Patterns of Transmission Ratio Distortion in Interspecific Lettuce Hybrids Reveal a Sex-Independent Gametophytic Barrier

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ABSTRACT Interspecific crosses can result in progeny with reduced vitality or fertility due to genetic incompatibilities between species, a phenomenon known as hybrid incompatibility (HI). HI is often caused by a bias against deleterious allele combinations, which results in transmission ratio distortion (TRD). Here, we determined the genome-wide distribution of HI between wild lettuce, *Lactuca saligna*, and cultivated lettuce, *L. sativa*, in a set of backcross inbred lines (BILs) with single introgression segments from *L. saligna* introgressed into a *L. sativa* genetic background. Almost all BILs contained an introgression segment in a homozygous state except a few BILs, for which we were able to obtain only a single heterozygous introgression. Their inbred progenies displayed severe TRD with a bias toward the *L. sativa* allele and complete nontransmission of the homozygous *L. saligna* introgression, *i.e.*, absolute HI. These HI might be caused by deleterious heterospecific allele combinations at two loci. We used an multilocus segregating interspecific F2 population to identify candidate conspecific loci that can nullify the HI in BILs. Segregation analysis of developed double-introgression progenies showed nullification of three HI and proved that these HI are explained by nuclear pairwise incompatibilities. One of these digenic HI showed 29% reduced seed set and its pattern of TRD pointed to a sex-independent gametophytic barrier. Namely, this HI was caused by complete nontransmission of one heterospecific allele combination at the haploid stage, surprisingly in both male and female gametophytes. Our study shows that two-locus incompatibility systems contribute to reproductive barriers among *Lactuca* species.

KEYWORDS hybrid sterility; transmission ratio distortion; Dobzhansky-Muller; postzygotic reproductive barrier; epistasis

UNDERSTANDING the genetic basis of speciation is an important topic in evolutionary biology. Speciation often starts with a reduction of gene flow between lineages due to reproductive barriers. These barriers can arise by divergent ecological or sexual selection, or by the evolution of genetic incompatibilities (Seehausen *et al.* 2014; Baack *et al.* 2015). Ecological adaptation may play only a minor role in the divergence between species, whereas purely mutational mechanisms play a larger role (Maheshwari and Barbash 2011). When diverging lineages hybridize, the genes underlying hybrid incompatibilities "meet" in the hybrid background. These heterospecific genetic incompatibilities can hamper proper development of the organism, resulting in hybrid inviability, sterility, weakness, or necrosis, collectively known as hybrid incompatibility (HI). In general, the genetic and molecular mechanisms behind HI are not well characterized. More insights into the underlying genetics of postzygotic HI may provide clues to the evolutionary forces and molecular mechanisms that lead to the formation of different species (Coyne and Orr 2004).

Postzygotic isolation can be caused by a difference in ploidy level, or by genetic mechanisms such as chromosomal rearrangements and mutational processes (Coyne and Orr 2004; Rieseberg and Willis 2007; Hoffmann and Rieseberg 2008). The latter is the most common mechanism that leads

Copyright © 2019 by the Genetics Society of America
doi: https://doi.org/10.1534/genetics.118.301566
Manuscript received August 31, 2018; accepted for publication October 30, 2018; published Early Online November 6, 2018.
Supplemental material available at Figshare: https://doi.org/10.25386/genetics.7296863.
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to the evolution of HI through the accumulation of dysfunctional genic interactions (Presgraves 2010; Maheshwari and Barbash 2011). A model that explains how HI can evolve without species themselves having a reduced fitness was formulated by Bateson, Dobzhansky, and Muller and is commonly referred to as the Dobzhansky-Muller (DM) model (Orr 1996; Bomblies 2013, Fishman and Sweigart 2018). The DM model states that each pair of interacting genes evolves independently in separate lineages, and deleterious interactions between them only occur in hybrids and/or in their derived progeny as a secondary consequence of intraspecific divergence (Bateson 1909; Dobzhansky 1937; Muller 1942). Pairwise incompatibilities are the result of epistasis—a phenomenon in which the effect of one gene is dependent on the presence of one or more other genes, *i.e.*, the genetic background. Sporadically single locus DM interactions also occur by deleterious interactions between variants of a single gene (Todesco *et al.* 2014). Genetic changes that are adaptive, or nearly neutral, in their own genomic background can be functionally incompatible with alleles from a foreign genomic background (Presgraves 2010).

