fate of the duplicated DNA, is either genetic deterioration of one of the gene copies, resulting in the formation of pseudogenes, or divergence in the function of the gene copies from each other (Schubert and Lysak 2011).

Inversion occurs when a chromosomal segment is excised and reintergrated in opposite orientation into the same position, causing a reversed gene order. Paracentric inversions do not include the centromere and both breaks occur in one arm of the chromosome. Pericentric inversions include the centromere and there is a break point in each arm. Homozygotes for an inversion can have new linkage relations. When synapsis occurs in an inversion heterozygote, a loop is often formed. This looping tendency has a key evolutionary effect on crossover suppression within the inverted region (Kirkpatrick 2010, chapter 1.4).

Reciprocal translocation is an aberration during which chromosomes mutually exchange segments. If it does not result in any loss or gain of chromosome material, the translocation is described as balanced (Schubert and Lysak 2011). Translocations have two genetic consequences. Translocation increases the linkage distance between two genes if a segment is translocated between the genes. Translocations can also define new linkage relationships among these genes (Rabkin and Janz 2008).

1.4 Chromosome rearrangements and speciation

In plants, polyploidy and chromosome rearrangements are principal evolutionary forces generating the karyotypic variation. Whereas the role of polyploidy in reproductive isolation and plant speciation was unequivocally recognised (e.g., Comai 2005, Soltis et al. 2007), the evolutionary significance of chromosome rearrangements is less clear.

A rare individual with a chromosome rearrangement generally shows reduced reproductive fitness (underdominance) and it will preferentially mate only with individuals not bearing the rearrangement. Hence, such plant is expected to be

eliminated from the population. Conversely, if the rearrangement is only weakly underdominant, it is not likely to cause a reproductive isolation leading to speciation. Regardless these caveats, chromosome repatterning has been often thought as key player in the intraspecific genetic diversification and plant speciation, having impact on the establishing of reproductive isolation barriers between populations (Faria and Navarro 2010, Levin 2002, Rieseberg 2001).

The role of chromosome rearrangements in reproductive isolation has been described by the suppressed recombination model of speciation (Faria and Navarro 2010, Navarro and Barton 2003, Rieseberg 2001). This model explains the formation of reproductive isolation barriers through chromosome rearrangements impeding or inhibiting recombination. In the rearranged regions, alleles involved in reproductive isolation are accumulated which leads to increased levels of divergence (Faria and Navarro 2010). The recombinational speciation model is mainly based on studies in dipteran insect (e.g., Navarro and Barton 2003, Noor et al. 2001, Ranz et al. 2007) and sunflowers of the genus *Helianthus* (e.g., Gross and Rieseberg 2005, Lai et al. 2005, Rieseberg 2001).

Lexer and Widmer (2008) reviewed the studies of the reproductive isolation genetics in monkeyflower, sunflower, iris, poplar and campion. They concluded that except sunflower, chromosome rearrangements are of limited importance in the origin of reproductive isolation barriers in plants. Recently, however, Lowry and Willis (2010) showed an inversion to be associated with the morphological and flowering time differences between populations of the yellow monkeyflower (*Mimulus guttatus*), and thus contributing to the adaptation and reproductive isolation between the annual and perennial ecotypes of the species.

Besides sunflowers and monkeyflower, there are still limited data prooving the impact of chromosome rearrangements on reproductive isolation and speciation in plants which is probably caused by a limited number of comparative genetic maps in several congeneric species and technical impediments. Due to the feasibility of comparative chromosome painting in the mustard family, crucifers represent a superior plant group to address questions on the causal link between chromosome rearragements, intraspecific karyotypic variation, potential reproductive isolation barriers and speciation.

1.5 Variation in chromosome number

An organism is considered as euploid when somatic cells contain two complete sets of homologous chromosomes (diploid, 2n) and gametes have half of this number (haploid, n). The ploidy level of cells can vary by an increase in complete chromosome sets (polyploidy) or by gain or loss of individual chromosomes (aneuploidy).

Polyploids have been classified on the basis of genetic similarity. If a polyploid has the same genome in multiple copies, Stebbins (1947) has described it as an autopolyploid whereas if it has two distinctive genomes it has been described as an allopolyploid. Grant (1981) and others (Ramsey and Schemske 1998, 2002) proposed that autopolyploids arise within populations, whereas allopolyploids are the products of crossings between species.

Polyploidy is more common in plants than in animals (Mable 2004). It can be restricted only to a specific tissue or group of cells, i.e., endopolyploidy (e.g., trichomes in plants or salivary glands of insect). Polyploidy can arise from *i*) a spontaneous somatic chromosome doubling during mitosis, *ii*) a non-disjunction/non-reduction of homologous chromosomes during meiosis resulting in unreduced gametes, or *iii*) in animals through the multiple fertilization of a single egg (reviewed by Ramsey and

Schemske 2002). Polyploidy can also be artificially induced by treatment with drugs inhibiting cell division (e.g., colchicine, an inhibitor of a microtubule polymerization).

1.6 The role of polyploidy in plant genome evolution

Polyploidy (i.e., whole genome duplication, WGD) is broadly considered as one of the most important forces driving the genome evolution of plants. Athough polyploidy increases the cost of genome replication, it also generates evolutionary advantageous genetic diversity and may result in speciation events (Wood et al. 2009). Although one of the paralogues can be lost because deletions are generally tolerated in polyploid genomes, paralogous gene copies can acquire new functions. A plethora of research reports and reviews have reported on the importance of polyploidization events for generating physiological and morphological innovations, colonization of new ecological niches etc. (e.g., Soltis et al. 2009, Fawcett et al. 2009, Van de Peer et al. 2009). There is a compelling evidence that polyploidy may have caused the dramatic increase in species richness in several angiosperm lineages (Fawcett and Van de Peer 2010, Soltis et al. 2009, Wood et al. 2009). Polyploidy is probably also responsible for the evolutionary dominance of angiosperms over gymnosperms (Leitch and Leitch 2012).

WGD events are followed by gene and genome-wide fractionation (diploidization process). Diploidization includes various processes, such as genome downsizing, gene diversification and loss, activation of transposable elements, epigenetic changes, chromosome rearrangements and/or chromosome number reduction (descending dysploidy). These processes can be specific for each polyploid population, species or clade, and result in reproductive isolation and speciation (Soltis et al. 2009).

Perhaps all land plants have experienced one or several rounds of WGD events in their evolutionary history. The current view on the plant evolutionary history is based on phylogenomic analysis of sequenced plant genomes and several million expressed sequence tag (EST) sequences (Jiao et al. 2011, Zuccolo et al. 2011). Jiao et al. (2011) identified two ancient WGD events, one in the common ancestor of extant seed plants [c. 319 million years ago (mya)] and the other in the ancestor of extant angiosperms (c. 192 mya). The evolution of the eudicots is marked by the third polyploidization, the gamma (γ) whole-genome triplication (WGT) event. Recently, Jiao et al. (2012) and Vekemans et al. (2012) revisited the phylogenetic position of γ, and placed this event after the divergence of the Ranunculales and core eudicots, i.e., c.117 mya.

Based on the sequence analysis of *A. thaliana* and papaya (*Carica papaya*) genomes, two additional WGD events, known as alpha (γ) and beta (β), have been uncovered as being specific for the Brassicales. Whereas all Brassicales families share the γ WGT, the younger β WGD occurred only in the core Brassicales clade, after its divergence from Caricaceae (papaya) (Barker et al. 2009). Interestingly, the occurrence of the β coincides with the mass K-T extinction caused by catastrophic events, such as an asteroid impact and/or volcanic activity, c. 65 mya (Van de Peer et al. 2009). It is hypothesized that duplicated Brassicales genomes had a greater adaptive potential to survive and diversify in the changed environment after the K-T extinction (Beilstein et al. 2010, Franzke et al. 2011). The alpha (α) WGD event, specific to the Brassicaceae, occurred 43 to 23 mya (Barker et al. 2009, Beilstein et al. 2010, Couvreur et al. 2010, Fawcett et al. 2009). Although the timing of the α event is debated, its position at the base of the family tree suggests its importance for promoting the radiation of the Brassicaceae genera and species (Franzke et al. 2011). An independent WGT event (c. 14 mya) has been identified in the Cleomaceae, a sister family to Brassicaceae (Barker et al. 2009, Schranz and Mitchell-Olds 2006).

2 Chromosome painting

Fluorescence *in situ* hybridization (FISH) is a method of microscopic detection of specific genome sequences on chromosomes using fluorescently labelled nucleic acid probes complementary to the target sequence.

The term **chromosome painting** (CP) was introduced by Pinkel et al. (1988) to describe fluorescence *in situ* visualization of large chromosome segments or whole chromosome arms using sequence-specific probes. For CP in its original sense as applied to vertebrates and humans, probes are obtained from chromosomes or chromosome segments isolated either by flow sorting (i.e., separation of chromosomes in a suspension on the basis of their relative fluorescence using a flow cytometer/sorter) or microdissection (i.e., isolation of DNA from a particular chromosome type or its part using a fine microcapillary pipette). The isolated DNA is amplified by degenerate oligonucleotide primed-polymerase chain (DOP-PCR) reaction, fluorescently labeled, hybridized and visualized on chromosome preparations.

The repetitive sequences often prevent the identification of complex DNA sequences. Because of their non-specificity, these sequences hybridize with their corresponding sequences present in the genome (reviewed by Sharma and Sharma 2001). To achieve chromosome specificity of hybridization signals, dispersed repeats contained in the painting probe and target chromosomes have to be prevented from hybridization by blocking with excess of unlabelled total genomic DNA or DNA enriched for repetitive fraction. Therefore, the term **chromosomal *in situ* suppression** was used as an equivalent of chromosome painting (Lichter et al. 1988).

A broad spectrum of different CP-based techniques has been developed for applications in research and clinical diagnostics (Langer 2004). CP enables the identification of chromosomes and chromosome rearrangements (Blenow 2004), mutagenicity testing (Cremer et al. 1990), analysis of the chromosome organization in interphase (Cremer and Cremer 2001).

Comparative chromosome painting (CCP) is based on cross-species hybridization of painting probes. It allows identification of entire chromosomes and large-scale chromosome regions shared among related species, study of chromosome collinearity, and reconstruction of ancestral karyotype structures (Ferguson-Smith and Trifonov 2007, Svartman et al. 2004).

2.1 Chromosome painting in plants

Although CP is commonly used in animal and human cytogenetics, all attempts to establish CP in plants have failed. CP using flow-sorted or microdissected probes is not directly applicable in plants due to the high proportion of complex dispersed repeats evenly distributed throughout plant genomes (Schubert et al. 2001). Thus plants require painting strategies different from the protocols working in animals and humans.

