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Karyotype evolution in apomictic *Boechera* and the origin of the aberrant chromosomes

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SUMMARY

Chromosome rearrangements may result in both decrease and increase of chromosome numbers. Here we have used comparative chromosome painting (CCP) to reconstruct the pathways of descending and ascending dysploidy in the genus *Boechera* (tribe Boechereae, Brassicaceae). We describe the origin and structure of three *Boechera* genomes and establish the origin of the previously described aberrant *Het* and *Del* chromosomes found in *Boechera* apomicts with euploid (2*n* = 14) and aneuploid (2*n* = 15) chromosome number. CCP analysis allowed us to reconstruct the origin of seven chromosomes in sexual *B. stricta* and apomictic *B. divaricarpa* from the ancestral karyotype (*n* = 8) of Brassicaceae lineage I. Whereas three chromosomes (BS4, BS6, and BS7) retained their ancestral structure, five chromosomes were reshuffled by reciprocal translocations to form chromosomes BS1-BS3 and BS5. The reduction of the chromosome number (from x = 8 to x = 7) was accomplished through the inactivation of a paleocentromere on chromosome BS5. In apomictic 2*n* = 14 plants, CCP identifies the largely *het*erochromatic chromosome (*Het*) being one of the BS1 homologues with the expansion of pericentromeric heterochromatin. In apomictic *B. polyantha* (2*n* = 15), the *Het* has undergone a centric fission resulting in two smaller chromosomes – the submetacentric *Het'* and telocentric *Del*. Here we show that new chromosomes can be formed by a centric fission and can be fixed in populations due to the apomictic mode of reproduction.

Keywords: *Boechera*, Brassicaceae, apomixis, chromosome fission, karyotype evolution, heterochromatin, centromere inactivation.

INTRODUCTION

The genus *Boechera* (Böcher's rock cress, formerly *Arabis*, Brassicaceae) is a member of the tribe Boechereae (127 species in nine genera, Al-Shehbaz, 2012) distributed throughout North America and Greenland (Koch *et al.*, 1999, 2003; Kiefer *et al.*, 2009). The genus with 110 species is monophyletic (Alexander *et al.*, 2013), has a basic chromosome number x = 7 (Koch *et al.*, 1999), and is characterized by diploid sexual (2*n* = 2x = 14), as well as diploid, aneuploid, and mostly triploid (2*n* = 3x = 21) apomictic forms (Böcher, 1951; Schranz *et al.*, 2005; Alexander *et al.*, 2015). Extensive morphological variations and corresponding problematic taxonomy of the genus are due to rampant hybridization among diploid species resulting in the origin of apomictic diploid and aneuploid hybrids. Triploids have arisen multiple times through hybridization between sexual and apomictic diploid species (Alexander *et al.*, 2015) as geographically and genetically distinct populations (Sharbel and Mitchell-Olds, 2001; Sharbel *et al.*, 2005).

The genus *Boechera* is an ideal model system to compare sexual and apomictic reproduction and associated life history traits, as it is characterized by naturally-occurring diploid sexual and diploid apomictic forms (Böcher, 1951; Aliyu *et al.*, 2010; Rushworth *et al.*, 2011). Using high throughput seed screening methods, in conjunction with custom-made high-density microarrays for tissue-targeted expression analyses, a single candidate factor each for meiotically unreduced egg (APOLLO; Corral *et al.*, 2013) and pollen (UPGRADE-2; Mau *et al.*, 2013) production in apomictic *Boechera* have been identified. More than 95% of seeds produced by diploid apomictic *Boechera* are characterized by a diploid embryo (100% maternally derived) and a hexaploid endosperm (four maternal ge-

nomes and two paternal genomes); less frequent are seeds with a diploid embryo and a pentaploid endosperm (Aliyu *et al.*, 2010). In plants producing seeds with a hexaploid endosperm, it is hypothesized that both maternal and parental factors are required in order to stabilize apomixis through the combined effects of producing a meiotically unreduced ovule with balanced (2 maternal:1 paternal) endosperm formation. Recent data on these candidates have demonstrated that both factors are highly correlated in apomictic *Boechera* from genetically (i.e. different taxa) and geographically (i.e. 1000s of kms) divergent genotypes (Mau *et al.*, 2015).

Apomictic *Boechera* plants with 15 chromosomes were already described as early as 1951 in the elaborate study of Tyge Böcher (Böcher, 1951), the cytologist whose name was later given to the genus. Later studies investigated the nature of supernumerary chromosomes in aneuploid diploid ($2n = 15$) and triploid ($2n = 22$) apomicts more closely (Sharbel *et al.*, 2004, 2005). But the findings from those studies failed to explain the nature and the origin of the additional, B-like chromosomes. Sharbel *et al.* (2004, 2005) described the chromosomes as being heterochromatic, often smaller than all the remaining chromosomes and of a different length in different populations. As these chromosomes were absent in the sexual diploids and present in the diploid ($2n = 15$) and triploid ($2n = 22$) apomictic cytotypes it was argued that the chromosomes may contain genetic elements associated with the apomictic trait (Sharbel *et al.*, 2005; Lovell *et al.*, 2013). Comparative analyses of the mitotic and meiotic cell complements of the sexual and apomictic accessions demonstrated that all diploid apomicts have a variable and highly heterochromatic (*Het*) chromosome with pericentromeric heterochromatin repeats from *Boechera stricta*, which suggests that this chromosome originated from this species. An additional smaller chromosome, referred to as *Del* (as presumed a deletion chromosome), was found in the $2n = 15$ and $2n = 22$ apomicts (Kantama *et al.*, 2007). Whereas GISH (genomic *in situ* hybridization) in diploid as well as aneuploid apomicts revealed the hybrid origin of these genomes, the nature of *Het* and *Del* chromosomes was not explained (Kantama *et al.*, 2007).

