Whole-genome triplication and species radiation in the southern African tribe Heliophileae (Brassicaceae)

Terezie Mandáková,[1] Klaus Mummenhoff,[2,5] Ihsan A. Al-Shehbaz,[3] Ladislav Mucina,[4] Andreas Mühlhausen[2] & Martin A. Lysak[1,5]

1 *RG Plant Cytogenomics, CEITEC - Central European Institute of Technology, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic*

2 *Osnabrück University, Biology Department, Botany, Barbarastrasse 11, 49076 Osnabrück, Germany*

3 *Missouri Botanical Garden, St. Louis, Missouri 63166-0299, U.S.A.*

4 *Curtin Institute for Biodiversity & Climate, Department of Environment & Agriculture, Curtin University, GPO Box U1987, Perth, 6845, Australia*

5 contributed equally to this work

Authors for correspondence: *Martin A. Lysak, martin.lysak@ceitec.muni.cz*
Klaus Mummenhoff, Mummenhoff@Biologie.Uni-Osnabrueck.de

Abstract The unigeneric tribe Heliophileae includes ca. 90 *Heliophila* species, all endemic to southern Africa. The tribe is morphologically the most diverse Brassicaceae lineage in every aspect of habit, foliage, flower and fruit morphology. Despite this diversity, virtually nothing is known about its origin and genome evolution. Here we present the first in-depth information on chromosome numbers, rDNA in situ localization, genome structure, and phylogenetic relationship within Heliophileae. Chromosome numbers determined in 27 *Heliophila* species range from $2n$ = 16 to $2n$ = ca. 88, but $2n$ = 20 and 22 prevail in 77% of the examined species. Chromosome-number variation largely follows three major lineages (A, B, and C) resolved in the ITS phylogeny. Clade A species mostly have a chromosome number of $2n$ = 20, whereas $2n$ = 22 is the dominant number in clade C ($2n$ = 16 and 22 were counted in two diploid species of clade B). The number and position of 5S and 45S rDNA loci vary between species and cannot be employed as phylogenetically informative characters. Seven species with different chromosome number and from the three ITS clades were analyzed by comparative chromosome painting. In all species analyzed, 90% of painting probes unveiled three homeologous chromosome regions in *Heliophila* haploid chromosome complements. These results suggest that all *Heliophila* species, and probably the entire tribe Heliophileae, experienced a whole-genome triplication (WGT) event. We hypothesize that the mesohexaploid ancestor arose through hybridization between genomes resembling the Ancestral Crucifer Karyotype with n = 8. The WGT has been followed by species-specific chromosome rearrangements (diploidization) resulting in descending dysploidy towards extant quasi-diploid genomes. More recent neopolyploidization events are reflected by higher chromosome numbers ($2n$ = 32–88). The WGT might have contributed to diversification and species radiation in Heliophileae. To our knowledge, this is the first study to document polyploidy as a potential major mechanism for the radiation of a Cape plant lineage.

Keywords Cape flora; chromosome painting; comparative phylogenomics; *Heliophila*; ITS; karyotype evolution; phylogenetics; polyploidy; rDNA; whole-genome duplication

Supplementary Material Figure S1 and Table S1 (both in the Electronic Supplement) and the alignment are available in the Supplementary Data section of the online version of this article (http://www.ingentaconnect.com/content/iapt/tax).

■ INTRODUCTION

There are multiple reasons why the unigeneric tribe Heliophileae DC. (hereafter alternatively referred to under its generic type *Heliophila* L.) is a truly remarkable group in the context of the entire Brassicaceae (Cruciferae). *Heliophila* (>90 species), the 9th-largest genus in Brassicaceae, is also one of the most complex genera from a taxonomic point of view, as suggested by more than 400 scientific names listed in the International Plant List Index (http://ipni.org), as well as 15 generic names currently placed in its synonymy (Al-Shehbaz, 2012). *Heliophila* is morphologically by far the most diverse genus in the family, especially in habit (tiny herbs to shrubs or lianas), foliage (entire to variously dissected), floral size (1.2–25 mm) and color, petal and stamen appendages, inflorescence position (terminal vs. intercalary; Marais 1970), flower number (few to numerous), ovule number (2–80), fruit size (2–120 mm long), shape and type of fruit (silique, silicle, samara, schizocarp), fruit flattening (terete, latiseptate, angustiseptate), and dehiscence (Mummenhoff & al., 2005).

Heliophila is mainly native to South Africa; the distribution ranges of three species extend into Lesotho, seven into Namibia, and one into Swaziland. The center of the greatest diversity is the Cape Floristic Region (CFR) which has 87 species (77 endemic), and only three species (*H. formosa* Hilliard & Burtt, *H. obibensis* Marais, *H. scandens* Harv.) are endemic

to KwaZulu-Natal Province (compiled from Marais, 1970). One species (*H. pusilla* L. f.) is naturalized in Australia.

Despite two molecular phylogenetic studies (Mummenhoff & al., 2005; Verboom & al., 2009) and recent taxonomic accounts (Marais, 1970; Al-Shehbaz & Mummenhoff, 2005), *Heliophila* remains under-explored in many respects, including genome evolution, pollination and reproductive biology, dispersal ecology, character development and evolution, rapid radiation and speciation, phylogeography, etc. Chromosome counts have been reported for only four unvouchered of the 90 species (*Heliophila africana* (L.) Marais, *H. amplexicaulis* L. f., *H. crithmifolia* Willd., and *H. linearis* DC.; Jaretzky, 1932; Manton, 1932). Assessing chromosome and genome evolution in the tribe thus remained unfeasible for the last eighty years. The closest relatives of Heliophileae remain unknown, as the tribe groups with many others in a basal hard polytomy (expanded lineage II) in a multi-gene Brassicaceae phylogeny (Couvreur & al., 2010; Franzke & al., 2011). These unexplored features, substantial taxonomic complexity and the very high endemism (97%) of the genus in a relatively small CFR (only ~90,000 km²; Goldblatt & Manning, 2000), all make *Heliophila* a challenging study system.

Here we present the first in-depth information on chromosome number variation, localization of ribosomal RNA genes (rDNA), and genome structure in the light of an updated taxonomy and a comprehensive phylogeny. We characterized the extent of chromosome number variation across the tribe by analyzing approximately one third of its species. Unexpectedly, a comparative chromosome painting (CCP) analysis revealed that diploid-like genomes of *Heliophila* species have undergone a whole-genome triplication (WGT) event, which along with other factors might have promoted the unique radiation of this Brassicaceae lineage.

■ MATERIALS AND METHODS

Plant material. — Plant material for the phylogenetic and chromosomal analyses (including CCP) was obtained from herbarium specimens and from two field expeditions in 2008 and 2009, respectively. Ninety accessions, representing 57 *Heliophila* species sensu Marais (1970) and Al-Shehbaz & Mummenhoff (2005), were analysed. *Chamira circaeoides* (L. f.) A. Zahlbr. the only species of this genus without tribal assignment (Al-Shehbaz, 2012), was chosen as outgroup for the Bayesian analysis as done in previous molecular phylogenetic studies (Mummenhoff & al., 2005). Origin and collection data of plant material are given in Table S1 (Electronic Supplement).