Alternative painting strategy is FISH with pools of large-insert DNA clones (Bacterial and Yeast Artificial Chromosomes, BACs and YACs). The BAC-FISH approach to identify specific chromosome region is successful in plant groups with small genome and relatively low content of repeats, e.g., *Brachypodium* (Febrer et al. 2010, Gu et al. 2009, Ma et al. 2010), Brassicaceae (Lysak et al. 2001, chapter 3.2), rice (Jiang et al. 1995), sorghum (Figueroa et al. 2011), potato and tomato (Szinay et al. 2008, Tang et al. 2009).

The first large-scale painting of plant chromosomes was achieved by Lysak et al. (2001) in *Arabidopsis thaliana*. CP became feasible due to the *A. thaliana* genomic

resources and the specific organization of the repeats in its genome (chapter 3.2). In later studies, CP was also successfully applied to other Brassicaceae species (chapter 3.3). Comparative chromosome painting (CCP) allowed unprecedented analyses of the cruciferous genome evolution in this group at the chromosomal level (Lysak et al. 2006, 2010, Mandáková and Lysak 2008, Mandáková et al. 2010a, b, 2012, chapter 3.3).

Brachypodium distachyon from the family Poaceae is a model system for temperate cereals and grasses. Like *A. thaliana*, *B. distachyon* possesses numerous model attributes, including small genome and low chromosome number (Draper et al. 2001, Garvin et al. 2008). The *B. distachyon* BAC DNA libraries and bioinformatic data from the completed genome sequencing project (International Brachypodium Initiative 2010) made it possible to paint entire *B. distachyon* chromosomes. CCP with BAC pools from *B. distachyon* was successfully used to investigate phylogenetic relationships between different *Brachypodium* species and the mechanisms which shaped their karyotypes (Wolny and Hasterok 2009). However, it has not been determined whether *B. distachyon*-specific BAC pools could be used for CCP of grasses outside the genus (Idziak et al. 2011).

3 Comparative cytogenetics in the Brassicaceae

3.1 Family Brassicaceae

The mustard family (Brassicaceae, Crucifereae), comprising approximately 321 genera and 3660 species (Al-Shehbaz 2012), belongs to the largest plant families. The inconspicuously beautiful crucifer plants are recognised by their cruciform corolla, tetradynamous stamens and their characteristic siliques. Family-wide phylogenetic analyses by Beilstein et al. (2006) revealed three major, well supported phylogenetic clades named lineages I, II and III (Fig. 1). Al-Shehbaz et al. (2006) based on the phylogenetic study of Beilstein (2006) introduced phylogenetic tribal classification of the family in which 25 monophyletic tribes were recognized. Subsequent molecular phylogenetic studies further specified the limits of many genera and tribes (Al-Shehbaz 2012). Lineage I harboured eight tribes (Boechereae, Camelineae including *A. thaliana*, Cardamineae, Descurainieae, Halimolobeae, Lepidieae, Physarieae, and Smelowskieae), lineage II comprised five (Arabideae, Brassiceae, Isatideae, Schizopetaleae, and Sisymbrieae), and lineage III four tribes (Anchonieae, Chorisporeae, Euclidieae, and Hesperideae) (Al-Shehbaz 2006). The genus *Aethionema* was recognized as the tribe Aethionemeae sister to the rest of the family (e.g., Beilstein et al. 2006, Al-Shehbaz et al. 2006).

With the doubling of the number of tribes in five years (German 2010, German and Al-Shehbaz 2010, Warwick et al. 2010, Franzke et al. 2011, Al-Shehbaz et al. 2011, Koch et al. 2012), lineage I includes 14 tribes (by the addition of Alyssopsideae, Crucihimalayeae, Erysimeae, Microlepidieae, Oreophytoneae, and Yinshanieae), and lineage II is expanded to include the recently recognized Stevenieae and all of the remaining 25 tribes of the family minus the Biscutelleae. Lineage III now includes seven tribes (by the addition of Anastaticeae, Buniadeae, and Dontostemoneae) which remain as part of the basal polytomy of the family (Fig. 1) (Franzke et al. 2011).

Before *A. thaliana*, the interest in the mustard family was focused primarily on its economically important genera such as *Brassica* (oilseed rape, cabbage, cauliflower, broccoli), *Raphanus* (radish), *Armoracia* (horse-radish), *Nasturtium* (water-cress), *Eutrema* (wasabi), and *Eruca* (salad rocket). For a long time, *Brassica* was the prime genus of crucifer cytogenetics. However, due to the small genome size and rapid life cycle, *A. thaliana* has been selected as a model organism and its genome has been sequenced (Arabidopsis Genome Initiative 2000, Koorneef et al. 2003). This revolutionized plant experimental biology and stimulated the interest in the genome analysis of other crucifer species. The *A. thaliana* genome is currently one of the most intensively studied genomes and many breakthrough findings came from the *A. thaliana* research (reviewed by Koorneef et al. 2003).

Although *A. thaliana* has been neglected by plant cytogeneticists for its tiny chromosomes, it has become a pre-eminent model in plant cytogenetics later on. It is due to the invent of FISH, substitution of the small mitotic chromosomes by extended meiotic chromosomes at the pachytene stage, availability of chromosome-specific BAC libraries, and intergration of cytogenetic data with genetic and whole-genome sequence data (Lysak et al. 2001).

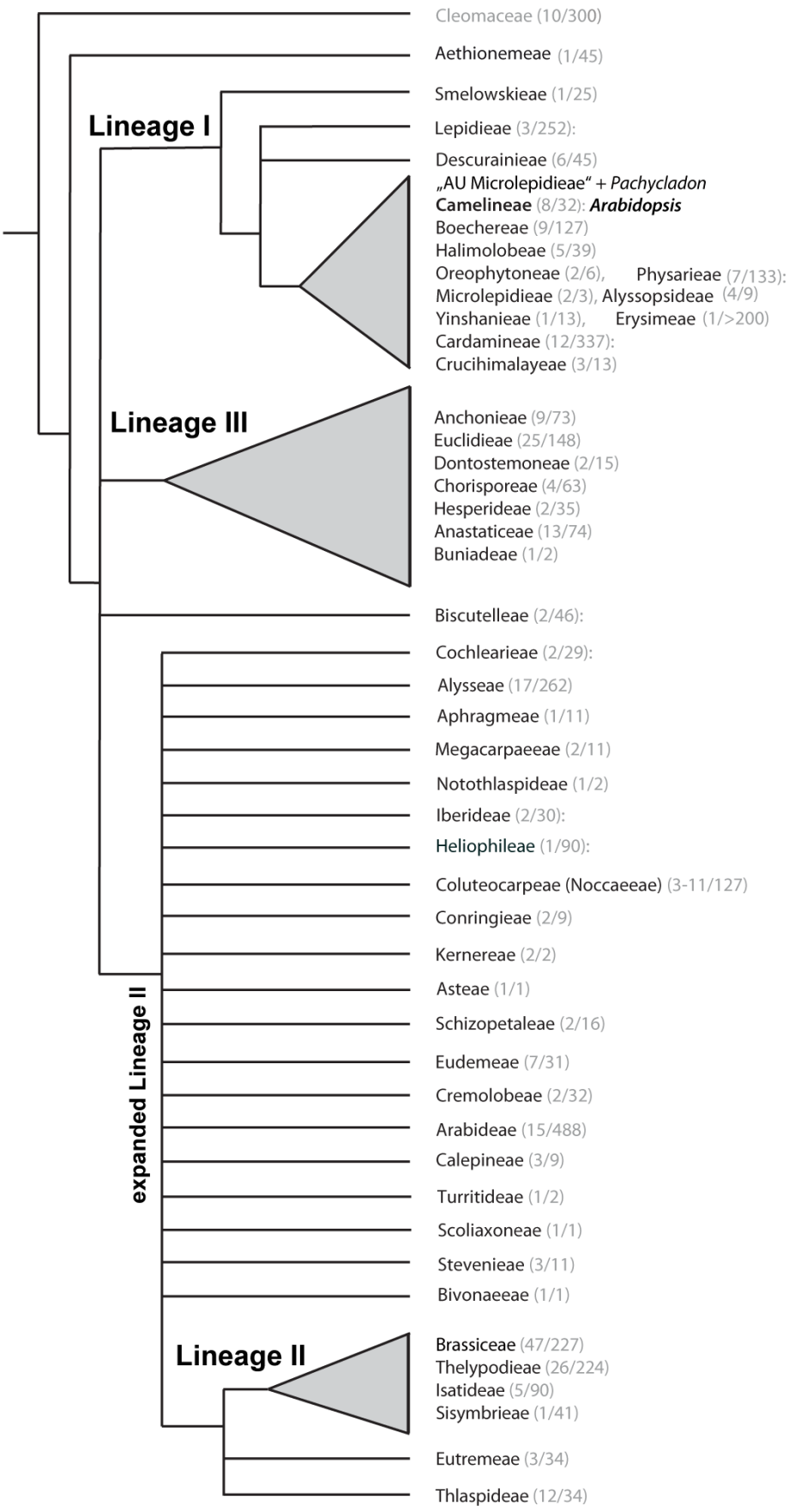


Fig. 1 The Brassicaceae family tree with the main lineages and tribes (from Franzke et al. 2011). Grey numbers in the brackets represent number of genera/species in the individual tribes.

3.2 Chromosome painting in *Arabidopsis thaliana*

The feasibility of CP in plants has dramatically changed when the *A. thaliana*-related genomic resources have become available. *A. thaliana*, like most Brassicaceae species, is favored for chromosome painting because of its small genome size, low amount of repetitive DNA (c. 15%) clustered mainly in the pericentromeric regions, low chromosome number (n = 5), and the public availability of chromosome-specific BAC libraries (Arabidopsis Genome Initiative 2000).

CP in *A. thaliana* is based on the method previously applied to paint chromosomes of yeast (Scherthan et al. 1992). CP in *A. thaliana* involves labelling of chromosome-specific repeat-free individual BAC clones followed by FISH. Typically, overlapping and differently labeled BAC clones are pooled and simultaneously hybridized to chromosome preparations (Lysak et al. 2001, Schubert et al. 2001, Lysak and Mandáková 2013). As the exact position of BAC clones on chromosomes is known, these can be combined and differently labelled according to a required experimental scheme. Chromosomes and nuclei at different developmental stages can be painted in this way, however, the breakthrough was accomplished by the application of extended pachytene chromosomes, providing high-resolution painting signals (Lysak et al. 2001).

A. thaliana chromosome 4 became the first entirely painted plant chromosome using different fluorochromes and chromosome-specific BAC contigs as painting probes (Lysak et al. 2001). Subsequently, all five *A. thaliana* chromosomes were painted, and *A. thaliana* became the first plant with an entirely painted karyotype (Pecinka et al. 2004, Lysak et al. 2006). Application of CP in *A. thaliana* includes chromosome structure analyses as well as studies of chromosome organization in interphase nuclei. CP represents a tool for study of intra- and interchromosomal rearrangements such as inversions and transloations (e.g., inversion after T-DNA insertion, Pecinka et al. 2005; a paracentric inversion identified in Shahdara × Columbia hybrid of *A. thaliana*, Lysak and Mandáková 2013). In interphase cytogenetics, CP enables the analysis of the spatial structure and association of individual chromosome territories (Berr et al. 2006, Berr and Schubert 2007, Pecinka et al. 2004, 2005).