The tribe Boechereae is one of the 49 well defined monophyletic tribes in Brassicaceae (Al-Shehbaz, 2012). The Boechereae along with tribes Cardamineae, Descurainieae, Halimolobeae, Lepidieae, Microlepidieae, Physarieae, Smelowskieae, and the paraphyletic Camelineae form one of the three major crucifer lineages – lineage I (Franzke *et al.*, 2011; Al-Shehbaz, 2012; Heenan *et al.*, 2012). Comparative genetic (e.g., Boivin *et al.*, 2004; Schranz *et al.*, 2007) and cytogenetic (Lysak *et al.*, 2006) studies showed that most likely all lineage I genomes have descended from an ancestral genome with eight chromosomes resembling the extant genome structure of *Arabidopsis lyrata* (Hu *et al.*, 2011) and *Capsella rubella* (Slotte *et al.*, 2013). Results of comparative genetic mapping and chromosome painting analyses in crucifer species have been integrated to establish a set of 24 conserved genomic blocks (GBs) building up eight chromosomes of the Ancestral Crucifer Karyotype (ACK; Schranz *et al.*, 2006a). These GBs have been used as the basis to elucidate chromosome rearrangements associated with descending dysploidies (reductions of chromosome number) across the Brassicaceae. For the Boechereae, Schranz *et al.* (2007) created a comparative genetic map of the diploid sexual *B. stricta* and outlined a tentative scenario of its evolution from the ACK. However, as the centromeres were only tentatively mapped onto *Boechera* chromosomes, neither the exact nature of chromosome rearrangements can be attained, nor it can be concluded if the genome of *B. stricta* is likely to represent an ancestral genome of the genus.

In this paper, we used comparative chromosome painting (CCP) with *A. thaliana* chromosome-specific BAC contigs representing 24 GBs of the ACK to resolve several questions about genome evolution in the genus *Boechera.* First, we used CCP to verify and specify the order and orientation of the GBs in *B. stricta* identified in our initial F_2 genetic mapping, and characterize chromosome regions, such as the centromeres and chromosome BS1, not resolved by genetic mapping due to a lack of recombination (Schranz *et al.*, 2007). Second, in comparison with the ACK, we reconstructed the origin of the seven chromosomes in *B. stricta* and *B. divaricarpa* from the eight ancestral chromosomes. Third, in the apomictic diploid *B. divaricarpa* ($2n = 14$) and the aneuploid apomictic *B. polyantha* ($2n = 15$) we elucidated the origin of aberrant chromosomes that have previously been referred to as the *Het* and *Del* chromosomes (Kantama *et al.*, 2007). The results of this study will contribute to better understanding of chromosome fissions in plants and may provide an invaluable comparative framework needed for high-quality genome assemblies in sequenced *Boechera* species.

RESULTS

CCP analysis confirms close phylogenomic relationship between the ACK $n = 8$ and the $n = 7$ *Boechera* genome

Based on the published genetic map of *B. stricta* (Schranz *et al.*, 2007) we have designed BAC painting probes to verify the position and orientation of all 24 GBs on the seven chromosomes of *B. stricta* (accessions ES06) and *B. divaricarpa* (accession ES09). CCP unambiguously identified all 24 GBs on pachytene chromosomes of both species (Figures 1a and S1), pointing to close phylogenetic relationship between the Boechereae and the core Camelineae (e.g., *A. lyrata*, *A. thaliana;* Figure 1b). Our results were