Chromosome counts. — Mitotic chromosomes were counted from enzymatically digested young flower buds collected in the field. They were fixed in 3 : 1 (ethanol : acetic acid) fixative and stored in 70% ethanol at −20°C until use. For details of the protocol, see Mandáková & al. (2010a). DAPI-stained (4′,6-diamidino-2-phenylindole in Vectashield, 2 µg/ml) chromosome figures were photographed with an Olympus BX-61 epifluorescence microscope and CoolCube CCD camera (Metasystem), processed in Adobe Photoshop CS2 (Adobe Systems), and counted. Multiple chromosome figures were analyzed per species. These chromosome spreads were probed with 5S and 45S rDNA probes to identify rRNA gene loci.

FISH: rDNA localization and comparative chromosome painting (CCP). — The *Arabidopsis thaliana* (L.) Heynh. BAC clone T15P10 (AF167571) containing 45S rRNA genes was used for fluorescence in situ localization of nucleolar organizing regions (45S rDNA). *Arabidopsis thaliana* clone pCT4.2 (M65137), corresponding to a 500-bp 5S rRNA repeat, was used to identify 5S rDNA loci. For CCP, we used *A. thaliana* chromosome-specific BAC contigs corresponding to ancestral genomic blocks on chromosomes AK1 and AK8 of the Ancestral Crucifer Karyotype ($n = 8$, Schranz & al., 2006). In the *A. thaliana* genome (http://www.arabidopsis.org/), AK1 corresponds to 17.1 Mb (block A: 6.7 Mb, B: 5.7 Mb, C: 4.7 Mb) and AK8 to 9.2 Mb (block V: 2.4 Mb, W: 4.3 Mb, X: 2.5 Mb). BAC contigs were differentially labeled with biotin-dUTP, digoxigenin-dUTP, and Cy3-dUTP by nick translation, ethanol precipitated, and hybridized to pachytene chromosomes of *Heliophila* species (for details see Mandáková & al., 2010a). Pachytene chromosome spreads were prepared as described by Mandáková & al. (2010a). DAPI-stained and painted chromosome complements were photographed, pseudo-colored and analyzed in Adobe Photoshop. Some chromosome images were straightened (Fig. 2) using the plugin 'Straighten Curved Objects' (Kocsis & al., 1991) in ImageJ v.1.38 program (Abramoff & al. 2004).

Phylogenetic analyses. — The ITS analysis presented here is based on the taxon sampling of Mummenhoff & al. (2005; 47 accessions) and 43 newly acquired accessions. Thus the current phylogeny comprises 90 accessions, representing 57 species. This represents 63% of the 90 known species.

Methods for DNA extraction, PCR, and ITS sequencing follow Mummenhoff & al. (2001, 2004). The DNA sequences were aligned automatically using the MAFFT v.6.0 multiple alignment programme (http://mafft.cbrc.jp/alignment/server/) and edited by hand. The model of nucleotide substitution for the Bayesian analysis was determined using MrModeltest v.2.2 software (Nylander, 2004; Posada, 2008) and the Akaike information criterion (AIC). Bayesian inference of phylogeny was performed using MrBayes v.3.1 (Ronquist & Huelsenbeck, 2003). Following MrModeltest, the General Time Reversible model with a proportion of invariable sites and a gamma-shaped distribution of rates across sites (GTR+R+I) was used in MrBayes. Two separate analyses were conducted, each starting from a different randomly chosen tree, on four parallel chains with a temperature for the heated chain set to 0.2. Each chain was run for 2,500,000 update cycles and every 100th cycle sampled. The initial 6250 samples (25%) were discarded as burn-in. Bayesian search results were summarized by a 85% majority-rule consensus tree. Tracer v.1.5 (Rambaut & Drummond, 2009) was used to check for convergence of the model likelihood and parameters after each run until reaching stationary status.

Molecular dating. — Node ages were estimated as follows: We incorporated the Cleomaceae/Brassicaceae split as a secondary calibration point using values of 19 Ma (Franzke

& al., 2009) and 32 Ma (Couvreur & al., 2010) for this split. In addition, we simultaneously applied a minimum age constraint of 2.58 and 5.33 Ma for the clade containing *Rorippa* Scop. and *Cardamine* L., as this corresponds to the lower and upper age estimate of Pliocene dated *Rorippa* fruit fossils (Mai, 1995).

A relaxed-clock approach was chosen to infer the ages of the Heliophileae radiation. *Tarenaya spinosa* (Jacq.) Raf. (= *Cleome spinosa* Jacq.), *Aethionema grandiflorum* Boiss. & Hohen., *Alliaria petiolata* (M. Bieb.) Cavara & Grande, *Rorippa amphibia* (L.) Besser and *Cardamine matthioli* Moretti were used as outgroup taxa. The analyses were performed in BEAST v.1.7.1 (Drummond & Rambaut, 2012) following an approach by Drummond & al. (2006) under a relaxed-clock model. The BEAST input file was created using the BEAST user interface BEAUti v.1.7.1. In this approach, a relaxed-clock model is incorporated into a Bayesian phylogenetic inference. The analyses were conducted with a relaxed molecular clock model drawing uncorrelated rates from a log-normal distribution. We created a normal prior with an age of 32/19 Ma for the Cleomaceae/Brassicaceae split and a normal prior with an age of 2.58/5.33 Ma for the *Rorippa*/*Cardamine* split, respectively. Two runs were calibrated with two calibration points (32 Ma and 5.33 Ma; 19 Ma and 2.58 Ma) in the same analysis. All analyses were started with a random tree. We used the General Time Reversible model of nucleotide substitution with a gamma (G) distribution with four gamma categories of rates and a proportion of invariant sites (I). After tuning the operators using the auto-optimization option, the final analysis was done with the MCMC chain-length set to thirty million. These settings resulted in ESS (effective sampling size) values, determined in Tracer, for all estimated parameters and node ages above 100, indicating a sufficient posterior distribution quality.

■ RESULTS

ITS phylogeny and dating. — Bayesian analysis of ITS data resulted in a robust phylogenetic tree of 57 *Heliophila* species, with a basal trichotomy comprising sublineages A (19 spp.), B (17 spp.) and C (19 spp.) (Fig. 1). Using different approaches to estimate and to calibrate the radiation of *Heliophila* in southern Africa, multiple previous analyses arrived at age estimations of 1.0–4.4 Ma for the crown group of Heliophileae (Table 1) (Mummenhoff & al., 2005; Verboom & al., 2009). The analysis of our new and expanded dataset using different calibration settings and BEAST confirms previous estimates, all of which date the crown group age of *Heliophila* in the Pliocene and Pleistocene (1.22–5.64 Ma).