3.3 Principles of comparative chromosome painting in the Brassicaceae

CCP within the Brassicaceae takes advantage of the established multicolor chromosome painting in *A. thaliana*, available phylogenetic information, and comparative genetic maps developed for several crucifer species (e.g., *A. thaliana*, *A. lyrata*, *Brassica rapa*, *Capsella rubella*). CCP in the Brassicaceae is based on cross-hybridization of *A. thaliana* chromosome-specific BAC contigs to chromosomes of other crucifer species (Lysak et al. 2006, Mandáková and Lysak 2008, Mandáková et al 2010a, b, 2012). Although CCP using other than *A. thaliana* BACs was tested and successfully established in crucifer species other than *A. thaliana* (Lysak et al. 2010), *A. thaliana* BACs are still first-choice painting probes for CCP in Brassicaceae because they have many advantages compared to those of other species. *A. thaliana* BAC clones are assembled on chromosomes, entire BAC libraries are available to the public, and more importantly, the evolutionary young *A. thaliana* genome contains only limited amount of repetitive sequences. The absence of the ancestral repeats enables the CCP with hight number of BACs.

CCP in the mustard family allows the identification of large-scale homeologous chromosome regions and entire chromosomes in different species, to reveal the extent of cross-species chromosomal homeology (chromosome collinearity), to identify chromosome rearrangements, to elucidate evolutionary mechanisms underlying the

extant karyotypic variation, and to acquire phylogenetically informative cytogenetic signatures (Lysak et al. 2006).

3.4 Karyotype evolution in the Brassicaceae

3.4.1 Concept of the Ancestral Crucifer Karyotype

To determine the evolutionary progression of karyotype alterations, it is necessary to distinguish ancestral from derived karyotypes.

The comparative genetic mapping between three species from the tribe Camelineae, *A. thaliana* (n = 5), *A. lyrata* (n = 8, Kuittinen et al. 2004), and *Capsella rubella* (n = 8, Boivin et al. 2004), revealed highly conserved genome structure shared between *A. lyrata* and *C. rubella*, and a relatively high level of chromosome collinearity between *A. thaliana* and the two n = 8 species (Koch and Kiefer 2005). Comparative genetic and cytogenetic analyses also showed that the compact *A. thaliana* genome is characterized by a highly reshuffled and derived karyotype (Boivin et al. 2004; Kuittinen et al. 2004; Koch and Kiefer 2005; Lysak et al. 2006), making this species inappropriate as a reference point for comparative studies across Brassicaceae (Schranz et al. 2006).

The overall similarity between karyotypes of *A. lyrata* and *C. rubella* and the fact that n = 8 is the most common chromosome number found in Camelineae as well as across Brassicaceae (Warwick and Al-Shehbaz 2006), resulted in the concept of a hypothetical Ancestral Crucifer Karyotype (ACK) with eight chromosomes and genome resembling that of *A. lyrata* and *C. rubella* (Lysak et al. 2006, Schranz et al. 2006).

The ACK concept was further expanded by defining apparently conserved genomic blocks (GBs) which make up individual ancestral chromosomes (Schranz et al. 2006). Conserved ancestral blocks were revealed through inter-specific genetic mapping between *A. thaliana* and *Brassica napus*. Parkin et al. (2005) identified a minimum of 21 conserved chromosomal segments within the *A. thaliana* genome, which can be duplicated and rearranged to build up the allopolyploid genome of *B. napus*. These chromosomal blocks were largely identical with the collinear chromosomal segments revealed by comparative genetic and cytogenetic mapping as shared between *A. thaliana*, *A. lyrata*, *C. rubella*, and other Camelineae and Descurainieae species (Boivin et al. 2004, Kuittinen et al. 2004, Lysak et al. 2006). These data have been integrated with Parkin et al.'s blocks into a set of 24 conserved genomic blocks (A to X) building up the eight AK chromosomes (Schranz et al. 2006; Fig. 2).

The ACK karyotype with 24 ancestral genomic blocks has been successfully adopted as an ancestral reference genome in comparative genomics and cytogenomics studies in the Brassicaceae (e.g., Ma et al. 2012, Mandáková et al. 2010a, b, 2012, Mandáková and Lysak 2008, Nelson et al. 2011, Schranz et al. 2007, Wang et al. 2011).

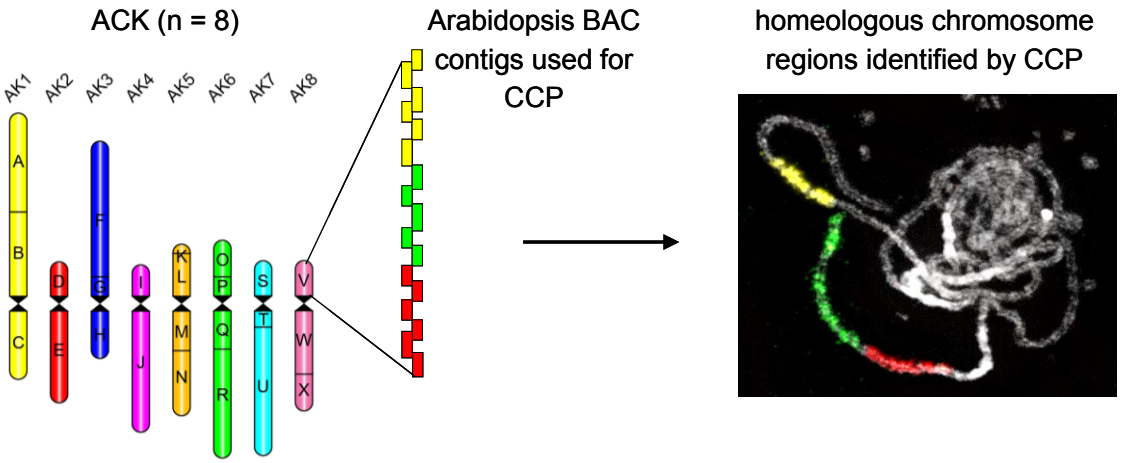


Fig. 2 Ancestral Crucifer Karyotype (ACK) comprising eight chromosomes (AK1-8) and 24 conserved genomic blocks (A-X) has been proposed by Lysak et al. (2006) and Schranz et al. (2006). The level of chromosome collinearity shared between the ACK and karyotypes of extant crucifer species is explored by comparative chromosome painting (CCP). Fluorescently labeled *A. thaliana* BAC contigs arranged according to ancestral genomic blocks are hybridized to pachytene chromosomes of a crucifer species.

3.4.2 Karyotype evolution in species from the Brassicaceae lineage I and II

The concept of the ACK has been for the first time applied in a multicolour CCP analysis of species from Brassicaceae lineage I with presumably reduced chromosome numbers from n = 8 towards n = 7, 6 and 5 (Lysak et al. 2006).

This study elucidated the evolution of the A. thaliana karyotype marked by the reduction of chromosome number from n = 8 towards n = 5 (Fig. 3) through three reciprocal translocation-mediated chromosome fusions and at least three inversions. Although belonging to the same genus as the extremely reshuffled *A. thaliana*, the karyotype of *A. lyrata* (n = 8), has not undergone the process of descending dysploidy. This demonstrates that karyotype dynamics in some Brassicaceae species contrasts with the stability in the others.

In species for which no genetic data were available, Neslia paniculata (n = 7, Camelineae), Turritis glabra (n = 6, Turritideae) and Hornungia alpina (n = 6, Descurainieae), CCP revealed largely preserved chromosomal colinearity between these species, *A. thaliana* and ACK. Despite some inversion events, six homeologous chromosomes in *N. paniculata*, and four in *H. alpina* and *T. glabra* resembled the structure of ancestral chromosomes (Fig. 3). Although some ancestral chromosomes participated in chromosome fusions more often than others, the chromosome fusion events were species-specific (Lysak et al. 2006). These data suggested that chromosome reductions from the ACK to evolutionary derived karyotypes with n = 7, 6 and 5 occurred independently and recurrently, and confirmed the ACK as a probable ancestral karyotype of lineage I (Fig. 3).

Subsequently, karyotype evolution in the expanded lineage II and closely related tribes has been investigated by Mandáková and Lysak (2008). Tribes of expanded lineage II are characterized by karyotypes based on n = 7. Hence, question on the modes of karyotype evolution leading to the same chromosome numbers (2n = 14/28) in different species and tribes has been addressed by means of CCP. This study includes eight species from six different tribes; *Calepina irregularis* and *Goldbachia laevigata* (Calepineae), *Conringia orientalis* (Conringieae), *Glastaria glastifolia* (Isatideae), *Noccaeae caerulescens* (Coluteocarpeae), *Ochthodium aegyptiacum* (Sisymbrieae), and *Thellungiella salsuginea* (Eutremeae). All analyzed species shared a complex structure of two translocation chromosomes resulting from recombination involving three

ancestral chromosomes. This represents a characteristic cytogenetic signature proving the existence of a common n = 7 ancestor named Proto-Calepineae Karyotype (PCK; Fig. 3 and 4). The PCK remained preserved in tribes Calepineae and Conringieae, and has become modified by secondary inversions in Coluteocarpeae. An additional translocation involving one PCK-specific translocation chromosome and AK2-like chromosome, resulted in the origin of the evolutionary younger translocation PCK (tPCK) genome in Isatideae, Sisymbrieae and Eutremeae (Mandáková and Lysak 2008). As the PCK shares five chromosomes and two chromosome arms with the ACK, it has been suggested that either the PCK and ACK are descended from a common ancestor or, more likely, that the seven PCK chromosomes are derived from the eight chromosomes of the ACK (Mandáková and Lysak 2008).

The tPCK was also tentatively suggested as an ancestral karyotype of the tribe Brassiceae (Mandáková and Lysak 2008). The released sequence of the triplicated *Brassica rapa* genome (chapter 3.4.3) allowed us to re-evaluate the genome evolution in the extant diploid *Brassica* species. We demonstrated that all three *B. rapa* paleogenomes resembled the n = 7 ancestral tPCK genome with seven chromosomes (Cheng at al., submitted).

Recently, genomes of *Schrenkiella* (*Thellungiella*) *parvula* (Dassanayake et al. 2011) and *T. salsuginea* (Wu et al. 2012) have been sequenced. The comparative cytogenetic map of *T. salsuginea* reconstructed by Mandáková and Lysak (2008) facilitated the assembly of *S. parvula* sequence clusters into its seven chromosomes. The tPCK genome structure was confirmed by *S. parvula* whole-genome sequencing. This work demonstrated the accuracy of comparative cytogenetic maps, and the importance of cytogenetics and cytogenomics in the era of genome assembly projects, particularly in species for which no genetic maps are available. Similarly, comparative cytogenetic maps of *Arabis alpina* and *Leavenworthia alabamica* will represent a basis for whole-genome assemblies (Mandáková and Lysak, unpublished data).