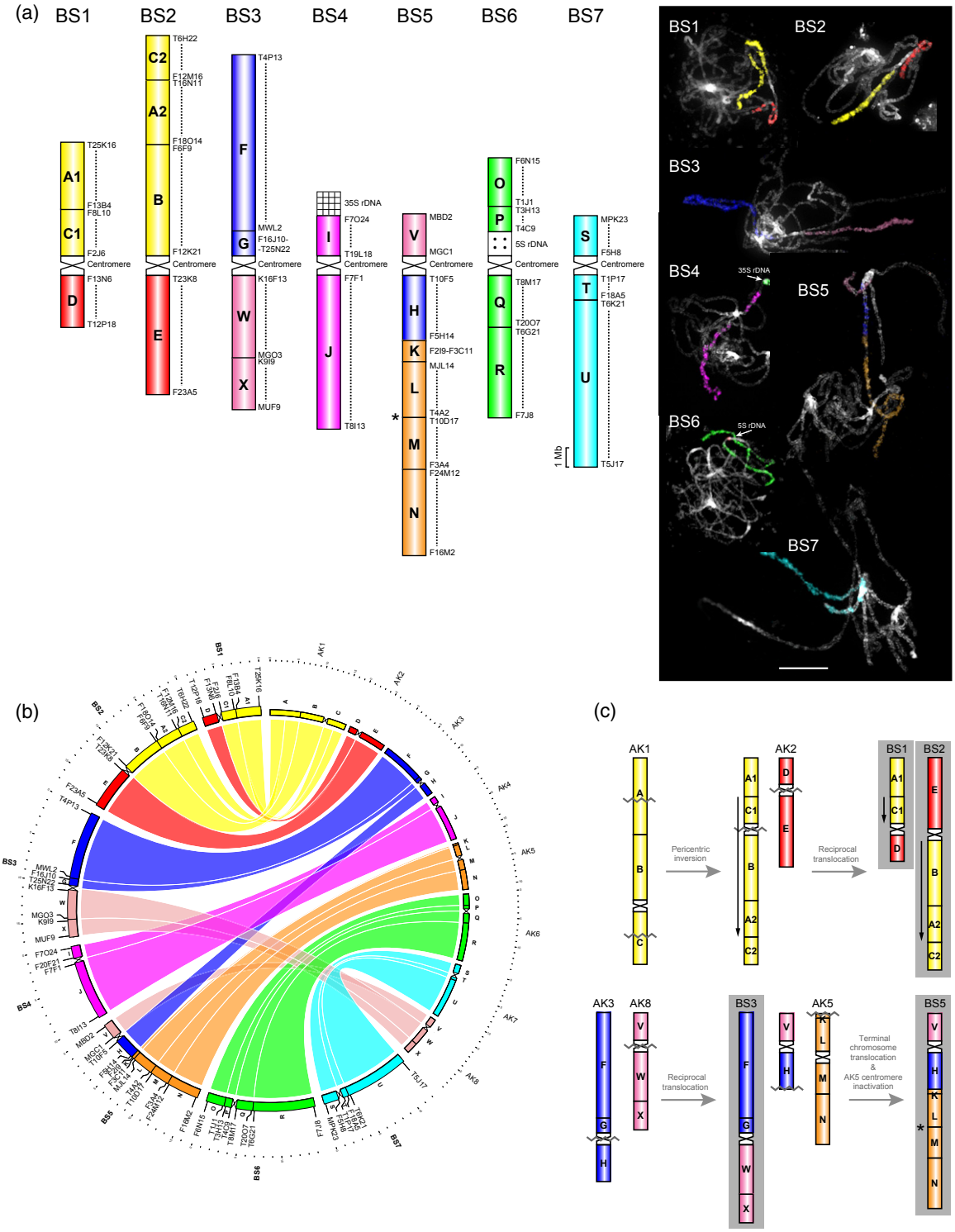

Figure 1. Comparative structure and origin of the *Boechera* genome.
(a) Cytomolecular map based on comparative chromosome painting in *B. stricta;* CCP of BS1 to BS7 in *B. stricta* on the right (scale bar, 10 μm).
(b) Circos diagram showing collinearity between chromosomes of *B. stricta*/*divaricarpa* (BS1–BS7) and chromosomes (AK1–AK8) of Ancestral Crucifer Karyotype (ACK; Schranz *et al.*, 2006a).
(c) Parsimonious scenario of the origin of ancestral *Boechera* chromosomes BS1, BS2, BS3, and BS5 from chromosomes AK1-AK3, AK5, and AK8. Capital letters mark 24 genomic blocks of the ACK; border *A. thaliana* BAC clones specify BAC contigs corresponding to genomic blocks identified on *Boechera* chromosomes. Downward-pointing arrows refer to genomic blocks inverted compared to their ancestral telomere-to-centromere orientation within the ACK. Asterisks in Figure (a, c) denote the inactive centromere on chromosome BS5 (see also Figure b).

congruent with the previous linkage mapping (Schranz *et al.*, 2007), except for block D forming the bottom arm of BS1/BD1 (Figures 1a and S1). *In situ* localization of GBs combined with DAPI staining for displaying (peri)centromeric heterochromatin, unequivocally identified the seven centromeres (Figures 1a and S1), which were only indirectly inferred by genetic mapping (Schranz *et al.*, 2007). The purported centromere between blocks L and M on BS5 (Schranz *et al.*, 2007) has been identified as an inactive/deleted ancestral AK5 centromere with no remaining traces of pericentromeric heterochromatin in its original position. In *B. stricta*, a single nucleolus organizing region (NOR) is located on the top arm of BS4 and a single 5S rDNA locus is near the pericentromere of BS6 (Figure 1a).