DM incompatibilities can evolve by various processes. One of these processes is gene duplication, followed by loss of function in one of the redundant gene copies (Bikard *et al.* 2009; Zuellig and Sweigart 2018). When functional genes end up in different genomic locations in related species, some individuals of interspecific hybrid-derived progenies may inherit only nonfunctional copies. Another process is internal genetic conflict, often caused by selfish genes (Presgraves 2010). Selfish genes can cause segregation distortion in their own favor, but with deleterious effects on their host, for instance, through meiotic drive and gamete-killing. Hosts can evolve suppressor genes to compensate for these negative effects. In interspecific hybrid-derived progenies, recombination may uncover the effects of a selfish gene when it is present without its suppressor (Presgraves 2010).

Many studies on the genetics of speciation have focused on *Drosophila* (Castillo and Barbash 2017), but studies have also been conducted on other organisms including yeast, nematode, parasitic wasp, salamander, African clawed frog, and plant species such as *Arabidopsis*, rice, wheat, and *Mimulus* (Hermsen 1963; Harushima *et al.* 2001; Niehuis *et al.* 2008; Wu *et al.* 2008, Leppälä *et al.* 2013; Snoek *et al.* 2014; Hou *et al.* 2015; Niedzicka *et al.* 2017; Gibeaux *et al.* 2018). DM genes that cause hybrid lethality or sterility have been reviewed by Presgraves (2010), Rieseberg and Blackman (2010) and Maheshwari and Barbash (2011). In plants, DM incompatibilities have been identified among genes involved in disease resistance and among cytonuclear interactive genes, leading to hybrid necrosis and cytoplasmic male sterility, respectively (Rieseberg and Blackman 2010; Fishman and Sweigart 2018). However, only a limited number of DM genes have been studied, and we are still only beginning to understand the molecular basis of HI (Fishman and Sweigart 2018).

Apart from a fundamental interest in reproductive barriers because of their influence on the evolution of species, reproductive barriers have a practical impact on the improvement of crops with genes from wild relatives. The narrow genetic base of many crops has become a major constraint in crop improvement. Introgression of genetic material from wild relatives or exotic accessions of the same species is an attractive natural means to broaden crop genetic resources (Hajjar and Hodgkin 2007). However, intraspecific or interspecific HI can result in a complete or incomplete nontransmission of certain genotypes. This is often observed through distortion of Mendelian segregation of genotypes and alleles in hybrid-derived progeny. Such transmission ratio distortion (TRD) is frequently observed in interspecific segregating populations (Harushima *et al.* 2001; Myburg *et al.* 2004; Moyle and Graham 2006; Chandnani *et al.* 2017). TRD can severely hamper the exchange of genetic variants between and within species. Therefore, understanding the mechanisms responsible for TRD is important for the introgression of agriculturally interesting alleles (Hajjar and Hodgkin 2007).

Transmission ratio-distorted loci (TRDL) colocate with HI loci more frequently than is expected by chance (Moyle and Graham 2006). Therefore, TRDL have been used as indicators of genetic incompatibilities in several plant species (Fishman *et al.* 2001; Leppälä *et al.* 2013; Brennan *et al.* 2014; Kerwin and Sweigart 2017). However, TRD is not always a sign of HI, but may also occur due to various other reasons before or after fertilization, like meiotic drive (Lyttle 1991), competition between gametes (Howard 1999), or competition between embryos (Korbecka *et al.* 2002). Genome scan approaches are also used to identify the candidate barrier loci involved in the reduction of gene flow between species (Seehausen *et al.* 2014). Regions of high genomic differentiation between species are often assumed to be related to reproductive barriers. However, in order to conclusively identify HI, additional experimental evidence, such as transgenic approaches or experimental crosses, is necessary (Ravinet *et al.* 2017).

Evidence of an association between hybrid sterility or inviability and a certain genotype can be provided by genetic mapping (*e.g.*, QTL analysis) in segregating populations or in libraries of single introgression lines, which contain a chromosomal segment of one species in the genetic background of another species (also referred to as backcross inbred lines, BILs). Because of an almost pure genetic background, single introgression lines can be very useful for validation and fine-mapping of HI genes, but cannot identify interlocus interactions in contrast to multilocus segregating populations (Maheshwari and Barbash 2011).