Considering 3,660 species in the mustard family, only negligible number of genomes has been reconstructed so far. No data on genome structure are currently available for lineage III species. However, some preliminary conclusions on karyotype evolution in lineage I and II could be drawn. The increasing body of evidence suggests the ACK with eight chromosomes and 24 genomic blocks is an ancestral genome of the lineage I and perhaps of the whole family. The PCK with seven chromosomes was identified as the ancestral genome of several tribes classified as or associated with expanded lineage II including the economically important tribe Brassiceae. The seven chromosomes of PCK were most likely derived from the eight chromosomes of the ACK (Fig. 3 and 4). Assuming the α WGD event at the base of the Brassicaceae family, the ACK genome itself most likely originated from a proto-genome with n = 4 via this genome duplication (Henry et al. 2006). Mechanisms of descending dysploidy from the ancestral n = 8 towards lower chromosome numbers are discussed in chapter 3.4.4.

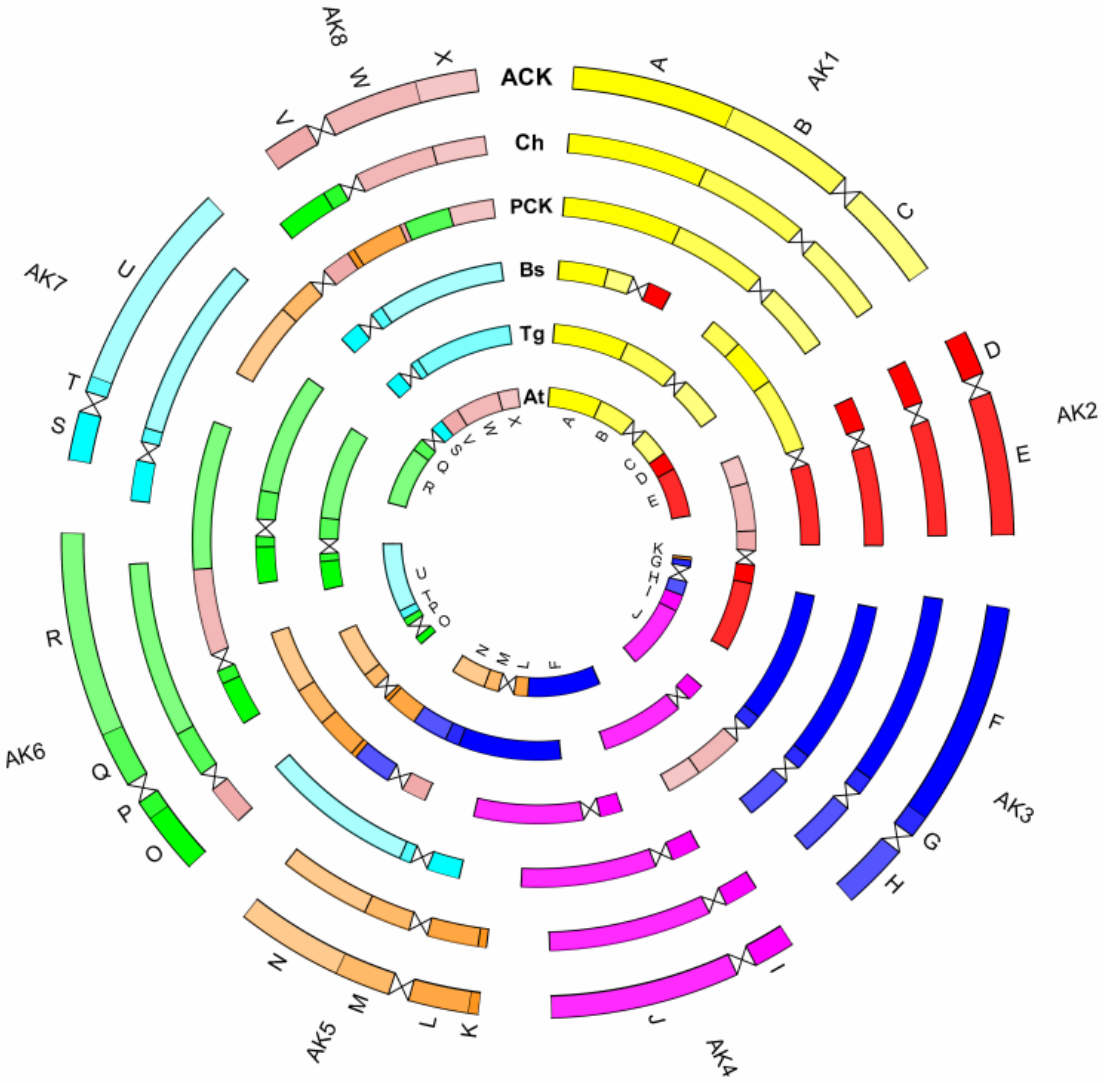
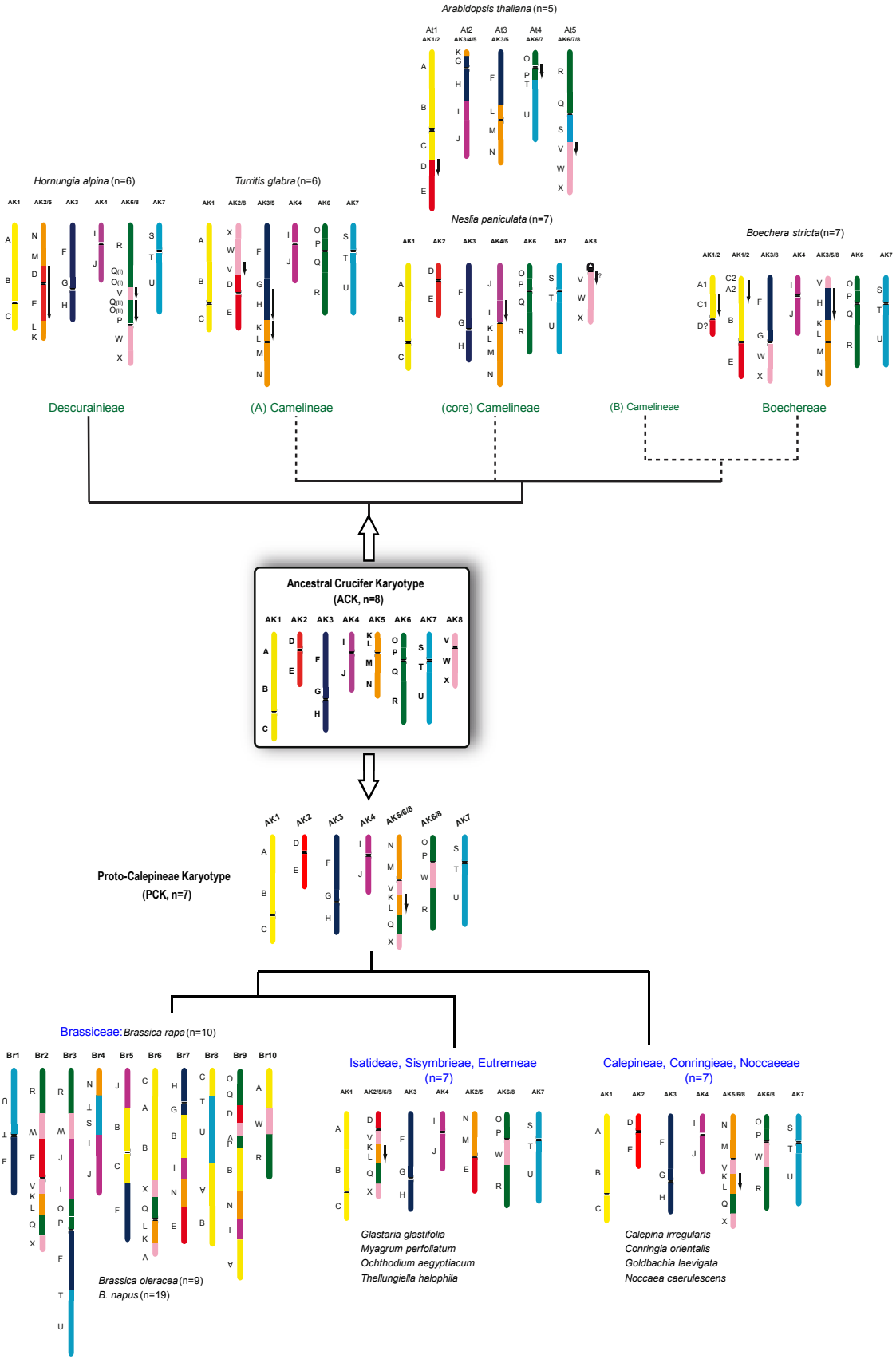


Figure 3 The Cabagge Circle displaying collinear relationships between chromosomes of ACK (n = 8; Schranz et al. 2006), PCK (n = 7; Mandáková and Lysak 2008), and the modern karyotypes of *Cardamine hirsuta* (n = 8, Cardamineae), *Boechera stricta* (n = 7, Boechereae), *Turritis glabra* (n = 6, Turritideae), and *A. thaliana* (n = 5, Camelineae). Based on Lysak et al. (2006), Mandáková and Lysak (2008), and unpublished data of T.M.

Figure 4 (next page) A tentative scenario of karyotype evolution in the Brassicaceae assuming the ACK being ancestral for taxa of lineage I and II. Lineage II genomes descended from ACK via a common intermediate PCK. The 24 genomic blocks are indicated by A-X and colored according to their position on chromosomes AK1-AK8 of the ACK (Schranz et al. 2006). Downward-pointing arrows indicate the opposite orientation of genomic blocks compared with the position in the ACK. Based on Lysak et al. 2006, Mandáková and Lysak 2008, Parkin et al. 2005, and unpublished data of T. M.; adopted from Lysak and Koch (2011).


Arabidopsis thaliana (n=5)
Hornungia alpina (n=6)
Turritis glabra (n=6)
Neslia paniculata (n=7)
Boechera stricta (n=7)
Descurainieae
(A) Camelineae
(core) Camelineae
(B) Camelineae
Boechereae
Ancestral Crucifer Karyotype
(ACK, n=8)
Proto-Calepineae Karyotype
(PCK, n=7)
Brassiceae: Brassica rapa (n=10)
Brassica oleracea (n=9)
B. napus (n=19)
Isatideae, Sisymbrieae, Eutremeae
(n=7)
Glastaria glastifolia
Myagrum perfoliatum
Ochthodium aegyptiacum
Thellungiella halophila
Calepineae, Conringieae, Noccaeeae
(n=7)
Calepina irregularis
Conringia orientalis
Goldbachia laevigata
Noccaea caerulescens

3.4.3 Mesopolyploid evolution in the Brassicaceae

When polyploidization has occurred recently in evolutionary time, the corresponding chromosome number multiplication is evident (neopolyploidy, Fig. 5). Ancient polyploid WGD events (paleopolyploidy; e.g., α, β and preceding WGDs) are masked by an extensive genome diploidization and can be revealed only by a bioinformatic search for orthologous and paralogous sequences (Fig. 5). Ancient polyploidization followed by diploidization including chromosome number reduction but still detectable by comparative genomics and/or chromosome painting has been recently described as **mesopolyploidy** (Mandáková et al. 2010a, Fig. 5).