Origin of *B. stricta* and *B. divaricarpa* karyotypes

Comparison between the ACK ($n = 8$) and the *B. stricta*/*B. divaricarpa* genome reveals extensive genome collinearity between both genomes (Figure 1b; NB: only the evolution of the *B. stricta* genome from ACK is described here, but the same events occurred in the ancestor of *B. divaricarpa*). Ancestral chromosomes AK4, AK6 and AK7 are entirely collinear with *Boechera* chromosomes BS4, BS6, and BS7. The origin of chromosomes BS1 and BS2 (Figure 1c) has likely been initialized by a large pericentric inversion on AK1 with breakpoints in blocks A and C. Then a whole-arm translocation between this chromosome and ancestral chromosome AK2 resulted in *Boechera* chromosomes BS1 (GBs A1, C1, and D) and BS2 (C2, A2, B, and E). As the breakpoints of the reciprocal translocation cannot be more specified, we are unable to assign the centromeres of BS1 and BS2 to ancestral chromosomes of AK1 and AK2, respectively. The evolution of chromosomes BS3 and BS5 (Figure 1c) can be reconstructed as a reciprocal whole-arm translocation between ancestral chromosomes AK3 and AK8, whereby we cannot infer the identity of BS3 and BS5 centromeres. This translocation has been followed by a terminal chromosome translocation between the AK3/8 translocation product (GBs V and H) and chromosome AK5, resulting in the origin of chromosome BS5. Because all AK5-derived GBs (K, L, M, and N) on the bottom arm of BS5 have preserved ancestral position, we infer an inactivation of the AK5 paleocentromere on chromosome BS5 (Figures 1a,c and S1).

Het and *Del* chromosomes

We address the origin of the previously described aberrant *Het* and *Del* chromosomes (Kantama *et al.*, 2007). A largely heterochromatic chromosome *Het* is present in the $2n = 14$ apomictic accessions (Figures 2a and 3a), whereas smaller heterochromatic *Het'* and *Del* chromosomes are known in the $2n = 15$ apomicts (Figures 2b and 3b). In the ES09 *B. divaricarpa* apomict ($2n = 14$), *Het* has been identified as one of the two BD1 chromosomes. Both homologues have the same structure of BD1 but differ by the expansion of pericentromeric heterochromatin in *Het* (Figures 2a and 3a). In the *B. polyantha* apomict ($2n = 15$), one BP1 homologue has the ancestral structure of genomic blocks, whereas GBs of the second homologue (i.e., presumably of the *Het*

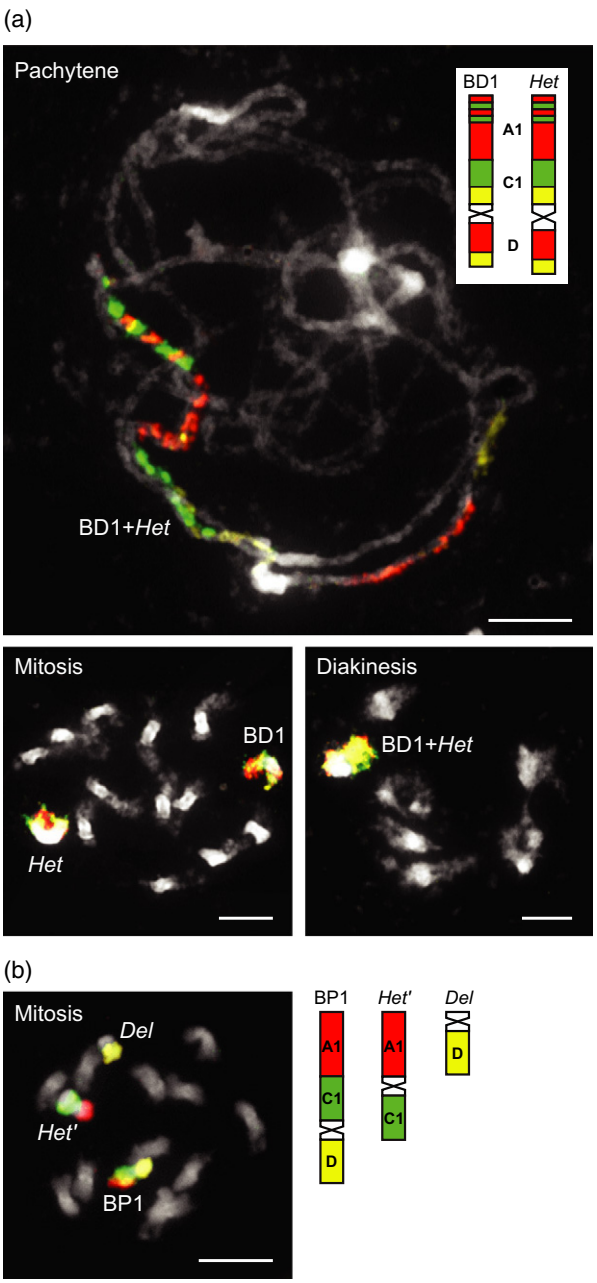

Figure 2. Behaviour of aberrant chromosomes during mitosis and meiosis.
(a) *B. divaricarpa* ($2n = 14$). Pairing of BD1 and *Het* homologues during meiotic prophase I (pachytene and diakinesis) and in mitosis.
(b) *B. polyantha* ($2n = 15$). BP1, *Het'* and *Del* chromosomes in mitotic metaphase. *A. thaliana* BAC contigs homeologous to ancestral chromosomes AK1 and AK2 were used as painting probes to identify BD1/BP1 and the aberrant chromosomes. All scale bars, 5 μm.