Chromosome-number variation (Figs. 1 & 2; Table 2). — Chromosome numbers were determined in 72 populations representing 27 *Heliophila* species, representing all three infratribal clades. Chromosome numbers range from $2n = 16$ in *H. juncea* (P.J. Bergius) Druce to $2n$ = ca. 88 in *H. pubescens* Burch. (Fig. 2C, D). Twenty-one species (78%) have $2n = 20$

Table 1. Age estimates for the Heliophileae (*Heliophila*) clade and its three main lineages A, B, and C using different methods and calibration options. Numbers indicate million years ago (Ma).

		Node		
Calibration	*Heliophila*	A	B	C
Mummenhoff & al. (2005)				
Wikström & al.[a]				
NPRS[b]	4.2/4.6	3.4/3.7	2.9/3.3	3.9/4.3
NPRS jackknife[c]	3.7–5.4	2.8–4.5	2.2–3.9	n.a.
Forced clock[d]	1.9	1.3	1.0	1.7
rDNA ITS rates[e]				
Non-ultrametric distances	2.7–5.8	1.1–2.3	1.5–3.2	2.2–3.8
Forced clock[d]	2.3–4.9	1.6–3.3	1.3–2.7	2.1–4.4
Current Study (expanded dataset)				
Franzke & al. (2009)[f]	3.77	1.70	1.22	2.03
Couvreur & al. (2010)[g]	7.84	5.60	3.99	5.64

[a] The *Brassica*/*Cleome* clade was dated to 22 Ma (from Wikström & al., 2001).
[b] Tree made ultrametric using NPRS (Non-Parametric Rate Smoothing); additional calibration by constraining the *Rorippa*/*Cardamine* clade to be minimally 2.5/5.0 Ma.
[c] Estimated on jackknife re-sampled branch lengths under NPRS.
[d] Tree made ultrametric assuming a global clock.
[e] (3.9–8.3) 3×10^{-9} substitutions/site/year (from Sang & al., 1994; Zhang & al., 2001).
[f] The Brassicaceae/Cleomaceae split was dated to 19 Ma; additional calibration by constraining the *Rorippa*/*Cardamine* clade to be minimally 2.58 Ma. This age of the *Rorippa*/*Cardamine* split corresponds to the lower limit of the minimal age estimate of a *Rorippa* macrofossil (Mai, 1995).
[g] The Brassicaceae/Cleomaceae split was dated to 32 Ma; additional calibration by constraining the *Rorippa*/*Cardamine* clade to be minimally 5.33 Ma. This age of the *Rorippa*/*Cardamine* split corresponds to the upper limit of the minimal age estimate of a *Rorippa* macrofossil (Mai, 1995)

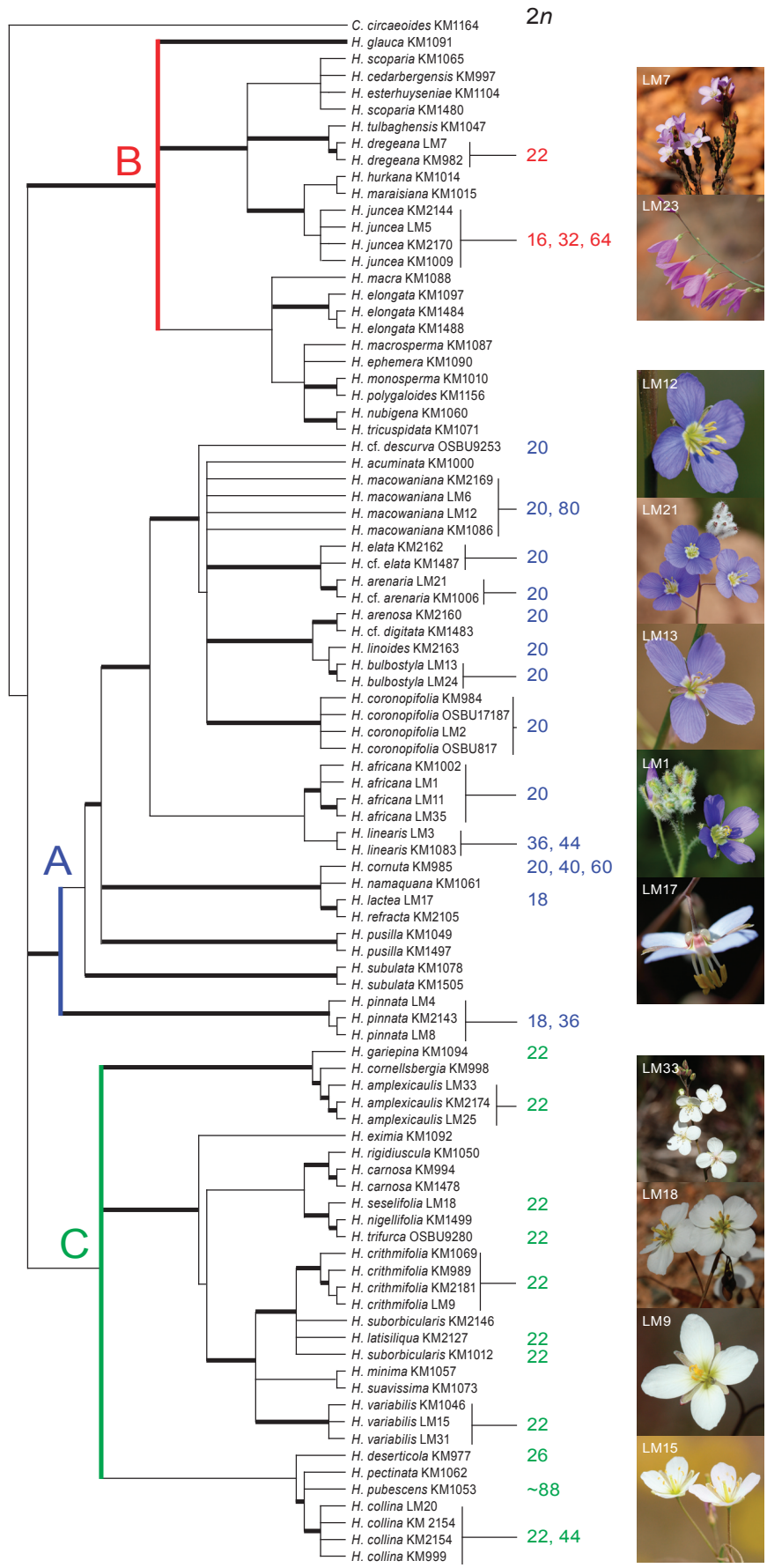


Fig. 1. Phylogeny of Heliophileae (*Heliophila*). 85% majority-rule consensus tree from a Bayesian analysis of ITS sequence data. This is a conservative estimate of the phylogeny, where nodes with posterior probability (PP) values <0.85 were collapsed. Nodes with PP 0.95–1.0 are shown as thick lines, and normal lines represent PP 0.94–0.85. Capital letters A, B, and C refer to the main phylogenetic lineages. *Chamira circaeoides* was used as outgroup. Chromosome numbers (2*n*) and images of flowers are given for selected species.