Genetically diploid species with chromosome numbers as low as n = 4, 5, or 6 have never been suspected to be ancient polyploids on the road to genome diploidization. However, during a few recent years, several diploid-like crucifer species were shown to have experienced mesopolyploid whole-genome duplications or triplications postdating the three paleopolyploid WGDs (α, β and γ). Due to the feasibility of CCP, mesopolyploid WGD events were detected in several crucifer taxa containing diploid-like species, namely in Brassiceae (Lysak et al. 2005), Australian and New Zealand Microlepidieae (Mandáková et al. 2010a, b), Heliophileae (Mandáková et al. 2012), and other tribes (Mandáková and Lysak, unpublished data).

The first characterized mesopolyploid event was the whole-genome triplication found at the base of the tribe Brassiceae through comparative genetic mapping (Parkin et al. 2005), cytogenetics (Lysak et al. 2005, 2007), and sequence genomics (Wang et al. 2011). Lysak et al. (2005, 2007) by analyzing several ancestral genomic blocks in eleven Brassiceae species spaning the entire range of chromosome numbers (from n = 7 to n = 34) gained compelling evidence for a tribe-specific triplication event. An independent mesotetraploid WGD has been identified in *Orychophragmus violac*eus, a species being frequently assigned to Brassiceae (Lysak et al. 2007).

Later on, two mesotetraploid events were inferred to occur in two groups from the tribe Microlepidieae endemic to Australia and New Zealand, respectively (Mandáková et al. 2010a, b, Fig. 6). CCP analysis unexpectedly revealed that genomes of Australian species (*Stenopetalum nutans*, *S. lineare*, and *Ballantinia antipoda*) with low chromosome numbers (n = 4, 5, and 6, respectively) descended from the eight ancestral chromosomes of the ACK through an allopolyploid WGD event followed by the species divergence and species-specific genome diploidization (Mandáková at al. 2010a). Nuclear and maternal gene phylogenies corroborated the hybrid origin of the mesotetraploid ancestor and suggested that the WGD occurred c. 6 to 9 mya. Similarly, the ancestral genome of ten *Pachycladon* species (all with n = 10) has been reconstructed and suggested to originate through a mesopolyploid event involving two ACK-like genomes. The diversification of *Pachycladon* species was estimated to occur c. 1 to 2 mya (Mandáková et al. 2010b). The two, presumably independent, allopolyploid WGD events were followed by diploidization process towards diploid-like genomes, with the less extensive genome repatterning in the evolutionary younger genus *Pachycladon*. The duplicated ancestral chromosome number (n = 16) was reduced towards n = 10 and n = 6, 5, 4 in *Pachycladon* and Australian Brassicaceae species, respectively. Chromosome number was reduced via chromosome rearrangements typically including peri- and paracentric inversions and reciprocal whole-arm translocations mediating centromere losses. Alternatively, translocations between chromosome ends of two non-homologous chromosomes led to the origin of fusion chromosomes with two centromeres, one being inactivated and/or deleted (Mandáková et al. 2010a, b, see chapter 3.4.4). Such dramatic rearrangements reshuffled the parental genomes and genomic blocks building them. Consequently,

extant diploidized genomes are built up as complex mosaics of duplicated genomic blocks (Fig. 6). In some *Stenopetalum* species (n = 8, 10), additional (neopolyploid) genome duplications have been identified.

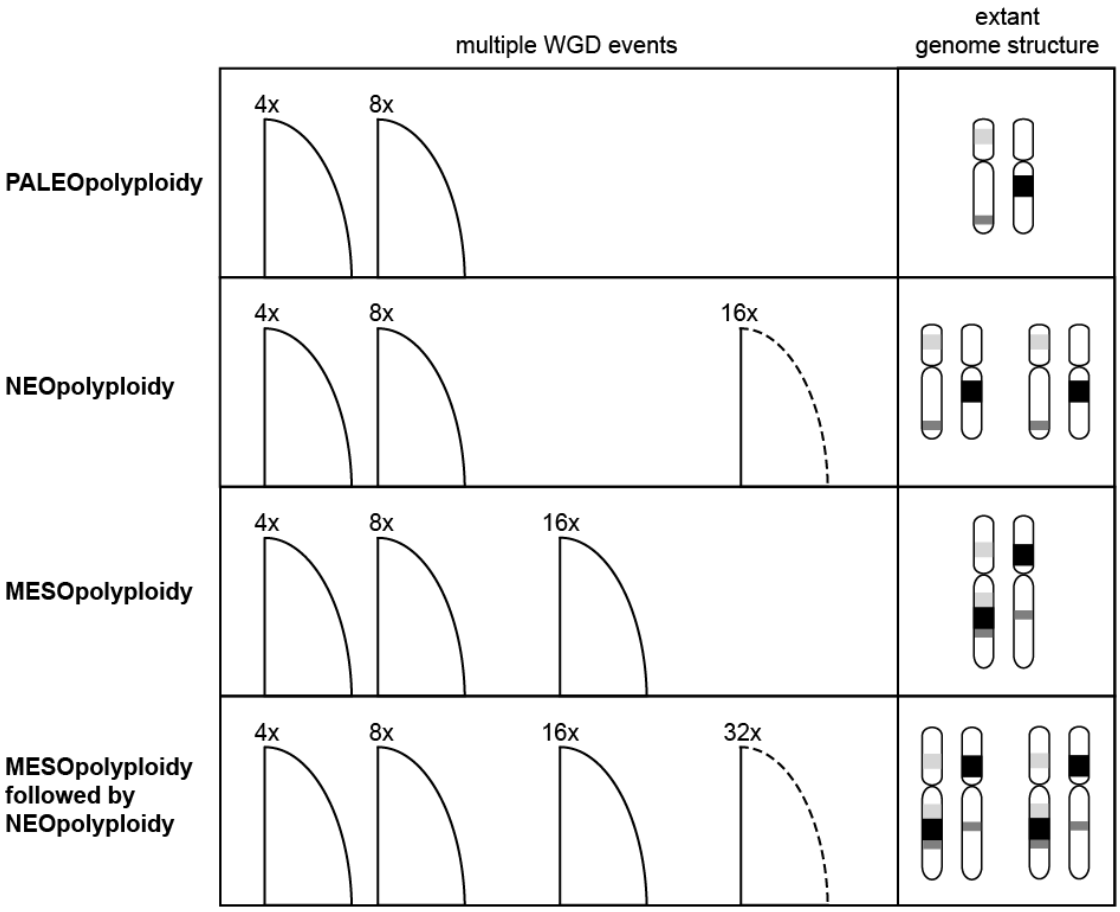


Fig. 5 A model of genome evolution through multiple WGD events (Mandáková et al. 2010a). Subsequent WGDs of different age shown as ploidy level increases (4x to 32x) are followed by genome diploidization towards diploid-like genomes. Chromosomes on the right represent duplicated and diploidized complements with three ancestral genomic blocks (GB) as revealed by comparative genetic and cytogenetic techniques. In paleopolyploids (e.g., *A. thaliana*, n = 5), paralogous regions are not detectable by (cyto)genetic analysis. In neopolyploids (e.g., *A. suecica*, n = 13), chromosome number is not reduced and most duplicated GBs are not reshuffled yet. In mesopolyploids (e.g., *Stenopetalum nutans*, n = 4), descending dysploidy results in diploid-like number of mosaic-like chromosomes and duplicated GBs are reshuffled by intra- and intergenomic rearrangements. The mesopolyploid genome experiences additional neopolyploidy (e.g., *S. velutinum*, n = 8).

Recently, seven *Heliophila* species from the endemic South African tribe Heliophileae possessing different diploid-like chromosome numbes (n = 8-11) and phylogenetic position within the tribe have been analyzed by CCP (Mandáková et al. 2012). In all species analyzed, 90% of painting probes unveiled three homeologous chromosome regions in *Heliophila* haploid chromosome complements. These results suggest that all the analyzed species, and probably the entire tribe Heliophileae, experienced a WGT event. As in Australian and New Zealand mesopolyploids, also WGT in *Heliophila* has been followed by species-specific chromosome rearrangements resulting in the extant diploid-like genomes with diverse chromosome numbers. More recent neopolyploid events in Heliophileae are reflected by increased chromosome

numbers (2n = 32-88). This study showed polyploidy as a potential major factor responsible for the diversification and species radiation in the species-rich tribe Heliophileae.

The incidence of successive WGD events in several crucifer lineages allowed us to postulate the WGD/diploidization cyclic model (Fig. 6).