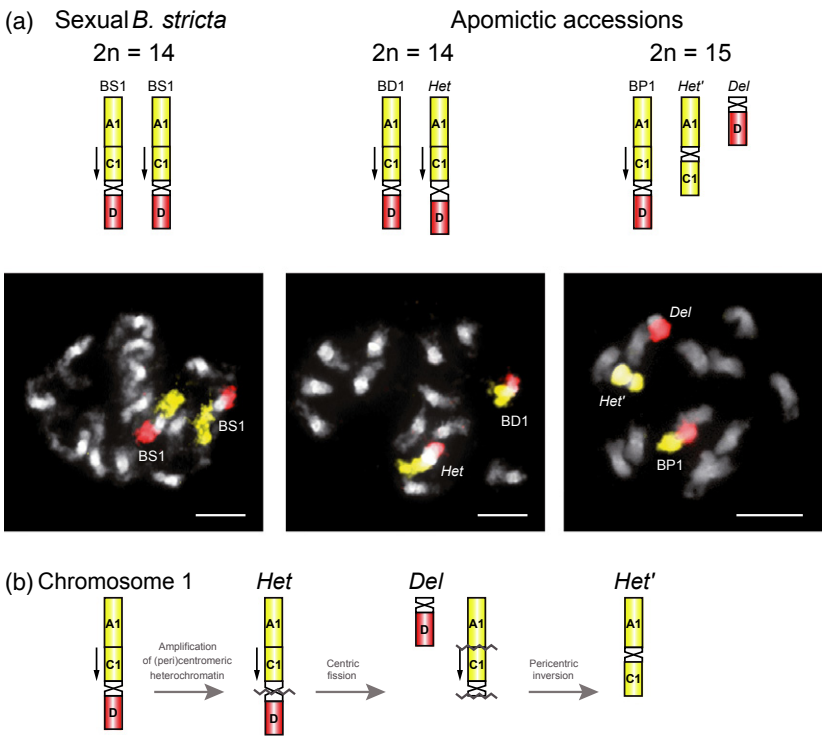

Figure 3. Structure and origin of aberrant chromosomes in apomictic *Boechera* taxa.
(a) Two BS1 homologues in the sexual *B. stricta* (2*n* = 14), BS1 and *Het* chromosome in the apomictic *B. divaricarpa* (2*n* = 14), and *Het*′ and *Del* chromosomes in the apomictic *B. polyantha* (2*n* = 15). Identification of BS1 and aberrant chromosomes within DAPI-stained mitotic metaphases using *A. thaliana* BAC contigs specific for chromosome BS1. All scale bars, 5 μm.
(b) Reconstructed origin of aberrant chromosomes from a BS1 homologue. Capital letters mark 24 genomic blocks of the Ancestral Crucifer Karyotype (ACK; Schranz *et al.*, 2006a). Downward-pointing arrows refer to genomic blocks inverted compared to their ancestral telomere-to-centromere orientation within ACK.

chromosome) are split between chromosomes *Het*′ and *Del*. The *Het*′ is a submetacentric chromosome with the centromere between blocks A1 and C1 due to a pericentric inversion comprising the entire block C1 and the centromere. The *Del* is a telocentric chromosome formed only by block D. Pericentromeres of *Het*′ and *Del* are characterized by heterochromatin expansion, although to a lesser extent than in *Het* in apomictic 2*n* = 14 accessions (Figures 2b and 3a).

DISCUSSION

The origin of the *Boechera* genome

Several phylogenetic studies (Bailey *et al.*, 2006; Beilstein *et al.*, 2006; Couvreur *et al.*, 2010; Warwick *et al.*, 2010) recognized the genus *Boechera* and later the tribe Boechereae to be closely related to the North American Halimolobeae and Physarieae, and to the paraphyletic tribe Camelineae, and thus well embedded among tribes of lineage I (Beilstein *et al.*, 2006). Both this study and comparative genetic mapping (Schranz *et al.*, 2007) corroborate the proposed phylogenetic ties by identifying the ACK (*n* = 8) as an ancestral genome of *Boechera.* CCP analysis of the sexual (*B. stricta*) and apomictic (*B. divaricarpa*) diploid species revealed that both taxa share three ancestral chromosomes with ACK (BS4, BD4 = AK4; BS6, BD6 = AK6 and BS7, BD7 = AK7) and four rearranged chromosomes of the identical structure (Figures 1a and S1). As Western Asia is the most probable cradle of the ACK ancestor and the Brassicaceae (Franzke *et al.*, 2009), the ancestor of Boechereae with *n* = 8 colonized North America from Asia *via* the Bering Land Bridge (Koch and Al-Shehbaz, 2004; Koch *et al.*, 2010). As the tribe Boechereae is monophyletic (Bailey *et al.*, 2006) and all taxa have x = 7, we suggest that the reduction of chromosome number from x = 8 to x = 7 occurred prior to the diversification of the Boechereae.

Centromere inactivation on BS5

The origin of modern *Boechera* chromosome 5 from three ancestral chromosomes was apparently a consequence of a terminal chromosome translocation (TCT) between a V-H chromosome intermediate and chromosome AK5 (Figure 1c). The completely conserved chromosome AK5 within the BS5/BD5 bottom arm suggests that its centromere was lost following the TCT event. This is explainable through a pericentric and paracentric inversion before and after TCT, restoring the ancestral collinearity of AK5, or more likely by centromere inactivation/deletion. In Brassicaceae, centromere inactivation and deletion were purported to be essential for the origin of composite chromosomes in several species (reviewed in Lysak, 2014). The mechanism of centromere inactivation/deletion, a necessary step to prevent the origin of dicentric chromosomes, remains elusive. Heterochromatinization, epigenetic modifications and recombination have been identified as the most probable agents (Stimpson *et al.*, 2012; Catania and Allshire, 2014; Lysak, 2014).