(11 spp.) or $2n = 22$ (10 spp.) chromosomes; two species have $2n = 18$ and one has $2n = 26$. Five species were found to have two or three intraspecific cytotypes. For example, the morphologically well-defined *H. juncea* has diploid ($2n = 16$), tetraploid ($2n = 32$) and octoploid ($2n = 64$) cytotypes, and in *H. cornuta* Sond. diploid ($2n = 20$), tetraploid ($2n = 40$) and hexaploid ($2n = 60$) populations were found. In *H. linearis*, two chromosome counts ($2n = 36$ and 44) apparently reflect the unsettled taxonomy of this species. The size of mitotic chromosomes (ca. 2–13 µm in *H. latisiliqua* E. Mey. ex Sond.) is largely dependent on the level of chromatin compaction, whereby less condensed prophase and pre-metaphase chromosomes are longer than metaphase chromosomes (Fig. 2). In most (if not all) species, mitotic chromosome complements comprise large and small chromosomes (e.g., Fig. 2A). Heterochromatin is localized in (peri)centromeric regions and, in some species, in distinct interstitial or terminal heterochromatic knobs (Figs. 2B & 3; Fig. S1 in the Electronic Supplement). Some trends in chromosome-number variation in *Heliophila* are detected when superimposing chromosome numbers on the ITS tree (Fig. 1). The lowest chromosome count ($2n = 16$) was found only in *H. juncea* (clade B). In clade A, $2n = 20$ is the dominating number (78% of species analyzed), and $2n = 18$ and 20 were found exclusively in this clade. Species of clade C mostly have $2n = 22$ (91% of species analyzed).

rDNA (Fig. 2; Table 2). — Within diploid chromosome complements, the number of 45S rDNA loci varies from one to four pairs. Six loci were observed in tetraploid (*H. collina* O.E. Schulz, $2n = 44$) and hexaploid (*H. cornuta*, $2n = 60$) cytotypes, and eigth loci in tetraploid (*H. pinnata* L. f., $2n = 36$) and octoploid (*H. macowaniana* Schltr., $2n = 80$) cytotypes. Several clade A species have two 45S rDNA loci, whereas four loci were detected in most clade C species. However, this pattern is inconsistent between the two clades. The same is true for the number of 5S rDNA loci, which varies from 1 to 14 pairs (in *H. pinnata*, $2n = 36$), although two pairs are found most frequently. The tetraploid cytotype of *H. pinnata* stands out from all other species as it has the highest number of both 5S and 45S rDNA loci. Interestingly, all 45S and 5S rDNA loci are localized interstitially on chromosomes in all *Heliophila* species analyzed (Fig. 2C–H). The size

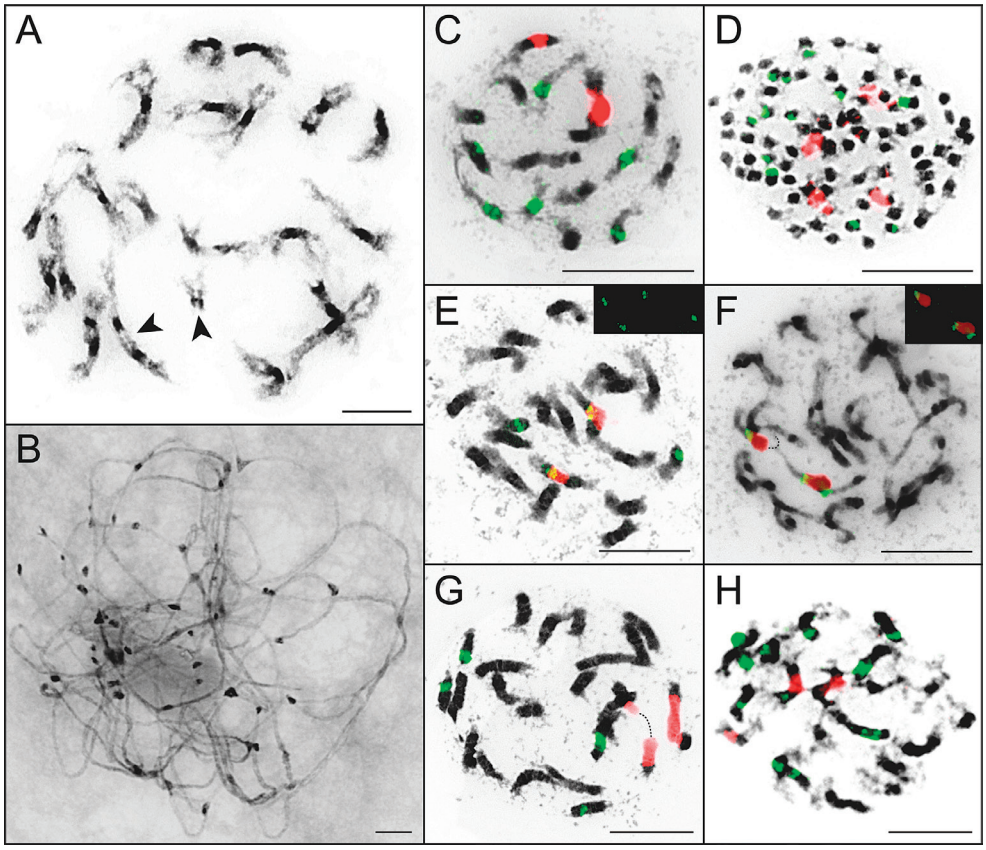


Fig. 2. Chromosome and rDNA variation in *Heliophila*. **A,** Differences in chromosome size in *H. latisiliqua* LM36 ($2n = 22$); arrowheads point to the smallest (4 µm) and largest (13 µm) chromosomes; **B,** numerous interstitial and terminal heterochromatic knobs on pachytene chromosomes of *H. linearis* LM3B ($2n = 44$); **C–H,** chromosomal localization of 5S rDNA (green) and 45S rDNA (red): **C,** one chromosome pair with interstitial 45S rDNA loci and six chromosomes with 5S rDNA in *H. juncea* KM2144 ($2n = 16$); **D,** twelve 5S rDNA loci in *H. pubescens* KM2137 ($2n = \sim 88$), the species with the highest chromosome number (the number of 45S rDNA loci has not been determined); **E,** *H. africana* LM11 ($2n = 20$); 5S and 45S rDNA loci are partly colocalized; **F,** *H. bulbostyla* LM24 ($2n = 20$); one chromosome pair with interstitial loci of 5S and 45S rDNA; one chromosome with a 45S locus flanked by 5S rDNA loci on both sides; **G,** unusually large 45S rDNA loci in *H. amplexicaulis* LM25 ($2n = 22$); **H,** eleven loci of 5S and three interstitial loci of 45S rDNA in *H. crithmifolia* KM2136 ($2n = 22$). — Chromosomes were counterstained with DAPI and B/W images inverted in Adobe Photoshop. Scale bars = 10 µm.

of the 45S rDNA locus can differ significantly between species (compare *H. amplexicaulis* and *H. crithmifolia*, Fig. 2G & H), but rarely differs within a chromosome complement (*H. juncea*, Fig. 2C). In two species, 5S and 45S rDNA loci are co-localized (*H. africana*, Fig. 2E) or reside in close proximity (*H. bulbostyla* P.E. Barnes, Fig. 2F). In *H. bulbostyla*, one 45S locus is flanked on both sides by 5S rDNA, probably due to an inversion within co-localized 5S and 45S rDNA loci.