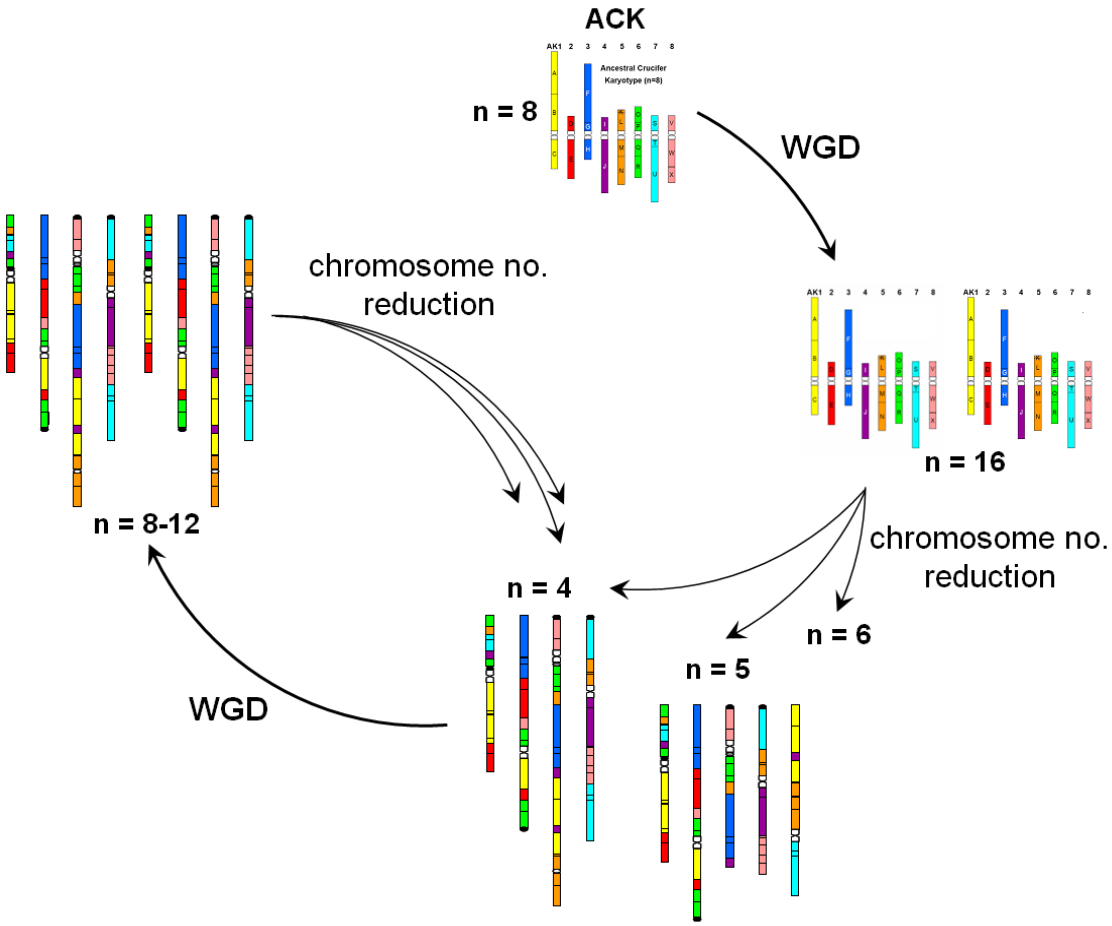


Fig. 6 Model of genome evolution in the Brassicaceae shaped by cyclic whole-genome duplication and diploidization events. A common paleopolyploid ancestor of the family, the ACK (n =8) experienced lineage-specific WGD event. The chromosome number of the tetraploid ACK-like karyotype (n = 16) was subsequently reduced during the process of diploidization towards genomes with diploid-like chromosome numbers (n = 6, 5, 4) and mosaic-like structure of the fusion chromosomes. Mesopolyploid karyotypes can undergo additional WGD and diploidization.

In Brassicaceae, several genera and tribes are polybasic (Fig. 7). Based on our published studies and preliminary data, we hypothesized that polybasic taxa have undergone mesopolyploid WGDs followed by extensive genome diploidization. From polybasic tribes listed in Fig. 7, WGDs have been already reported to occur in Australian and New Zealand Microlepidieae, Brassiceae and Heliophileae (Lysak et al. 2005, 2007, Mandáková et al. 2010a, b, 2012). Additional evidence of mesopolyploid WGD was revealed by CCP in the tribes Biscutelleae, Cardamineae, Cochlearieae, Erysimeae, Iberideae and Physarieae (Fig. 8, Mandáková and Lysak, unpublished data). These data support our hypothesis of a direct link between genome duplication events and the variation in basic chromosome numbers.

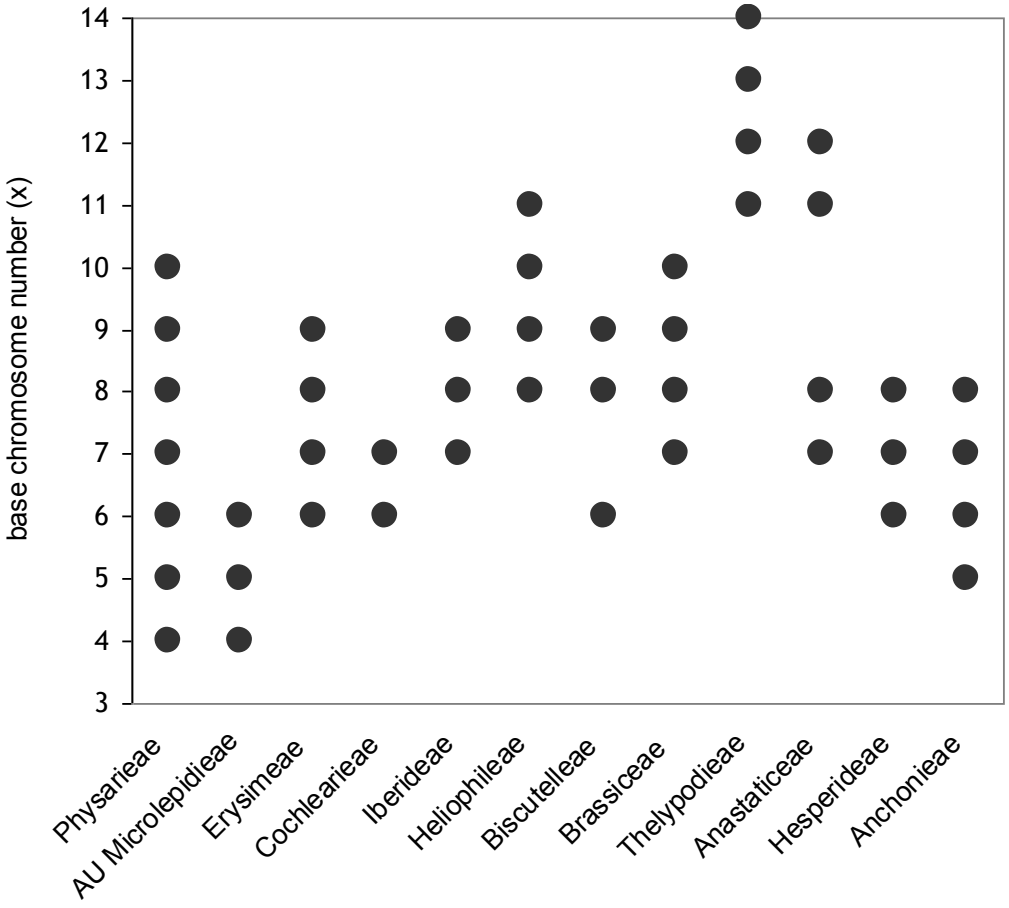


Fig. 7 Selected polybasic cruciferous tribes based on Warwick and Al-Shehbaz (2006).

The available data on mesopolyploid events across Brassicaceae are sparse and yet insufficient to draw general conclusions about the frequency of lineage-specfic genome duplications and their long-term impact on crucifer genome evolution. However, recently uncovered mesopolyploid events *i*) indicate that ancient polyploidy events are more frequent than previously thought, *ii*) highlight the importance of multiple whole-genome duplication events in the angiosperm genome evolution, *iii*) elucidate diploidization mechanisms transforming polyploid into quasi-diploid genomes, and *iv*) demonstrate that chromosome number *per se* is not a reliable indicator of ploidy level.

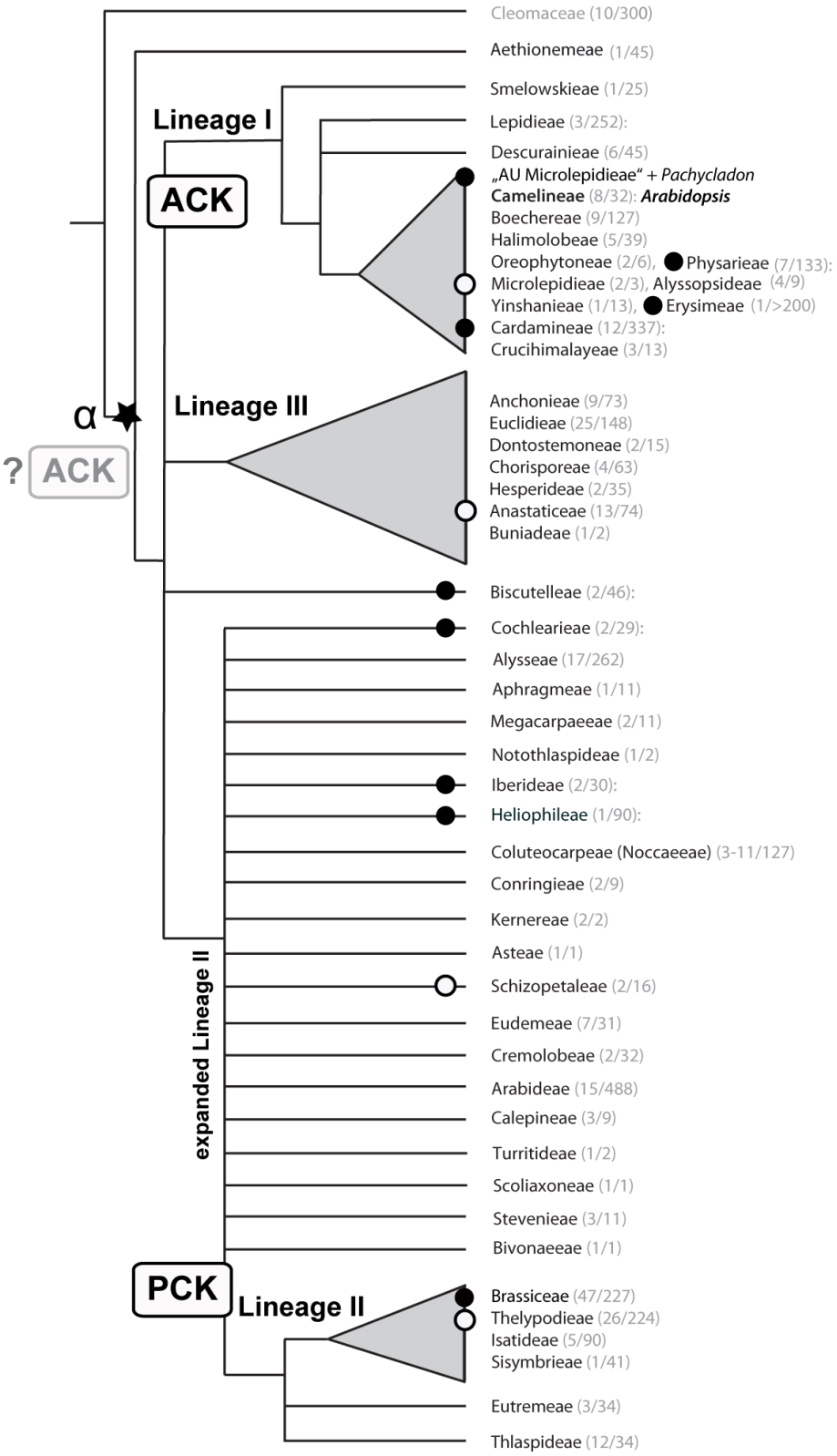


Fig. 8 The Brassicaceae family tree, main lineages and tribes (from Franzke et al. 2011). Published or verified and purported tribe-specific mesopolyploid duplications are indicated as black and empty circles, respectively. Grey numbers in the brackets represent number of genera/species in the individual tribes (based on data from Lysak et al. 2005, 2007; Mandáková et al. 2010a, b, 2012; Mandáková and Lysak, unpublished data).

3.4.4 Mechanisms of chromosome number reduction in the Brassicaceae

Uncovering the genetic mechanisms responsible for the descending dysploidy (i.e., reduction of the chromosome number) has been one of the most chalenging tasks of comparative cytogenetics in the Brassicaceae.