Here, we studied an interspecific cross between two distantly related autogamous, diploid *Lactuca* species: wild lettuce *L. saligna* and cultivated lettuce *L. sativa*. Apart from fundamental insights into the genetic basis of *Lactuca* divergence, knowledge of HI between wild and cultivated *Lactuca* species might be useful for breeding horticulturally interesting wild lettuce alleles into cultivated lettuce. Previously an

easily recognizable form of HI, hybrid necrosis, has been observed in an interspecific F2 population and in a BIL of the cross *L. saligna* × *L. sativa*. This hybrid necrosis was explained by a digenic epistatic interaction that acted as a zygotic barrier and resulted in reduced viability by autoimmunity (Jeuken *et al.* 2009).

Many studies use a multilocus segregating population to identify TRD loci and/or hybrid sterility loci as an indicator of the number of DM incompatibilities. Our starting point was a set of BILs, each BIL containing one or few chromosomal segment(s) from the wild parent *L. saligna* in the genomic background of the cultivated parent *L. sativa*. While monitoring the set of BILs for introgression of *L. saligna* segments into *L. sativa* DNA, we came across regions that were associated with distorted segregation and showed an absolute absence of the occurrence of homozygous *L. saligna* segments in a homozygous *L. sativa* background. We hypothesized that this exclusion of specific homozygous *L. saligna* introgression segments was explained by a complete nontransmission of heterospecific allele combinations, which can be considered as HI. We validated whether HI loci in BILs overlapped with TRDL in interspecific F2 and BC1 populations. Next, the F2 population was used to find potential conspecific genetic loci or "dance partners" (Moyle and Graham 2006) that can nullify HI, and double introgression lines were developed to validate these conspecific loci.

Here, we investigated the occurrence and the cause of HI in regard to the introgression of *L. saligna* DNA into a *L. sativa* genetic background. We addressed the following main questions: how many HI loci are present in the set of BILs? How many TRDL are present in the F2 and BC1 populations, and which of these TRDL were predictive for absolute HI in our set of BILs? By which genetic mechanism can these cases of HI be explained? Through answering these questions, we have taken the first step towards identifying the genes that cause reproductive isolation between *Lactuca* spp.

Materials and Methods

Plant materials

An F2 population of 126 plants was derived from a cross between wild parent *L. saligna* CGN05271 (mother) and cultivated parent *L. sativa* cv Olof (Jeuken *et al.* 2001). F1 plants of the same cross were backcrossed as a mother to the cultivated parent, resulting in a BC1 population of 88 plants designated as BC1cult, and to the wild parent, resulting in a BC1 population of 33 plants designated as BC1wild.

BILs with wild parent chromosomal segments introgressed into cultivated lettuce were developed by four to five generations of backcrossing to the cultivated parent followed by a minimum of one generation of selfing (Jeuken and Lindhout 2004). In this paper, a BIL number reflects the linkage group (LG) number where the wild lettuce introgression segment resides, according to the *L. sativa* cv. Salinas genome map (Reyes-Chin-Wo *et al.* 2017), and letters discriminate among segments on the same LG (Supplemental Material, Figure S1).

Genetic nomenclature

Alleles of wild lettuce, *L. saligna*, are referred to as "w" and alleles of cultivated lettuce, *L. sativa*, as "c." Consequently, genotypes are "cc": homozygous *L. sativa*, "cw" or "wc": heterozygous, and "ww": homozygous *L. saligna*.

DNA isolation and genotyping

DNA was isolated by either a high-throughput NaOH method (Wang *et al.* 1993) or a CTAB method (van der Beek *et al.* 1992). For genotyping, we used expressed sequence tag (EST)-based markers and KASPar markers based on single nucleotide polymorphisms (SNP)s between *L. sativa* and *L. saligna*. The SNPs were obtained by mapping Illumina paired-end reads from *L. sativa* cv. Olof and a pool of five *L. saligna* accessions (CGN05304, CGN05318, CGN15705, CGN15726, and 275-5) against the *L. sativa* cv. Salinas genome version 8 (Reyes-Chin-Wo *et al.* 2017) using BWA-mem, version 0.6.3 (Li and Durbin 2009), with default settings. SNP calling was performed using Freebayes, version v1.0.2-29 (Garrison and Marth 2012) with default parameters. Subsequently, the SNPs were filtered with SNPsift version 4.3 (Cingolani *et al.* 2012), with parameters: RPL&RPR >1, SAF&SAR >1, PAIRED&PAIREDR >0.8, $6 <$ DP > 20, isHom&isRef for the *L. sativa* cv. Olof reads and isHom&isVariant for the *L. saligna* pooled reads. Flanking sequences were checked against the reference genome (*L. sativa* v8) using BLASTn (Altschul *et al.* 1990) to select for unique sites. The criterion for a SNP was: the same base for cv Salinas and cv Olof and the same alternative base in all reads of *L. saligna* accessions. From a collection of 9000 identified SNPs, we selected 293 genome-wide SNPs (with an average distance of 3.7 cM between markers) and seven chloroplastic SNPs for KASPar assays. For EST-based markers (Table S1), polymorphisms between PCR products of *L. saligna* and *L. sativa* alleles were visualized by high-resolution melting curve differences on a LightScanner System (den Boer *et al.* 2014) or by gel electrophoresis. KASPar markers (Table S2) were designed and used for genotyping by Dr. Van Haeringen Laboratorium B.V., Wageningen, The Netherlands.