Chromosome painting in diploids (Fig. 3; Fig. S1). — Two chromosomes of the Ancestral Crucifer Karyotype (ACK), AK1 and AK8, were chosen to get a glimpse of genome structure and karyotype evolution in seven *Heliophila* species. These species were chosen such that they would represent the three ITS clades and have both different ploidy levels and chromosome numbers (Fig. 1). Chromosome-specific *A. thaliana* BAC contigs, representing the two AK chromosomes and six

Table 2. Chromosome numbers and numbers of rDNA loci (vouchers at MO and OSBU).

Accession	Species	2*n*	rDNA loci		Chromosomes with 45S and 5S
			45S	5S	
KM2166	*H. africana*	20	2	4	2
LM1	*H. africana*	20	2	4	2
LM11	*H. africana*	20	2	4	2
LM35	*H. africana*	20	2	4	2
KM2161	*H. africana*	20	2	4	2
LM25	*H. amplexicaulis*	22	2	4	0
LM33	*H. amplexicaulis*	22	2	4	0
LM16	*H. arenaria*	20	2	4	2
LM21	*H. arenaria*	20	2	4	2
KM2156	*H. arenaria* var. *acocksii*	20	2	4	2
KM2160	*H. arenosa*	20	2	2	0
LM13	*H. bulbostyla*	20	2	4	2
LM24	*H. bulbostyla*	20	2	3	2
LM20	*H. collina*	22	2	4	0
KM2101	*H. collina*	22	4	–	–
KM2154	*H. collina*	44	6	4	0
KM2094	*H. cornuta*	20	2	–	–
KM2117	*H. cornuta*	60	6	–	–
KM2134	*H. cornuta*	40	4	–	–
LM2	*H. coronopifolia*	20	2	4	2
KM2116	*H. crithmifolia*	22	4	2	0
KM2119	*H. crithmifolia*	22	4	4	2
KM2136	*H. crithmifolia*	22	3	8	0
KM2147	*H. crithmifolia*	22	4	8	1
KM2247	*H. crithmifolia*	22	4	4	0
LM9	*H. crithmifolia*	22	4	10	0
LM14	*H. descurva*	20	2	4	2
KM2106	*H. deserticola*	26	4	4	2
KM2108	*H. deserticola*	26	4	–	–
LM7	*H. dregeana*	22	–	–	–
KM2162	*H. elata*	20	2	4	2
KM2115	*H. gariepina*	22	2	–	–
KM2121	*H. gariepina*	22	2	4	2
KM2129	*H. juncea*	32	4	4	0
KM2144	*H. juncea*	16	2	6	0
LM23	*H. juncea*	32	4	9	0
KM2120	*H. lactea*	18	2	4	2
LM17	*H. lactea*	18	2	2	2
KM2127	*H. latisiliqua*	22	4	4	2
LM34	*H. latisiliqua*	22	4	2	0
LM3B	*H. linearis* agg.	44	6	4	–
LM3A	*H. linearis* agg.	>30	4	10	4
KM2163	*H. linoides*	20	2	2	2
KM2140	*H. macowaniana*	20	2	4	2
KM2169	*H. macowaniana*	80	8	>8	0
LM12	*H. macowaniana*	20	2	4	2
LM6	*H. macowaniana*	20	2	4	2
KM2143	*H. pinnata*	18	2	8	2
KM2150	*H. pinnata*	18	4	>6	4
LM10	*H. pinnata*	36	–	–	–
LM4	*H. pinnata*	36	8	14	8
LM8	*H. pinnata*	18	4	4	2
KM2137	*H. pubescens*	~88	>22	18	–
KM2130	*H. seselifolia*	22	4	–	–
KM2131	*H. seselifolia*	22	–	–	–
KM2133	*H. seselifolia*	22	4	4	2
KM2135	*H. seselifolia*	22	4	–	–
KM2148	*H. seselifolia*	22	4	4	2
LM18	*H. seselifolia*	22	4	4	2
KM2138	*H. schulzii*	20	2	–	–
KM2146	*H. suborbicularis*	22	4	4	2
KM2107	*H. trifurca*	22	2	4	2
KM2100	*H. variabilis*	22	4	4	–
KM2112	*H. variabilis*	22	4	–	–
KM2113	*H. variabilis*	22	4	–	–
KM2139	*H. variabilis*	22	4	4 or 6	0
LM15	*H. variabilis*	22	4	4	0
LM22	*H. variabilis*	22	–	–	–
LM30	*H. variabilis*	22	4	6	0
LM31	*H. variabilis*	22	4	6	0
LM32	*H. variabilis*	22	4	4	0