The **pericentric inversion - reciprocal translocation mechanism** involves a pericentric inversion which moves the centromere of the (sub)metacentric chromosome towards the chromosome end, creating an acrocentric chromosome. A reciprocal translocation occurs between the centric end of the acrocentric and a subtelomeric region of another chromosome and results in the origin of a large fusion chromosome and small minichromosome. It is hypothesized that the minichromosome is free of essential genes, meiotically unstable, and hence eliminated (Fig. 9A). This Robertsonian-like translocation eliminating one ancestral centromere and decreasing chromosome number has been described for several crucifer species (Lysak et al. 2006, Mandáková and Lysak 2008, Mandáková et al. 2010a, b,) and represents a common mechanism of descending dysploidy in the Brassicaceae. Karyotypes are reshuffled independently in different clades, however, an apparent trend can be observerd in ancestral chromosomes AK5, AK8, and AK6 being more frequently involved in rearrangements than the other ancestral chromosomes (described for taxa of lineage I and II; Lysak et al. 2006, Mandáková and Lysak 2008, Mandáková et al. 2010a, b)
Mandáková et al. (2010a, b) identified several ancestral chromosomes within the mosaic-like chromosomes in the mesopolyploid Australian and New Zealand Microlepidieae species. Ancestral associations of GBs in the karyotypes exhibit preserved collinearity corresponding to the structure of ancestral chromosomes. It cannot be ruled out that the ancestral associations of GBs were generated by the mechanism of a pericentric inversion-reciprocal translocation event but involving an additional inversion (Lysak et al. 2006, Schubert 2007). However, the occurrence of entire ancestral chromosomes within the composite chromosomes might more probably result from tandem **end-to-end fusion of two chromosomes**, i.e., end-to-end translocation with the breakpoints within the subtelomeric chromosome regions, producing a dicentric chromosome. Subsequent **centromere inactivation** and/or loss via recombination ensures regular meiotic segregation and stabilizes the composite chromosome (Fig. 9B).

A similar telomere-to-telomere translocation has been assumed for an origin of a large metacentric chromosome of ants (Imai and Taylor 1989). Several cases of inactivated ancestral centromeres were reported in mammalian species (e.g., Ferreri et al. 2005, Ventura et al. 2007), and in the human chromosome 2 (Ijdo et al. 1991). Only a few examples of centromere inactivation are known in plants, including dicentric chromosomes of Trititiceae (Luo et al. 2009), maize B chromosomes (F. Han et al. 2006), and chromosomes of two cucurbit species (Y. Han et al. 2009).

Besides Australian and New Zealand mesopolyploid Brassicaceae (Mandáková et al. 2010a, b), end-to-end chromosome fusions were not previously described in the family. Assuming an alternative scenario to explain the extant karyotype structures, centromere inactivation of AK4 can be suggested for the origin of chromosome At2 in *A. thaliana* (Lysak et al. 2006, Hu et al. 2011), centromere inactivation of AK5 for the origin of Bst5 in *Boechera stricta* (Schranz et al. 2007), and chromosome AK4/5 in *Neslia paniculata* (Lysak et al. 2006). Recently, new examples of putative paleocentromere inactivation were revealed in *Arabis alpina* (Arabideae; Mandáková and Lysak, unpublished data) and *Cardamine rivularis* (Cardamineae; Mandáková et al., submitted). Putting the evidence of paleocentromere inactivation in the Brassicaceae

together, centromere of the ancestral chromosomes AK5, AK3 and AK4 (in this order) seem to be inactivated preferentially.

Except one heterochromatic knob in *Stenopetalum nutans* (Mandáková et al. 2010a), no heterochromatin was observed at sites of presumably inactivated centromeres in the Brassicaceae species (Mandáková et al. 2010a, b). Illegitimate recombination between (peri)centric repeats is supposed to gradually remove the repeats and heterochromatin from those regions (Ventura et al. 2004). Similarly, centromere inactivation accompanied by the loss of heterochromatin has been reportedted in cucumber (Y. Han et al. 2009).

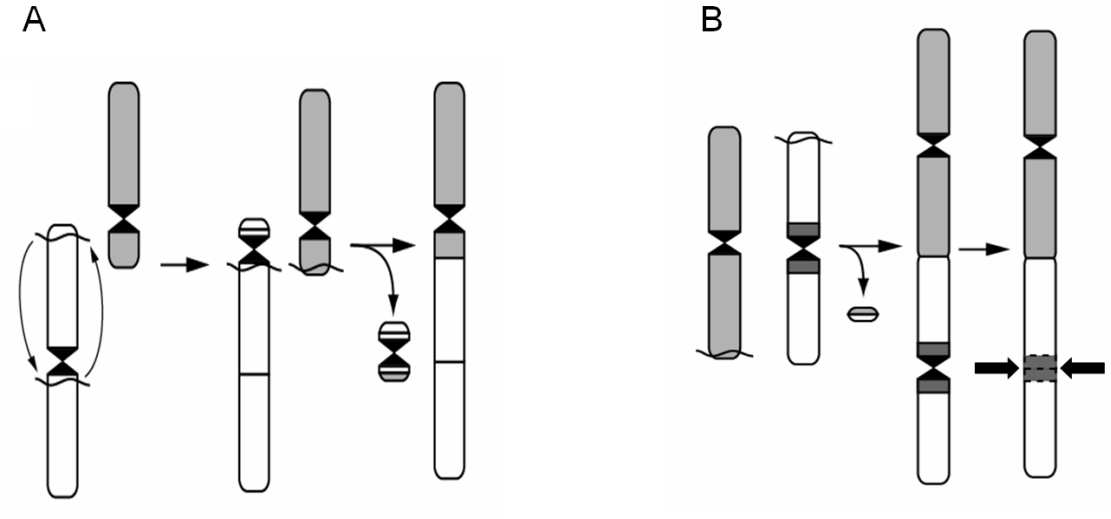


Fig. 9 Putative mechanisms of chromosome number reduction in the Brassicaceae. (A) Pericentric inversion - reciprocal translocation mechanism. (B) End-to-end chromosome fusion accompanied by centromere inactivation (Schubert and Lysak 2011, modified).

3.4.5 Conclusions on the karyotype evolution in the Brassicaceae

It can be concluded that descending dysploidy, one of the crucial features of the karyotype evolution in the Brassicaceae, is typically mediated by pericentric inversions, and reciprocal translocations resulting in centromere losses and origin of fusion chromosomes. Alternatively, translocations between chromosome ends lead to the formation of so-called fusion chromosomes with two centromeres, with one of the two being inactivated and/or deleted.

Our data summarized above suggest, that rearrangement breakpoints were predominantly located in (peri)centromeric and subtelomeric regions. The rearrangements involving the whole chromosome arms played the prevalent role in the reduction of chromosome number. Clustering of breakpoints at (peri)centromeres and chromosome termini indicates a preferential involvement of repetitive sequences in the genome repatterning processes. Centromeres and telomeres can be considered as fragile sites and hotspots of structural rearrangements.

The ACK karyotype is assumed to originated from a proto-genome (n = 4) by the Brassicaceae-specific α WGD (Henry et al. 2006). In *A. thaliana* (n = 5), the pre-α ancestral chromosome number has been almost restored within 23-43 mya (Barker et al. 2009, Fawcett et al. 2009) via mechanisms described above. In the clade of mesopolyploid Australian Microlepidieae genera the evolutionary tempo was faster compared to *A. thaliana*. Despite an additional WGD in the ancestry of endemic Australian crucifers, the pre-α chromosome number has been re-established in the same time frame as in *A. thaliana.*

4 Trends in angiosperm karyotype evolution

An intensive genetic mapping and genomics research has been carried out across other angiosperm families, particularly those with several crops, such as the Poaceae (Murat et al. 2010, Salse et al. 2008, 2012), Solanaceae (Mueller et al. 2005, Prince et al. 1993, Tanksley et al. 1992, Wu et al. 2009a, b), Fabaceae (Cronk et al. 2006, Zhu et al. 2005, Schoemaker et al. 2006), and Rosaceae (Considine et al. 2012, Dirlewanger 2004, Jung et al. 2012).

4.1 Genome evolution in the Poaceae

Comparative genetic mapping in the grass family (Poaceae), which includes many important cereal and forage crops, resulted in the synthesis of the Crop Circle (Devos 2005, Devos and Gale 1997, 2000, Feuillet and Keller 2002, Moore et al. 1995). The Crop Circle demonstrates a large degree of chromosome collinearity among the grass genomes (Gaut 2002). The small-sized rice genome (n = 12) subdivided into c. 30 blocks was placed to the center of the Circle. Although rice has been shown to retain the genome structure of its n = 12 ancestor (Murat et al. 2010, Salse et al. 2008), extensive genome reshuffling marked the evolution of other grass genomes, such as maize, sorghum, *Brachypodium*, Triticeae species or sugar cane.

The 30 ancestral blocks of rice represent an equivalent to the 24 genomic blocks of the Ancestral Crucifer Karyotype. In the grasses, the ancestral GBs have been shown as conserved in the extant karyotypes (Devos 2005). Similarly, cruciferous karyotypes are very stable in some clades (e.g., PCK-like karyotypes of lineage II, *Arabidopsis lyrata, Capsella rubella*), however, in the other clades rapidly evolving dynamic structures with GBs frequently disrupted via inversions and translocations, particularly in those with mesopolyploid WGD events.

In grasses, genes and repeats are organized differently in small and large genomes. In small genomes, there is a clear partioning between gene-rich euchromatin and repeat-rich heterochromatin (Devos 2009). This kind of genome organization is similar to that frequently found in Brassicaceae. In grass species with larger genomes, repeats are interspersed between genes. As larger grass genomes have been considered as more dynamic compared to the small ones, dispersed repeats may be the source of chromosome rearrangements (Devos 2009).

A common grass ancestor is reported to have n = 5 (or 7) chromosomes forming an allopolyploid genome of n = 10 (or 14) chromosomes (Devos 2009, Murat et al. 2010, Salse et al. 2008). The allopolyploidy in Poaceae was followed by diploidization process resulted in the origin of n = 12 karyotype of similar stucture as that of rice (Devos 2009, Murat et al. 2010, Salse et al. 2008). Multiple post-(n = 12) WGDs and subsequent chromosome number reduction responsible for the mosaic of parental genomic blocks have been well documented in grasses (Luo et al. 2009, Murat et al. 2010, Salse et al. 2008, 2012).

Descending dysploidy in the Poaceae is caused mainly by so called **nested chromosome fusions** (Luo et al. 2009, Murat et al. 2010). Intra-chromosome cross-over, produces ring chromosome, may form a chromosome with two telomere-free termini and a minichromosome containing two telomeres. The minichromosome is often lost. The major chromosome without telomeres attaches to another chromosome, often in a (peri)centromeric repet-rich region via illegitimate recombination between nonhomologous chromosomes. The attachment comprises an insertion, i.e., breakage of the recipient chromosome in its (peri)centromeric region and incorporation of the insertion chromosome. The nested chromosome fusion of one chromosome into

another's (peri)centromeric region results in repeat-rich boundaries representing the traces of the split ancestral (peri)centromere of the recipient chromosome. In all observed nested fusions, centromeres of insertion but not recipient chromosome were preserved (Luo et al. 2009, Murat et al. 2010, Schubert and Lysak 2011).