The origin of the *Het* chromosome

Here we described the structure and reconstructed the origin of aberrant chromosomes repeatedly found within chromosome complements of *Boechera* species (Böcher,

1951; Sharbel *et al.*, 2004, 2005; Kantama *et al.*, 2007). Our results agreed well with earlier observations of Kantama *et al.* (2007), except for the colocalization of 5S and 35S rDNA loci, which cannot be confirmed in the sexual and apomictic diploids analyzed. In diploid apomicts, the *Het* chromosome gained its name according to its bright DAPI fluorescence of a large heterochromatin block (Kantama *et al.*, 2007). Large blocks of pericentromeric heterochromatin visually constitute more than half of the chromosome within mitotic metaphase spreads (Figures 2a and 3a). Despite the difference in the amount of heterochromatin between the *Het* and its (BS1/BD1) homologue, both chromosomes exhibit a regular bivalent pairing during meiosis I (Kantama *et al.*, 2007; Figure 2a). This observation is concordant with the observed regular meiotic pairing and segregation of heterochromatin divergent homeologues in *Drosophila* (Gilliland *et al.*, 2015). The *Het* chromosome is derived from *B. stricta* as based on hybridization of pericentromeric repeats (Schranz *et al.*, 2006b; Kantama *et al.*, 2007). Genetic analysis has demonstrated that the *Het* chromosome can be crossed into *B. stricta* without conferring a dominant apomictic phenotype (Schranz *et al.*, 2006b). These results suggest that *Het* chromosomes might have originated and be present within the sexually reproducing *B. stricta* lineage. Here we found that the *Het* is one of the BS1 homologues with expanded pericentromeric heterochromatin. The reasons for the asymmetric heterochromatin expansion on the *Het* remain unclear as both BS1/BD1 and *Het* homologues have the same GB composition, and thus, no large-scale inversion mediating heterochromatin relocation and spreading on *Het* cannot be suspected. Nevertheless, a 'hemicentric' inversion with one breakpoint in the centromere resulting in two closely spaced centromeres and subsequent amplification of pericentric heterochromatin can be considered (Lamb *et al.*, 2007). In humans, most whole-arm (Robertsonian) translocations bring centromeres of both participating chromosomes together onto the translocation chromosome (Jones, 1976; Perry *et al.*, 2004 and references therein) and in plants 'hybrid centromeres' have been described to be formed through recombination between two broken centromeres (Zhang *et al.*, 2001). Therefore, it is conceivable that *Boechera* chromosome BS1, formed by a reciprocal translocation between the ancestral chromosomes AK1 and AK2, gained a 'hybrid centromere' along with pericentric heterochromatin of both original centromeres. This naturally does not explain why only one and not both BS1/BD1 homologues has become a *Het* chromosome.

Heterochromatin accumulation on *Het* could be promoted by meiotic drive, if *Het* would be preferentially included into the egg due to a potential pseudokinetochore activity of the expanded heterochromatin. This would be a scenario analogous to preferential transgenerational transmission of the maize knob-bearing chromosome Ab10 with heterochromatin at the long arm roughly the size of the short arm of a normal chromosome 10 (Hiatt and Dawe, 2003).

Potential link between the aberrant chromosomes and apomixis

One of the most common hypotheses on the inception of apomixis is the so-called hybridization-derived floral asynchrony (Carman, 1997), which describes that hybridization and polyploidization plays a key role in leading to competition of nearly complete sets of asynchronously-expressed duplicated genes involved in gametophyte and embryo development. More recently, such a reproductive deregulation was supported by heterochronic expression patterns during ovule development in sexual and apomictic *Boechera* spp. (Sharbel *et al.*, 2010), and points to a network of epigenetic and posttranscriptional regulation during germline specification (Twell, 2011). GISH revealed that all analyzed *Boechera* apomicts are interspecific hybrids but can contain different combinations of *holboellii* and *stricta* chromosomes in their cell complements, suggesting large-scale chromosome substitutions (Kantama *et al.*, 2007). Meiosis in these hybrid *Boechera* apomicts is variable with disturbed or incomplete synapsis (Kantama *et al.*, 2007). Homeologous recombination between the *holboellii* and *stricta* chromosomes can potentially generate novel epialleles and gene combinations, induce structural chromosome rearrangements, chromosome substitutions or aneuploidy, and can destabilize meiosis and affect fertility, a phenomenon referred to as 'polyploid ratchet' by Gaeta and Pires (2010). Despite apomeiotic egg formation, homeologous recombination in somatic tissues can potentially modify the organization of parental GBs towards novel block associations (e.g., Mandáková *et al.*, 2010). Hence, the genome structure in a broader spectrum of apomictic allopolyploid *Bochera* species should be analyzed in the future. In addition, differences in epigenetic marks of the maternal and paternal genomes in the hybrid may induce changes in DNA methylation and RNAi divergence at gametogenesis and embryo development of next generations (Köhler and Grossniklaus, 2005; Martienssen, 2010; and reviewed in De Storme and Mason, 2014). It is now tempting to believe that the aforementioned epigenetic changes may also explain the formation of the unique APOLLO (APOmixis-Linked LOcus) (Corral *et al.*, 2013) and BspUPG2 apoalleles (Mau *et al.*, 2013). However, direct experimental evidence for their positions on the *Het* or any other chromosome is still lacking.