Genetic map of interspecific F2 population

KASPar markers were added to our latest genetic linkage map [based on EST and amplified fragment length polymorphism (AFLP)-markers] of the F2 population from the cross *L. saligna* CGN05271 × *L. sativa* cv. Olof (Jeuken *et al.* 2001). Linkage analyses were performed using JoinMap v5 software (Van Ooijen 2006). A new consensus genetic linkage map was calculated per LG using regression mapping and Kosambi's mapping function with default settings: linkages with a recombination frequency <0.40, LOD scores >1, a jump threshold of 5, and a third round. Marker intervals for studied traits in all populations were based on this F2 population consensus map. Physical map locations refer to the *L. sativa*

cv. Salinas reference lettuce genome v8 (Reyes-Chin-Wo *et al.* 2017); https://lgr.genomecenter.ucdavis.edu/). Here, we use the LG numbering and orientation of that reference *L. sativa* physical map, which differs from the numbering used in our previous publications. In order to relate previously reported gene and marker locations to the mapped loci in the current study, we present a conversion table (Table S3).

Detection of TRD in segregating populations

Observed genotype frequencies were compared to the expected Mendelian ratio of 1:2:1 in the F2 population, or to the Mendelian 1:1 ratio in BC1 populations. Assuming that the nine homologous chromosomes of lettuce contain at least two independent regions, at least 18 independent genomic regions were expected. To correct for genome-wide testing, we applied a threshold of $\alpha = 0.05/18 = 0.003$ for assigning TRD. For the F2 population, chi-square tests were performed per marker. Regions with three or more distorted consecutive markers were considered as regions with TRD. As BC1 populations were genotyped with only ~80 markers, our criterion of at least three distorted markers may be too strict; therefore, regions with at least one distorted marker were considered as TRD regions.

Indication of zygotic or gametophytic barriers

Reproductive barriers may be zygotic or gametophytic. As explained in Figure S2, distortion of heterozygote frequency may indicate a zygotic barrier (or possibly two gametophytic barriers, one affecting the male and the other affecting the female gametophyte), whereas nondistortion of heterozygote frequency may indicate a gametophytic barrier (Figure S2). For each representative marker in a distorted region in the F2 population, the observed heterozygote frequency and the sum of the two homozygote frequencies were compared to the expected Mendelian ratio of 1:1 in a chi-square test at $\alpha = 0.05$.

Identification of digenic interactions

The genotype dataset of the F2 population was used to identify candidate conspecific loci that can nullify the TRD that led to HI in specific BILs. F2 individuals with a homozygous *L. saligna* introgression were selected for each HI region separately from a total of 126 F2 plants. Within these selected subsets of F2 plants, we scanned the F2 genotype data marker-by-marker for all LGs in search of loci with at least one conspecific (*L. saligna*) allele for each F2 individual. These loci were considered as candidate interacting partners of the HI gene (*i.e.*, "dance partners," Moyle and Graham 2006). Crosses were made between BIL plants with a heterozygous segment at an HI region and the BILs containing their candidate interacting locus in a homozygous wild parent segment. A limited number of candidates were tested per HI. After selfing, the F1 plant of each cross, segregation of the HI region was assessed in a subset of inbred plants that were homozygous *L. saligna* for the candidate interactive locus. If segregation was Mendelian (no TRD) at the HI locus, we considered this as proof of nullification of HI through the presence of a conspecific digenic interaction.