In the Brassicaceae, only two cases of chromosomal structure affected by nested fusion has been reported in *Pachycladon* (Mandáková et al. 2010b) and *Hornungia alpina* (Lysak et al. 2006). It suggests that although nested fusions have a major role in Poaceae, they represent an inconsiderable mechanism of the karyotype evolution in the Brassicaceae. The transposon-rich breakpoins are still detectable after the nested fusions in grasses (after ~70 mya) (Murat et al. 2010). This is in contrast to the evolutionary breakpoints in Brassicaceae in which are not reported to be enriched by repetitive sequences and thus detectable only based on the collapsed genome collinearity (Hu et al. 2011).

The mechanism of end-to-end fusion accompanied by centromere inactivation is, however, reported to be quite rare in the Poaceae (Murat et al. 2010). By contrast, this mechanism is described to be the second most frequent type of chromosome rearrangement in the mustard family.

4.2 Genome evolution in the Solanaceae

The Solanaceae is the third larger economically important plant family. Features and outcomes of chromosomal evolution in the family could be deduced from the extensive comparative mapping studies which have been performed for several major solanaceous crops relative to tomato (Prince et al. 1993, Tanksley et al. 1992, Wu et al. 2009a, b), from genome sequence of potato and tomato (Potato Genome Sequencing Consortium et al. 2012, Tomato Genome Consortium 2012), and from cross-species multicolor cytogenetic mapping using BAC clones from potato and tomato (Tang et al. 2008). The chromosome number in the Solanaceae is stable with actual karyotypes of eggplant, *Nicotiana*, pepper, potato and tomato sharing 12 chromosome pairs. Tomato and potato differ by 6 inversions, tomato and eggplant by 24 inversions and 5 translocations, tomato and pepper by 19 inversions and 6 translocations, tomato and *Nicotiana* by at least 10 inversions and 11 translocations (Prince et al. 1993, Tanksley et al. 1992, Wu et al. 2009a, b). This suggests that in the Solanaceae, inversions occur at a consistently higher rate than translocations. No complex chromosome changes as described in Brassicaceae or Poaceae were reported to occur in the Solanaceae. The major solanaceous crops diverged c. 30 mya and no recent WGD events played an important role in the genome evolution of the Solanaceae. Given the constant chromosome number across the Solanaceae, a relatively low rate of chromosome repatterning and absence of recent genome duplications, the family has experienced chromosomal changes at a moderate rate compared to Brassicaceae and Poaceae.

4.3 Genome evolution in the Fabaceae

In legumes, genomes of soybean, *Medicago truncata*, pea and lotus have been sequenced, and detailed comparative genetic maps are available for bean and *Mimosa*. The legume crop species originated from a common ancestor which experienced a WGD c. 59 mya. The Fabaceae genomes underwent an additional, more recent genome doubling c. 13 mya (Schoemaker et al. 2006). Mosaic synteny blocks can be identified in the genomes of legumes. However, the genome synteny was shown to be limited compared to conserved large-scale genomic blocks in Brassicaceae, Poaceae and Solanaceae. This suggests substantial genome rearrangements to occur shortly after the ancestral legume-specific WGD event.

4.4 Genome evolution in the Rosaceae

In the Rosaceae, genome sequencing of three crop species [apple (n = 17), peach (n = 8) and strawberry (n = 7)] showed different evolutionary patterns of genome evolution (Jung et al. 2012). The hypothetical ancestral genome of Rosaceae had seven chromosomes. The genome evolution in the family is characterized by shared conserved ancestral genomic blocks reshuffled by chromosome **fusions and fissions**. Chromosomal fusion-fission cycle was described as a reversible fusion of two telocentrics giving rise to the metacentric chromosome. The next step is a fission of this chromosome into two stable telocentrics eventually followed by a new fusion reconstituting the metacentric chromosome (Schubert et al. 1995). Jung et al. (2012) described strawberry lineage to experience at least five fission and seven fusion events, the peach lineage at least three fission and four fusion events. The apple genome to experienced a WGD after the divergence from peach and strawberry, and subsequently has undergone seven fission and nine fusion events (Jung et al. 2012).

Thus, Rosaceae represents the only plant family in which chromosome fissions presumably were the crucial mechanism of karyotype evolution. Recently, however, the genome study in apple shed light on the mechanism responsible for the odd-numbered basic chromosome numbere (Considine et al. 2012). Considine et al. (2012) showed that diploid apple species produce gametes with different chromosome numbers. Fusions of these gametes result the formation of different aneuploid basic chromosome numbers, previously explained as the consequence of chromosome fissions (Jung et al. 2012).

4.5 Contrasting karyotype evolution in angiosperms

These are great times for scientists as high-throughput genomic sequencing provides powerful new approaches to solve questions that puzzled scientists for centuries. Each crop species represents a model that offers unique opportunities to make a new progress in comparative plant biology.

In the early 2000s, *A. thaliana* and rice genomes have been sequenced and since that time, 16 more plant genomes have become available (apple, barley, *Brachypodium*, cacao, cotton, grapevine, maize, papaya, pea, peach, poplar, sorghum, soybeen, strawberry, wheat) and high-resolution genetic and/or cytogenetic maps have been reconstructed for a number of species (Salse 2012). Such data provide an important insight into the plant genome organization and evolution.

Although polyploidization has been repeatedly documented as the central process directing the genome evolution in land plants, there are obvious counteracting evolutionary forces towards lower chromosome numbers. Several rounds of WGD events have been followed by clade- and species-specific rearrangements leading to the pre-polyploid level. It is known that the rate with which genomes undergo and fix chromosome rearrangements varies between different lineages (Devos and Gale 2000, Ilic et al. 2003). The strong selection for diploid chromosome number restored via different mechanisms of chromosome number reduction after polyploidization is probably caused by the fact that massive structural and functional changes following WGD might provide an organism with a chance to adapt to changing environment.

The available data on karyotype and genome evolution in plants support the widespread opinion that genomes evolve mainly by duplicating and/or reorganizing the existing genomic blocks rather than by inventing new ones (Salse 2012).

Chromosome fissions probably do not occur at all because they produce potentially unstable chromosomes without telomere ends. In each analyzed angiosperm family, genomes were reshuffled by a different predominant type of chromosome

rearrangements. In the Brassicaceae, pericentric inversion-reciprocal translocation together with end-to-end chromosome fusions play the key role in chromosome number reductions, whereas nested chromosome fusions typical for Poaceae are very rare. Solanaceae karyotypes are stable, compared to other plant families, modified predominantly by inversions.

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6 Aims of the dissertation & author's contribution

Author's scientific contribution to the field of comparative cytogenetics in the Brassicaceae is documented by enclosed publications. The principal author's contribution is summarized as follows:

6.1 Optimization and application of the large-scale comparative chromosome painting in different species from the family Brassicaceae... I, II

I. Lysak and Mandáková (2013)
Step-by-step protocols of chromosome painting and comparative chromosome painting in plants.

II. Lysak et al. (2010)
Fist large-scale comparative chromosome painting using non-*Arabidopsis* BAC contigs was established and tested in several crucifer species.

6.2 Identification of the mechanisms responsible for descending dysploidy in crucifer plants... III

III. Mandáková and Lysak (2008)
This study represents the most comprehensive CCP study published in Brassicaceae. The whole-karyotype comparative analyses of species having identical chromosome number n = 7 from crucifer lineage II and affiliated tribes. The mechanism of chromosome number reduction from n = 8 to n = 7 is described and ancestral karyotype with n = 7 proposed.

6.3 Evaluation of the role of hybridization and polyploidy in karyotype and genome evolution... IV - VII

IV. Dierschke et al. (2009)
Antient inter-species hybridization and polyploid evolution in the genus *Lepidium* revealed by genomic *in situ* hybridization.

V. Mandáková et al. (2010a)
Mesopolyploid WGD in Australian crucifers revealed by CCP. It is demonstrated that the diploid-like genomes are originated from a common ancestral genome through a WGD followed by multiple rounds of chromosome rearrangements. Centromere inactivation was for the first time proposed as an important and common mechanism of karyotype and genome evolution in the Brassicaceae.

VI. Mandáková et al. (2012)
Mesopolyploid WGT in the evolutionary history in the tribe Heliophileae, the most diversified Brassicaceae lineage, uncovered by CCP. This is the first study to reported polyploidy as a potential major mechanism for the radiation of a Cape plant group.

VII. Cheng et al., submitted.
The reconstruction of the three diploid *Brassica* genomes with seven chromosomes involved in the origin of the hexaploid ancestror with 42 chromosomes.

6.4 The role of chromosome rearrangements in plant speciation... VIII

VIII. Mandáková et al. (2010b)
An evidence that large-scale chromosomal rearrangements often thought as key players in plant speciation did not play a major role in species diversification in the genus *Pachycladon* endemic to New Zealand.

7 Selected publications of the author

IX. Lysak M.A., **Mandáková T.** 2013. Analysis of plant meiotic chromosomes by chromosome painting. In: Pawlowski W.P, Grelon M. (Eds.). Plant Meiosis: Methods and protocols. Humana Press. NY.

X. Lysak M.A., **Mandáková T.**, Lacombe E. 2010. Reciprocal and multi-species chromosome BAC painting in crucifers (Brassicaceae). Cytogenetic and Genome Research 129: 184-189.

XI. **Mandáková T.**, Lysak M.A. 2008. Chromosomal phylogeny and karyotype evolution in x = 7 crucifer species (Brassicaceae). Plant Cell 20: 2559-2570.

XII. Dierschke T., **Mandáková T.**, Lysak M.A., Mummenhoff K. 2009. A bicontinental origin of polyploid Australian/New Zealand *Lepidium* species (Brassicaceae)? Evidence from genomic *in situ* hybridization. Annals of Botany 104: 681-688.

XIII. **Mandáková T.**, Joly S., Krzywinski M., Mummenhoff K., Lysak M.A. 2010. Fast diploidization in close mesopolyploid relatives of *Arabidopsis*. Plant Cell 22: 2277-2290.

XIV. **Mandáková T.**, Mummenhoff K., Al-Shehbaz I.A., Mucina L., Mühlhausen A., Lysak M.A. 2012. Whole-genome triplication and species radiation in the southern African tribe Heliophileae (Brassicaceae). Taxon 64: 989-1000.

XV. Cheng F., **Mandáková T.**, Wu J., Xie Q., Lysak M.A., Wang X. Deciphering the diploid ancestral genome of the mesohexaploid *Brassica rapa*. Submitted.

XVI. **Mandáková T.**, Heenan P.B., Lysak M.A. 2010. Island species radiation and karyotypic stasis in *Pachycladon* allopolyploids. BMC Evolutionary Biology 10: 367.