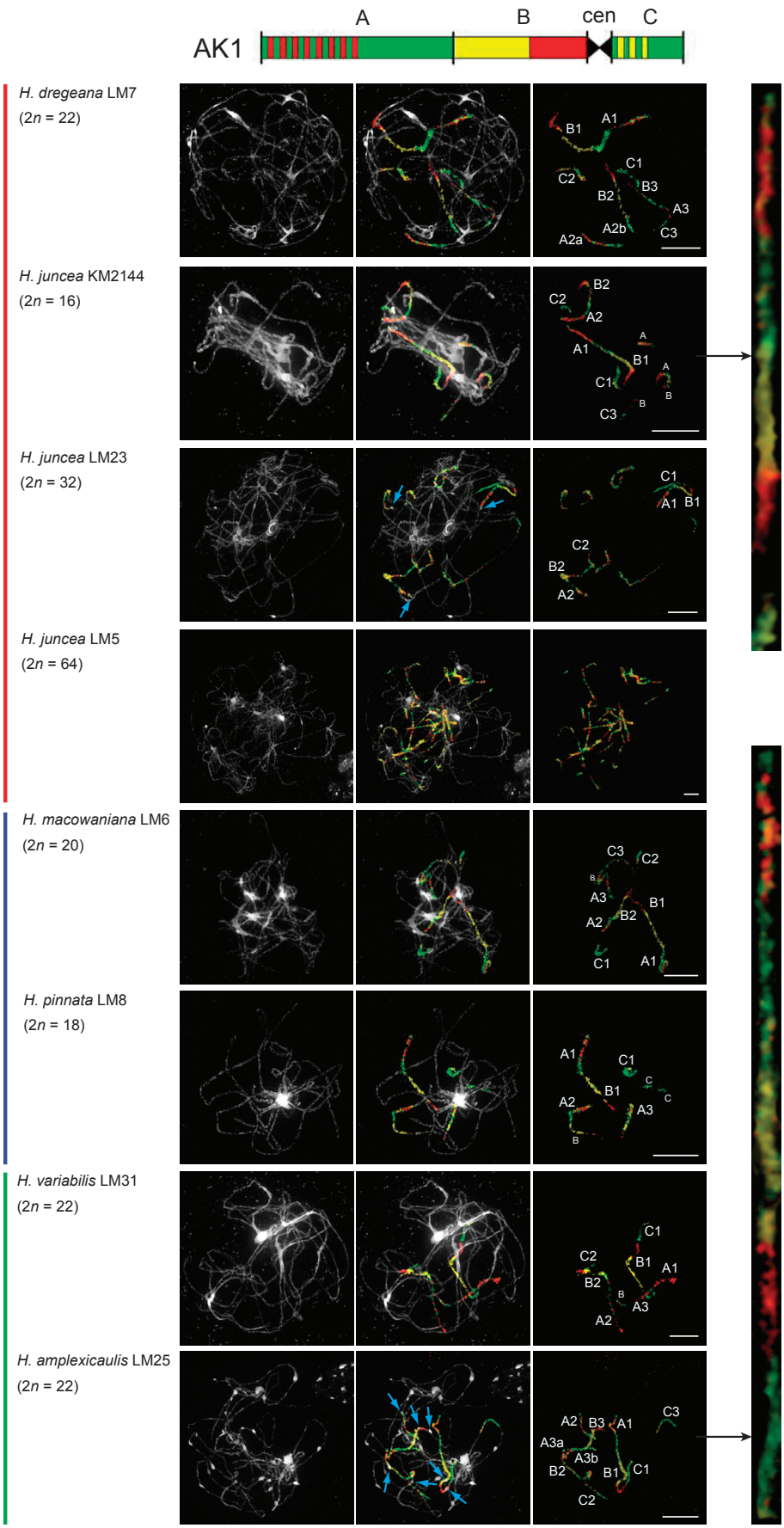


Fig. 3. Examples of comparative chromosome painting in *Heliophila* species/cytotypes with different chromosome numbers ($2n$ = 16–64) and from the three infratribal clades (A–C). *Arabidopsis thaliana* BAC contigs corresponding to AK1-specific genomic blocks A, B, and C were differentially labeled and polarized, and used as painting probes on pachytene bivalents. Triplicated GBs were tentatively assigned, for example, as A1, A2, and A3. In *H. juncea* with $2n$ = 64, the complex hybridization pattern has not been analyzed. Small capitals refer to rearranged/split blocks. Blue arrows mark heterochromatic knobs. The AK1 homeologue in the diploid *H. juncea* and in *H. amplexicaulis* was straightened and shown as enlarged images on the right. Chromosomes were counterstained with DAPI. Scale bars = 10 µm.

ancestral genomic blocks (GB), were used as painting probes on *Heliophila* pachytene chromosomes. Painting probes were polarized and differentially labeled to facilitate identification of GBs building up the ancestral chromosomes (AK1: blocks A, B, and C, Fig. 3; AK8: V, W, and X, Fig. S1). The fluorescent painting signals were strong and unambiguously identified respective homeologous chromosome regions. Most GBs were found triplicated in the analysed *Heliophila* accessions with $2n = 16$, 18, 20, and 22 (100% of A and W, 97% of C, V and X, and 93% of B were triplicated). In most species, one homeologous copy was on average longer than the other two genomic copies (see the diploid *H. juncea* cytotype in Fig. 3). In 37% of AK1 copies, A and B form the ancestral association corresponding to the upper arm of AK1. Less frequently (13% of AK1 copies) the whole AK1-like chromosome structure remained preserved (see the straightened chromosomes of *H. juncea* and *H. amplexicaulis* in Fig. 3). AK8 painting probe revealed one conserved VW and one rearranged WX association, respectively, as well as partly rearranged AK8-like chromosome in *H. macowaniana*. In both the AK1- and AK8-like chromosomes, the position of the functional centromere does not match the position of the ancestral centromere in AK1 and AK8. The ancestral structure of AK1 and AK8 in *Heliophila* was frequently altered by intrachromosomal rearrangements and translocations with other (unpainted) chromosomes, whereby AK8 homeologous copies are significantly more often rearranged than segments homeologous to AK1. Interestingly, in *H. dregeana* Sond., *H. pinnata*, *H. variabilis* Burch. ex DC. and *H. amplexicaulis*, in addition to three homeologous copies of blocks X and W, one or two very short extra copies of these blocks were also found (Fig. S1).

Chromosome painting in polyploids (Fig. 3). — Patterns of inter-species chromosome homeology to AK1 were analyzed in two polyploid accessions of *H. juncea* ($2n = 4x$, $8x = 32$, 64) and in the tetraploid accession of *H. pinnata* ($2n = 4x = 36$). In the tetraploid *H. juncea* genome, one AK1-like knob-bearing chromosome and one AB association observed in the diploid cytotype were found as two identical genomic copies. In the octoploid cytotype of *H. juncea*, at least twelve homeologous copies of blocks A, B and C were found. At least six copies of these blocks were observed in the tetraploid accession of *H. pinnata*. However, the complexity of painting results prevented us from clear conclusions on the exact number and structure of homeologous segments in these polyploid accessions.

■ DISCUSSION

Infratribal phylogeny. — The present ITS study corroborates the results published by Mummenhoff & al. (2005) and demonstrates the monophyly of the unigeneric tribe Heliophileae, with *Chamira circaeiodea* as sister species. Monophyly of Heliophileae was recently also confirmed by Couvreur & al. (2010) based on a multi-gene dataset representing the nuclear, chloroplast and mitochondrial genomes. The phylogeny presented here recognizes three main lineages (A, B and C), which correspond to the main groups detected by Mummenhoff & al. (2005) based on a smaller taxon sampling. Aspects of infrageneric classification, morphological character evolution, and eco-geographical evolution of *Heliophila* have already been discussed in detail by Mummenhoff & al. (2005).

Chromosome-number variation. — While chromosome numbers of only four *Heliophila* species have been published in the last eighty years (Jaretzky, 1932; Manton, 1932), the present study reports chromosome counts for 27 species. This still only represents less than one-third of all known Heliophileae species. Similar to many other Brassicaceae groups with small chromosomes, heterochromatic knobs and nucleolar organizing regions (often detached from chromosomes) are often erroneously counted as chromosomes or interphase chromocenters. Jaretzky (1932) already reported on difficulties with chromosome counting "in cells filled with an achromatic mass" (= chromocenters). Although chromosome numbers are known for only one-third of Heliophileae species, counts of $2n = 20$ and 22 dominate (77% of species). These chromosome numbers, though not exceptional in Brassicaceae, are restricted only to a few tribes and genera such as Anastaticeae, Brassiceae, *Iberis* L., *Leavenworthia* Torr., *Menonvillea* DC. and *Pachycladon* Hook. f. (Warwick & Al-Shehbaz, 2006). Interestingly, as in Heliophileae, $2n = 20$ and 22 are found in taxa of mesopolyploid origin, i.e., in Brassiceae (Lysak & al. 2005), *Pachycladon* (Mandáková & al. 2010a) and *Leavenworthia* (T. Mandáková and M.A. Lysak, unpub. data). Therefore, other species and genera with $n = 11$ might also have experienced mesopolyploid evolution.