Chromosomes pairing failure and epigenetic changes themselves do not explain the formation of the largely heterochromatic *Het* chromosome, but such events can be the starting point of a stepwise degeneration that resembles the evolutionary origin of heteromorphic sex chromo-

somes (Kantama *et al.*, 2007). Such erosion processes include accumulation of deleterious mutations, non-coding DNA, transposon accumulation and retrotransposable elements expansions (reviewed in Bachtrog, 2006). A comparable process may have taken place with the formation of the supernumerary chromatin in one of the chromosomes of apomictic *Pennisetum squamulatum* and *Cenchrus ciliaris* (Akiyama *et al.*, 2005).

The origin of *Het'* and *Del* by centric fission

Current genomic and cytogenetic data suggest that the whole-genome duplications followed by descending dysploidy is the dominating trend in the evolution of angiosperm plants. However, the common acro- and telocentric chromosomes that prevail in the karyotypes of several plant groups, were traditionally interpreted as having resulted from chromosome fission events (see Jones, 1998). Indeed, when karyomorphological data are plotted on phylogenetic trees, fission events seem to occur, for example, during genome evolution in cycads (Olson and Gorelick, 2011), *Crocus* (Brighton, 1978), the slipper orchids (Cox *et al.*, 1998), *Lycoris* (Shi *et al.*, 2014) or in the classical example of *Campanula persicifolia* (Darlington and La Cour, 1950). Whereas these fission events in plants were deduced only from chromosome numbers, chromosome morphology and/or meiotic pairing configurations (Jones, 1998), data on the molecular mechanism of centric fissions as well as on the detailed genomic composition of metacentric chromosomes and their telocentric derivatives are scarce.

Here we have shown that two aberrant chromosomes, *Het'* and *Del*, originated by a centric fission of the heterochromatin-rich *Het* chromosome in the apomictic *B. polyantha* (given as *B. holboellii* in Kantama *et al.*, 2007). The fission event is reflected in the size of pericentromeric heterochromatin blocks, which are larger on *Het* than on *Del* and *Het'* chromosomes in $2n = 15$ apomicts (Kantama *et al.*, 2007 and this study). In the case of *Het'*, the fission was most likely followed by a pericentric inversion further reshaping the telocentric chromosome into a submetacentric one (Figures 2b and 3b). The stable inheritance of both heterochromatic fission chromosomes is probably due to the apomictic mode of reproduction. We hypothesize that the fission might have occurred during meiotic anaphase/telophase I through merotelic kinetochore attachment (Gregan *et al.*, 2011) and opposite pulling forces of meiotic spindles. Darlington (1939), and later Friebe *et al.* (2005), showed that chromosome laggards and univalents, respectively, are prone to misdivision at meiosis I. Kaszás *et al.* (2002) showed that centromere misdivision of a univalent chromosome can occur during meiosis in maize and that the separated chromosome arms may become telocentric chromosomes through healing of broken ends (telomere capping). We further suggest that the accumulation of large arrays of pericentromeric heterochromatin on *Het* could have been an important prerequisite for the centric fission and subsequent stabilization of telocentrics by *de novo* telomere capping. As the *Het* resulted from a whole-arm translocation between AK1 and AK2 chromosomes, the *Het* centromere can be potentially prone to fission due to its 'hybrid' nature and/or breakpoint reuse.

EXPERIMENTAL PROCEDURES

Plant materials

The sexual diploid *B. stricta* (Graham) Al-Shehbaz, genotype ES06, was a parental line (SAD12) of the genetic mapping population in Schranz *et al.* (2007) and Anderson *et al.* (2011). The apomictic diploid ($2n = 14$) line ES09 has been cytologically investigated in Kantama *et al.* (2007) and referred to as *B. divaricarpa.* This species name is temporary used for apomicts of multiple hybrid origins (Koch *et al.*, 2003; Kantama *et al.*, 2007) and it is used here for the sake of consistency. An apomictic aneuploid ($2n = 15$) genotype of *B. polyantha* (Greene) Windham & Al-Shehbaz was grown from seeds collected in Birch Creek, Ravalli Co., Montana, USA (accession no. BH115, determined as *B. holboellii* in Kantama *et al.*, 2007).