HI on LG 8, segment 8A

Segregation analysis: Segregation of segment 8A and 4A was studied in an inbred progeny ($n = 691$) of an F1 plant (double heterozygote, genotype "4cw8cw") from the cross: BIL4A +8A × *L. sativa* cv Olof. This inbred population is named "F2_4A8A". Individuals of "F2_4A8A" were genotyped with two markers per segment to determine segregation ratios of the nine expected genotypes: markers NL1151 and NL0897 on LG4 and markers M7120 and LE1211 on LG8. Individuals of "F2_4A8A" with crossovers between either pair of markers ($n = 56$) were excluded. We also determined the segregation of segments 8A and 4A individually in separate lines with a *L. sativa* background. Segregation of segment 8A was assessed in inbred progeny ($n = 545$) of a plant with a heterozygous 8A segment and a homozygous 4A segment (genotype "4ww8cw"). Segregation of segment 4A was assessed in inbred progeny ($n = 118$) of a plant that contains only the 4A segment in a heterozygous state (genotype "4cw8cc").

Hypothesis testing: The pattern of TRD in the "F2_4A8A" may reveal an explanation for the HI associated with the 8A segment. Six hypotheses for the HI based on gametophytic and/or zygotic barriers were tested by chi-square tests at $\alpha = 0.05$. The hypotheses are: hypothesis 1 (H1) Mendelian segregation of 1:2:1 with expected allele frequencies of 0.5 for both loci; hypothesis 2 (H2) distorted segregation by nontransmission of male and female gametophytes with the heterospecific genotype "4c8w"; hypothesis 3 (H3) distorted segregation by lethality of three absent genotypes "4cc8cw", "4cc8ww", and "4cw8ww"; hypothesis 4 (H4) distorted segregation by nontransmission of either male or female gametophytes with heterospecific genotype "4c8w"; hypothesis 5 (H5) hypotheses 3 and 4 combined; hypothesis 6 (H6) distorted segregation by observed allele frequencies that were deviant from expected allele frequencies of 0.5 on both loci.

To validate the nonrejected hypothesis (number 2) of distorted segregation in "F2_4A8A", we tested for TRD in two backcross populations from a reciprocal cross: the double heterozygote "4cw8cw" was crossed to BIL4A ("4ww8cc") reciprocally. Only the gametophytes of the double heterozygote segregate, and, therefore, their maternal or paternal effects on the segregation ratios, can be observed separately in these backcross populations. Observed segregation ratios of these two BC1 populations ($n = 117$ and $n = 140$ plants) were tested against the hypothesized segregation by a chi-square test ($\alpha = 0.05$).

Pollen vitality and seed set were assessed as a phenotypic validation of hypothesis 2. To test pollen vitality, capitula that had just starting flowering were collected from the double heterozygote "4cw8cw" and from the control genotype *L. sativa* cv Olof. From the same genotypes, developing capitula (flower buds) of 2–3 mm in length were collected to observe tetrads. Capitula were dissected individually and examined

microscopically after treatment with Alexander stain, which differentially stains aborted and nonaborted pollen (-Alexander 1980; Peterson *et al.* 2010). Seed set was assessed in *L. saligna* CGN05271, *L. sativa* cv Olof, the double heterozygote “4cw8cw”, and recombinants of the double heterozygote. Per plant, at least five capitula that flowered on the same day were labeled. For unique recombinant genotypes, at least 10 capitula that flowered on the same day were labeled. The number of seeds (achenes) per labeled capitulum was counted to determine the percentage of aborted and nonaborted seeds. An aborted seed was distinguished as a thin, empty seed coat. Statistical differences were tested by ANOVA followed by a Tukey HSD test in Genstat 18th edition.

Mapping: The HI conferred by a deleterious heterospecific combination of genes on segment 4A and 8A was first identified in the “F2_4A8A” (F2 of cross BIL4A8A × *L. sativa* cv. Olof) and delineated by the borders of the introgression segments 4A and 8A. Map intervals were reduced by mapping the specific pattern of TRD in the interspecific F2 population (*L. saligna* CGN05271 × *L. sativa* cv. Olof) with additional markers. Phenotyping (seed set analysis) and/or genotyping the recombinant offspring of population “F2_4A8A” further reduced the map interval by two approaches described in the *Results*.

Data availability

The Illumina raw read files for *L. sativa* cv. Olof and for pooled *L. saligna* accessions are available through the NCBI Short Read Archive (BioProject ID PRJNA434185). Table S2 lists SNPs sequences for genotyping. All other data necessary for confirming the conclusions of this article are presented fully within the article and its tables and figures. Supplemental material available at Figshare: https://doi.org/10.25386/genetics.7296863.