Higher chromosome counts ($2n = 32$, 36, 40, 44, 60, 64, 80 and 88) represent tetra-, hexa- and octopolyploids derived from mesopolyploid diploidized cytotypes, as was shown for *H. juncea* and *H. pinnata* by our CCP results. We consider these accessions as very recent neopolyploids, representing intraspecific karyological variation. The geographic distribution of the neopolyploid cytotypes remains to be studied, and it needs to be established whether there is gene flow between different ploidy levels.

rDNA. — We analyzed the number and position of rDNA loci in 72 accessions of 27 *Heliophila* species with the aim of obtaining phylogenetically informative markers. Although some differences in the number of 45S rDNA loci between clades and individual species were observed, often identical numbers of rDNA loci preclude these markers as species-specific characters. The interstitial position of 45S rDNA, otherwise rare in Brassicaceae (Ali & al., 2005), apparently is common in Heliophileae.

Whole-genome triplication. — Triplicated homeologous chromosome regions corresponding to ancestral chromosomes AK1 and AK8 found in *Heliophila* quasi-diploid species ($2n =$ 16–22) argue for a whole-genome triplication (WGT) shared by all three clades. This was an unexpected finding, as all known chromosome numbers ($2n = 20$ and 22) as well as $2n = 16$ and 18 were implicitly considered diploid. Also, the genome size (ca. 400 Mb) of two *Heliophila* species with $2n = 20$ and 22 is comparable to many truly diploid crucifer taxa (Lysak & al., 2009). As the WGT was detected by CCP in seven *Heliophila* species from all three clades, it is apparently shared by the whole tribe and predates the diversification of Heliophileae (Fig. 4). As the homeologous chromosome regions identified correspond to

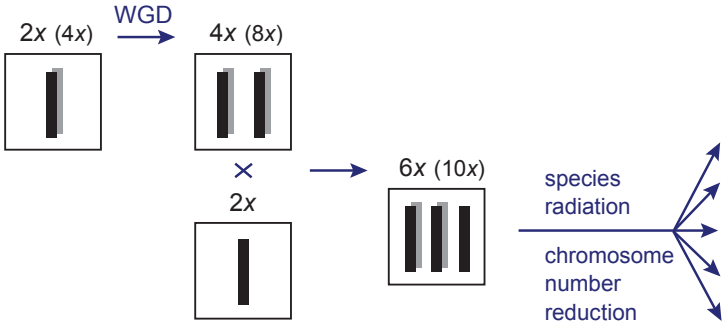
ancestral GBs and GB associations (i.e., AB of AK1 and VW of AK8) in several species, we assume that the hexaploid ancestor of Heliophileae was derived from hybridization events between two or three species with genomes resembling the Ancestral Crucifer Karyotype ($n = 8$; Schranz & al., 2006). The fact that the two homeologous copies are on average shorter than the third one, and that the chromosomes are of different size in the triplicated genomes (Jaretzky, 1932; this study), argues for an allopolyploid origin of the mesohexaploid ancestor. Similarly, the different size of homeologous chromosome segments was also reported in other mesopolyploid taxa, such as Brassiceae (Lysak & al., 2005) and Australian crucifers (Mandáková & al., 2010b). Although biased (sub)genome fractionation that usually follows ancient polyploidization events (e.g., Schnable & al., 2011) may explain the subgenome difference in *Heliophila*, we argue that a more likely scenario is that proposed for the three subgenomes in *Brassica rapa* L. (Tang & al., 2012). Analogous to *B. rapa*, the two more fractionated ("shorter") genomic copies might represent a more diploidized tetraploid ancestral genome prior the WGT, whereas the least fractionated ("longer") subgenome was brought into a hexaploid *Heliophila* by subsequent hybridization (Fig. 4). One or two very short extra copies of blocks X and W found in four diploid *Heliophila* species are more intriguing. We hypothesize that these segments may be relics of a whole-genome duplication (WGD) event predating the triplication or large segmental duplications. Clearly, more detailed analyses are needed to disentangle the genome structure of ancient diploids and the hexaploid ancestor.

The Heliophileae-specific WGT is yet another example of a lineage-specific mesopolyploid event recently identified in Brassicaceae. Mesopolyploid triplication has been described in the ancestry of Brassiceae (Lysak & al., 2005, 2007; Tang & al., 2012), whereas mesotetraploid events mark the evolution of *Orychophragmus* Bunge (Lysak & al., 2007), Australian endemic crucifer genera (Mandáková & al., 2010b) and *Pachycladon* Hook. f. (Mandáková & al., 2010a).

WGT and karyotypic diploidization. — The WGT has been followed by descending dysploidy (chromosome-number reduction) towards the diploid-like chromosome complements found in extant *Heliophila* species. The diploidization process during which the presumably more than 40 chromosomes of the mesohexaploid ancestor were reduced to only 16 to 22 must have been accompanied by extensive chromosome rearrangements, centromere loss, and by changes at the epigenetic and sequence levels. Similar to the Australian and New Zealand crucifers (Mandáková & al., 2010a, b), the overall collinearity of AK1 and AK8 in *Heliophila* species was retained, whereas the ancestral centromeres were deleted or lost their function. In some species analyzed, the positions of inactive paleocentromeres coincide with interstitial heterochromatic knobs (Fig. 3; Fig. S1), and knobs on several other chromosomes may also be remnants of inactive centromeres (e.g., Fig. 2B). The exact mechanism of centromere elimination in Brassicaceae taxa remains to be studied.

Has the whole-genome triplication been a driver of the species radiation in Heliophileae? — Factors that might promote speciation and/or determine speciation rates of Cape clades are intrinsic and extrinsic (Barraclough, 2006). These factors include climatic changes supposed to generate an array of responses including phenology shifts and migration facilitating both fragmentation as well as re-union of populations, potential geographical (topographic in the first place) isolating barriers (e.g., mountain ranges, deep river valleys), steepness of ecological and altitudinal gradients, regional edaphic (e.g., soil types, geology) and disturbance heterogeneity (specially fire regimes differing in spatial distribution, frequency and severity) and last but not least a plethora of biotic interactions including mycorrhiza and related phenomena and animal–plant interactions especially pollinators, pollination and dispersal (Linder, 2005; Barraclough, 2006; Verboom & al., 2009; Warren & al., 2011; Schnitzler & al., 2011; Mucina & Majer, 2012), but generally also intrinsic factors such as WGD events (Franzke & al., 2011). Consequently, the speciation rate of a particular lineage will depend on the interaction between a wide range of intrinsic and extrinsic factors.