Chromosome preparations

Cytogenetic analyses carried out in this study mostly followed protocols published by Mandáková and Lysak (2008) with minor modifications as detailed below. Entire inflorescences with the prevalence of closed flower buds were fixed in ethanol:acetic acid (3:1) overnight and stored in 70% ethanol at −20°C until use. The youngest flower buds were rinsed in distilled water (2 × 5 min) and in 1 × citrate buffer (10 × citrate buffer: 40 ml of 100 mM citric acid and 60 ml of 100 mM trisodium citrate, pH 4.8; 2 × 5 min) and incubated in a pectolytic enzyme mixture (0.3% cellulase, cytohelicase, and pectolyase; all Sigma Aldrich; http://www.sigmaaldrich.com/) in 1× citrate buffer at 37°C for 3–6 h, transferred into citrate buffer and processed or kept at 4°C until the next day. Individual anthers were put on a microscope slide and disintegrated by dissection needles in a drop of 1× citrate buffer until a fine suspension was formed. Then the suspension was softened by adding 15–30 μl of 60% acetic acid and spread by stirring with a needle on a hot plate at 50°C for 0.5–2 min. Chromosomes were fixed by pipetting 100 μl of ethanol:acetic acid (3:1) around the suspension drop. The slide was tilted to remove the fixative and dried using a hair dryer. The dried preparation was examined using a phase -contrast light microscope and suitable slides postfixed in a Coplin jar with 4% formaldehyde in distilled water for 10 min and left to air dry. To remove cytoplasm, the slides were treated with pepsin (0.1 mg ml^{-1}; Sigma Aldrich) in 0.01 M HCl for 3–6 min, postfixed in 4% formaldehyde in 2× SSC (20× saline sodium citrate: 3 M sodium chloride, 300 mM trisodium citrate, pH 7.0) for 10 min, dehydrated in an ethanol series (70, 80, and 96%; 3 min each), and left to air dry.

Painting probes

Arabidopsis thaliana BAC contigs used for CCP were referred to by Mandáková and Lysak (2008). For CCP, on average each third BAC was used and contigs were arranged and differentially labeled according to the position of GBs in the ACK (Schranz *et al.*, 2006a) and the *B. stricta* genetic map (Schranz *et al.*, 2007). To characterize inter-chromosome and intra-chromosome rearrangements (translocation and inversion breakpoints), the respective BAC con-

tigs were split into smaller subcontigs, differentially labeled and used as CCP probes. The BAC clone T15P10 (AF167571) bearing 35S rRNA genes was used for *in situ* localization of nucleolar organizer regions, and clone pCT 4.2, corresponding to a 500-bp 5S rRNA repeat (M65137), was used for localization of 5S rDNA loci.

All DNA probes were labeled by nick translation with biotin-dUTP, digoxigenin-dUTP, or Cy3-dUTP as follows: 1 μg of DNA diluted in distilled water to 29 μl, 5 μl of nucleotide mixture (2 mM dATP, dCTP, and dGTP, 400 μM dTTP; all Roche; http://www.roche.com/), 5 μl of 10 × NT buffer (0.5 M Tris–HCl, pH 7.5, 50 mM $MgCl_2$, and 0.05% bovine serum albumin), 4 μl of 1 mM custom-made *x*-dUTP (in which *x* was biotin, digoxigenin, or Cy3), 5 μl of 0.1 M β-mercaptoethanol, 1 μl of DNase I (2000 U mg^{-1} diluted to 4 μg ml^{-1}; Roche), and 1 μl of DNA polymerase I (10 U μl^{-1}, Fermentas; http://www.thermofisher.com/). The nick translation mixture was incubated at 15°C for 90 min (or longer) to obtain a fragment length of ~200–500 bp. The nick translation reaction was stopped by adding 1 μl of 0.5 M EDTA, pH 8.0, and by incubation at 65°C for 10 min. Labeled probes were stored at −20°C until use.

Comparative chromosome painting

To reduce the probe volume and remove unincorporated nucleotides, labeled BAC clones were pipetted together and the DNA precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.5 volume of ice-cold 96% ethanol, kept at −20°C for at least 30 min, and centrifuged at 13 000 ***g*** at 4°C for 30 min. The pellet was dried using a desiccator and resuspended in 20 μl of hybridization buffer (50% formamide and 10% dextran sulfate in 2× SSC) per slide. The probe was covered with a cover slip and sealed with rubber cement around the edges. The probe and chromosomes were denatured together on a hot plate at 80°C for 2 min and incubated in a moist chamber at 37°C for 40–63 h.

Post-hybridization washing was performed in 20% formamide in 2 × SSC at 42°C. Signal detection and amplification were as follows: biotin-dUTP was detected by avidin–Texas Red (1:1000; Vector Laboratories; http://www.vectorlabs.com/) and amplified by goat anti-avidin–biotin (1:200, Vector Laboratories; http://www.jacksonimmuno.com/) and avidin–Texas Red; digoxigenin-dUTP was detected by mouse anti-digoxigenin (1:250; Jackson ImmunoResearch Laboratories) and goat anti-mouse Alexa Fluor 488 (1:200; Molecular Probes; http://www.lifetechnologies.com/), and Cy3-dUTP labeled probes were observed directly. Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (2 μg ml^{-1}) in Vectashield (Vector Laboratories).

Fluorescence hybridization signals were analyzed with an Olympus BX-61 epifluorescence microscope equipped with Zeiss AxioCam charged-coupled device (CCD) camera (http://www.zeiss.com/). Images were acquired separately for all four fluorochromes using appropriate excitation and emission filters (AHF Analysentechnik; https://www.ahf.de/). The monochromatic images were pseudocoloured, processed and merged using the ADOBE PHOTOSHOP CS5 software (Adobe Systems; https://www.ahf.de/). Eight different pseudocolours, corresponding to the eight chromosomes of the ACK, were applied in Adobe Photoshop to display the chromosome homeology between *Boechera* and AK chromosomes (Figures 1a and 2a).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Cytomolecular map of *B. divaricarpa* based on comparative chromosome painting on pachytene chromosomes.

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