Results

HI in single introgression lines (BILs)

Previously, BILs with wild (*L. saligna*) segments in cultivar (*L. sativa*) background have been produced by several generations of backcrossing with *L. sativa*, starting from the F1 generation and ending with at least one generation of selfing (Jeuken and Lindhout 2004). Recently, one newly developed BIL was added (BIL6D). Each BIL contains one, or a few, homozygous *L. saligna* introgression segments in a *L. sativa* background, except four lines that harbor only a heterozygous introgression segment at one region. Genotyping with 300 new KASPar markers, including chloroplast markers, gave a more detailed picture of the *L. saligna* introgressions and the cytoplasmic genotype present in the BILs (Figure S1). The majority of the BILs contained *L. sativa* cytoplasm. A few missing *L. saligna* segments were revealed at distal chromosomal ends that went unnoticed during earlier BIL development: the top of LG2 and the bottom of LG5. Absence of an introgression at the top of LG7 was already observed by Jeuken and Lindhout (2004). Probably it was already lost in one of the early backcross generations (BC2 or BC3). We did not study the top of LG7 further.

For four genomic regions, we were only able to obtain a line with a single heterozygous introgression in a *L. sativa* background, *i.e.*, introgression segments: 7B, 8B, 9A, and 9C (Figure S1). Their inbred progenies showed TRD with a complete lack of individuals with a homozygous *L. saligna* segment (Table S4). This indicated a complete nontransmission of a heterospecific allele combination, *i.e.*, homozygous *L. saligna*-genotype at HI locus and homozygous *L. sativa*-genotype at an unknown interacting locus. We consider this complete nontransmission of a homozygous *L. saligna* segment as absolute HI. HI regions were based on the borders of the heterozygous segment.

The *L. saligna* introgression segments (homozygous or heterozygous) in our set of BILs cover 90% of the *L. saligna* genome. Consequently, in 10% of the genome, HI could not be determined due to absence of a *L. saligna* introgression. This may be caused by a lack of genetic markers in this region at the time of selection and/or due to loss of the introgression segment in an early backcross generation. Therefore, the current number of identified HI may be an underestimate.

HI in the interspecific F1 hybrid and its offspring

Our strongest evidence of HI was observed in the set of BILs, but symptoms of hybrid inviability and sterility were already visible in the F1 and/or F2 generation. F1 seeds from the cross *L. saligna* CGN05271 (female) × *L. sativa* cv. Olof (male) showed normal germination, and the F1 plants were phenotypically intermediate between the parents (Figure 1A). However, the F1 fertility was severely decreased as it showed only 2% of the seed set of the parents (Figure 1B and Table S5). The F2 progeny (162 seeds) of one F1 plant was further characterized for symptoms of HI; 22% of F2 seeds did not result in adult plants due to seed death (no germination) or hybrid inviability (early plant death). Of the 126 adult F2 plants, 11% showed hybrid necrosis and ~10% showed malformed growth (hybrid weakness). The F2 plants showed variation in fertility, from complete sterility to severely reduced seed sets of 2–13% of the parental seed set (Table S6). All these aberrant F2 plant phenotypes are likely associated with genetic incompatibilities between the two species.

TRDL in segregating populations F2 and BC1

Marker TRDs in segregating populations are usually a consequence of deleterious allele combinations that cause HI. To verify whether TRDs in segregating populations would have been predictive for HI in our set of BILs, we characterized TRD in available F2 and BC1 populations, which all contained *L. saligna* cytoplasm (confirmed with chloroplast markers). Ten TRDL were indicated by chi-square tests ($P < 0.003$) after genome-wide genotyping of the F2 population ($n = 126$) with 492 markers (Figure 2 and Table 1). Heterozygote frequency analysis indicated that five TRDL may be due to a zygotic (or possibly sex-independent gametophytic) barrier,