Heliophila, as many other lineages in the CFR, has probably undergone an adaptive radiation and has diversified at the highest rates calculated for a dated Cape radiation (Warren & Hawkins, 2006). Using different methodologies and calibration concepts, the radiation was dated to 1.0–5.64 Ma (Mummenhoff & al., 2005; Verboom & al., 2009; this study, see Table 1), and all datings indicate a recent diversification against a background of increased aridity in the Late Pliocene and of alternation of cold and warm (= dry and wet, respectively)



	meso-		neo-mesopolyploid genomes		
clade	6x (10x)	WGD →	12x (20x)	WGD →	24x (40x)
B	2n = 16		2n = 32		2n = 64
A	2n = 18		2n = 36		
A	2n = 20		2n = 40		2n = 80
B+C	2n = 22		2n = 44		2n = 88
C	2n = 26				

Fig. 4. Model of genome evolution in Heliophileae (see Discussion for details). A haploid genome is shown for each putative ancestral species. Additional genome copies detected in some diploid species by CCP (Fig. S1) are depicted as grey bars and reflected by ploidy levels in parentheses.

climatic periods of the Pleistocene. As suggested by Linder (2003), the radiation of many lineages in the Cape region resembles evolutionary processes of island-species radiation. The Cape seed plant endemism of almost 70% is comparable to that found on islands. Furthermore, a high contribution by a very small number of clades to overall species richness is also typical of island floras (Linder, 2003). This indicates a great degree of isolation of the CFR. While islands are isolated by water, the CFR differs in climate, topography and soil types from the rest of southern Africa and is surrounded by oceans on three sides (Marloth, 1929; Linder, 2003). To our knowledge, no other example in Brassicaceae exhibits such a species-rich radiation confined to a relatively small region as does tribe Heliophileae.

Similar to the WGT event specific for tribe Brassiceae and its apparent impact on the infratribal diversification (238 spp. in 47 genera), we link cladogenesis, species radiation and the WGT event in Heliophileae. Ancient polyploidization and subsequent diploidization have been repeatedly shown as driving forces of genetic diversification and radiation in plant lineages (e.g., Barker & al., 2008; Soltis & al., 2009; Jiao & al., 2011). For example, gene duplication, neofunctionalization or loss may facilitate reproductive isolation and speciation. The paleopolyploid event (α) that occurred very early in the history of Brassicaceae provided the genetic raw material for radiation, and diversification rates that are among the highest reported for flowering plants (Franzke & al., 2011). We suggest that the Miocene onset of global aridity (Zachos & al., 2001; Guo & al., 2002; Liu & al., 2009) heralded the advent of a prolonged climatically unstable period punctuated by periods of enhanced aridity and later (towards Pliocene) associated with climatic oscillations. These dynamics, driven by changes in periodicity of Milankovich oscillations (Raymo & Huybers, 2008), and boasts of increased aridity during the Pleistocene (deMenocal, 2004), set the scene for the remarkable radiation of *Heliophila*. The progressing aridity presumably resulted in opening of the formerly forested landscapes (Axelrod & Raven, 1978) and the creation of more sun-exposed ('heliophilous') habitats, which underwent further changes (including alterations such as expansion, contraction, obliteration, formation) in reaction to the dry-wet cycles of the Pleistocene. Repeated isolation of populations (fragmentation) and putative reunion of migrating populations could then have acted as an 'evolutionary pump' (see also Plana, 2004 and Compton, 2011 for applications of this concept). Environmental heterogeneity, including geological and topographical complexity of the region, steep ecological gradients (see Linder, 2003; Linder & al., 2010), various large-scale disturbance factors (Rebelo & al., 2006) and small-scale animal–plant interactions (Van der Niet & al., 2006) are presumed to have been of vital importance in propelling this evolutionary pump. Phenological responses, i.e., shifts in mean flowering time (from summer towards spring) and flowering time duration, consistent with wet/dry climatic cycles, may also have contributed to the species diversity in *Heliophila* and other Cape lineages (Warren & al., 2011). We argue that the purported whole-genome triplication occurred prior to the origin of the three *Heliophila* lineages and facilitated radiation in changing environment.

Dating the radiation of Heliophileae. — Molecular dating within Brassicaceae has been limited and controversial due to a lack of fossils and calibration options. Different age estimates for the origin of Brassicaceae and its major lineages are given in Beilstein & al. (2010) and Franzke & al. (2011). Using different approaches to calibrate phylogenetic trees, or different algorithms, Mummenhoff & al. (2005) arrived at age estimates of 1.0–4.4 Ma for the crown group age of Heliophileae (Table 1). Our new and expanded ITS dataset was dated with an uncorrelated relaxed molecular clock approach (BEAST) using different calibration settings, e.g., direct or primary fossil calibration (*Rorippa*/*Cardamine* split) and secondary calibration (age of the Cleomaceae/Brassicaceae split). The age of the radiation of *Heliophila* was inferred to be ca. 3.4 (1.22–5.64) Ma, which agrees with previous studies (Table 1), all dating the diversification of *Heliophila* to the Pliocene and Pleistocene. This corresponds with findings by Warren & Hawkins (2006) who suggested that *Heliophila* diversified at the highest rates calculated for a dated Cape radiation.

Phylogenetic relationships of Heliophileae. — Nothing is known about the closest relatives of Heliophileae. However, based on the average age of the origin of *Heliophila* (1.9–7.8 Ma; Table 1), one might speculate that ancestors of *Heliophila*—like those of South African endemic *Lepidium* species—entered the African continent from southwest Asia, the presumed cradle of Brassicaceae (Franzke & al., 2011). From here they may have reached southern Africa by several putative routes, including the East African route (Hedge, 1976) and the (more probable) Arid Corridor (Verdcourt, 1969). The latter may have functioned as an intermittent link between the semi-deserts and deserts of North Africa and Arabia and the emerging arid regions of southern Africa since the Late Miocene (about 7 Ma) when the Sahara Desert started to emerge as a major arid biome (Schuster & al., 2006; Mummenhoff & al., 2001). Clearly, the closest relatives of Heliophileae are in the expanded lineage II sensu Franzke & al. (2011), and of the other 24 tribes included in that lineage, Brassiceae and Sisymbrieae, which also show some diversification in South Africa, are more likely candidates than other tribes.

Polyploidy in the evolution of Cape flora. — Based on available surveys of chromosome numbers and ploidy levels, polyploidy was considered to have only minor importance for radiations of genera centered in the CFR (Goldblatt & al., 1993; Goldblatt & Takei, 1997; Goldblatt & Manning, 2011; Prebble & al., 2011; P. Goldblatt, pers. comm.). The Heliophileae is the first group of the Cape flora where the prominent role of ancient and more recent polyploidization events has been demonstrated. Multiple base numbers in Bruniaceae (Goldblatt 1981) suggest that ancient WGD events played an important role in the evolution of this family, an endemic to the CFR. In *Oxalis* L., remarkable intraspecific ploidy variation was found in nearly 30% of Cape species investigated (Suda & al., 2012). However, it is premature to draw major conclusions on the role of WGD events in the evolution of Cape genera as karyological data are limited and no in-depth genomic studies are available. Prior to this study only four chromosome numbers were known for *Heliophila* (>90 spp.), nine species have been

counted in *Erica* (ca. 680 Cape spp., Pirie & al., 2011), and only three chromosome counts are available for the 350 species of Restionaceae (Linder, 2003 and references therein).

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