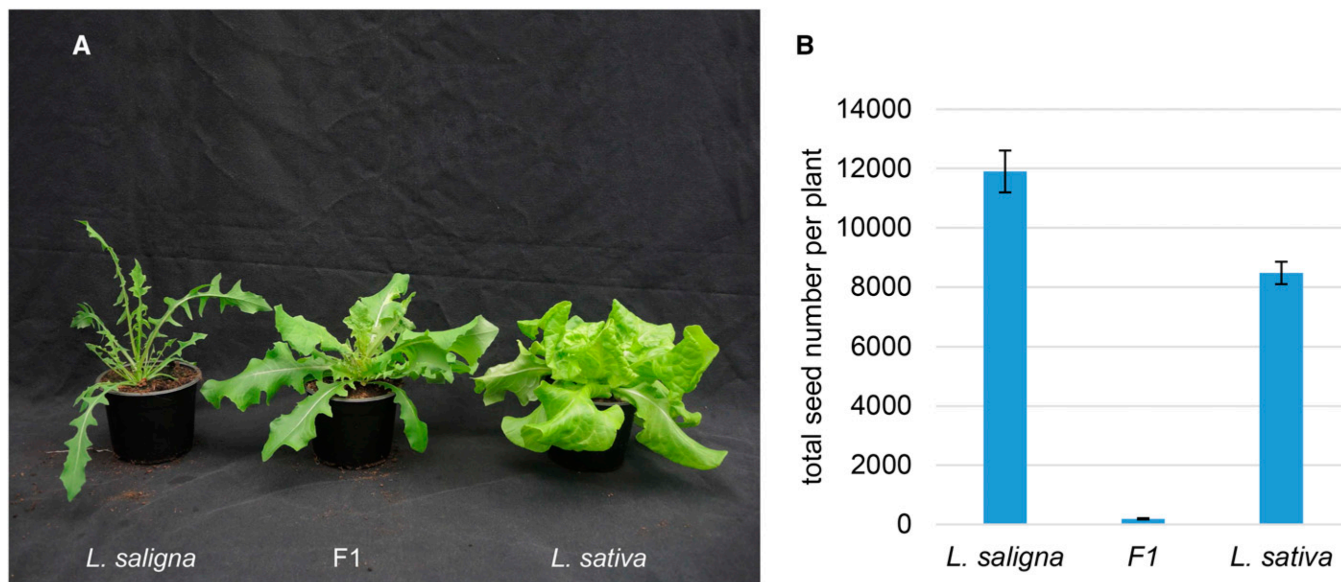

Figure 1 Viability and fertility of the interspecific F1 and its parental lines. (A) Viability at 5 weeks of age, (B) average estimated total number of seeds per plant, based on three plants per genotype (details in Table S5).

and five TRDL may be due to a male or female gametophytic barrier (Figure S2 and Table 1). Of the 10 TRDL in the F2 population, 4 were validated in one or both BC1 populations (Figure S3, Table S4, and Table 1). Each BC1 population displayed two additional unique TRDL (Table S4). The 10 TRDL from the F2 population were distributed over six chromosomes, leaving only LG1, LG2, and LG6 free of TRD. Large deviations from Mendelian segregation ratios were detected in the F2 population. We observed genotype frequencies for: (1) homozygotes ranging from almost zero to 0.65, while 0.25 is expected under Mendelian segregation; (2) heterozygotes ranging from 0.35 to 0.70, while 0.50 is expected under Mendelian segregation. Eight TRDL had a bias toward *L. sativa* alleles and two TRDL had a bias toward *L. saligna* alleles. The two TRDL with a bias in favor of *L. saligna* alleles were identified on LG5 and LG4 (Table 1). The TRD on LG5 was observed in all three segregating populations (F2, BC1cult, and BC1wild). Interestingly, this region of TRD overlapped with a recombination coldspot between *L. saligna* and *L. sativa* (Figure S4). The TRD on the top of LG4 (Table 1) can be explained by its interaction with the top of LG8, and is described below. Four of the eight TRDL with a bias toward *L. sativa* allele in the F2 population overlapped with HI intervals in the set of BILs. One HI interval in the BILs, segment 8B, displayed a Mendelian segregation in the F2 and BC1 populations (Table 1). To summarize, four of the five genomic regions that are associated with absolute HI in the set of BILs, display a similar, but less severe TRD in a multilocus segregating F2 population.

Detection of digenic interactions

According to the DM model, TRD in hybrid-derived progeny may be due to deleterious interactions between heterospecific genes. HI caused by such a heterospecific gene pair can therefore be nullified by the presence of conspecific genes at each interacting locus. Genotypes with homozygous *L. saligna* introgressions at HI regions can only be present in plants that also carry the *L. saligna* allele for a corresponding interacting locus. As the interspecific F2 population segregates for all loci, some plants obtain *L. saligna* alleles at both interacting loci, in contrast to BILs with a single *L. saligna* introgression in a purely *L. sativa* background.

We used the genotype dataset of the F2 population to identify candidate loci of interactive genes that can nullify the TRD leading to HI in specific BILs. Per HI locus, we selected F2 plant genotypes with a homozygous *L. saligna* introgression in the specific HI region (defined by the borders of the heterozygous segment in the BIL). Candidate interacting partners were loci with at least one conspecific (*L. saligna*) allele in all of these selected F2 plants (Table S7). The number of candidate interacting loci per HI locus varied from 1 to >10. Crosses between four BILs with a heterozygous introgression and five BILs containing a segment with a candidate interacting gene resulted in several plants with two introgression segments in heterozygous state. In inbred progenies from those plants, HI was nullified at three HI loci, and eventually resulted in three double-introgression lines with a homozygous *L. saligna* introgression in the HI region and a conspecific introgression at the interactive locus (Figure S1 and Table S7). This showed that the presence of the conspecific allele elsewhere on the genome nullified the previously observed TRD at the HI locus (Table S7). This indicated a two-locus hybrid incompatibility between heterospecific alleles, most probably caused by a digenic interaction.

The digenic interaction of segment 9A with segment 8C (shown for subsegment 9A-1 in Figure S1b) has been

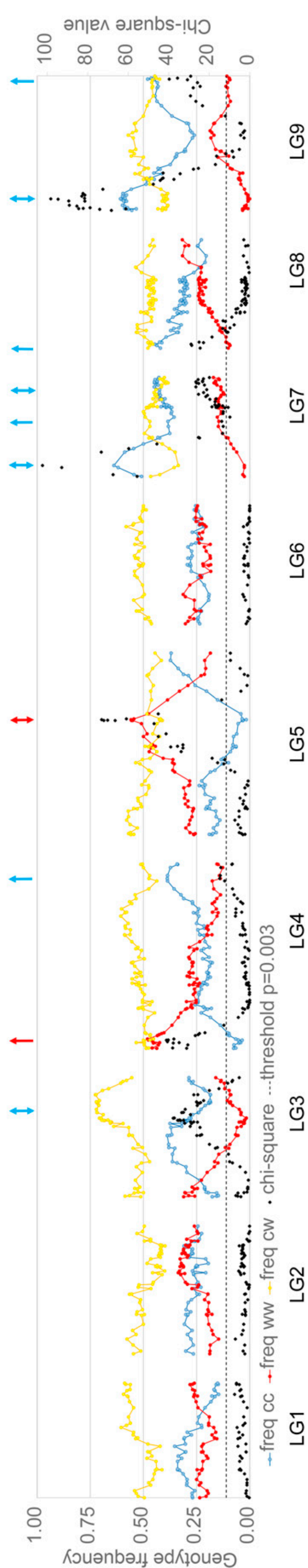

Figure 2 Genotype frequencies and transmission ratio distortion (TRD) per marker in an interspecific F2 population (cross *L. saligna* CGN05271 × *L. sativa* cv Olof). Homozygous *L. saligna* (red), heterozygous (yellow), homozygous *L. sativa* (blue) genotypes. Black dots above the dashed threshold line (at $Chi^2 = 11.6$) indicate TRD with a deviation from a 1:2:1 Mendelian segregation ($P < 0.003$). X-axis: nine LGs in centimorgan. Arrows indicate peak loci with significant transmission ratio distortion (TRDL) with a bias toward *L. sativa* (in blue) or toward *L. saligna* (in red). Single-headed arrows refer to nondistortion of heterozygote frequency (indication of gametophytic barrier). Double-headed arrows point at TRDL with distortion of heterozygote frequency (indication of zygotic barrier). Three TRDL peaks were identified on LG7 based on a switch of the most prominent genotype.

characterized previously (Jeuken *et al.* 2009). Here we have identified a two-locus interaction for segment 8A with segment 4A and for segment 7B (by subsegment BIL7C) with segment 3A (Figure S1b). Below, we characterized the hybrid incompatibility between heterospecific alleles at introgression segments 8A and 4A.

HI by segment 8A: a sex-independent gametophytic barrier

The 26-cM interval of segment 8A in BIL4A+8A overlaps with a TRDL with bias toward the *L. sativa* allele in the F2 population. Likewise, the 27-cM interval of segment 4A overlaps with a TRDL in the F2 population, albeit with a bias in favor of the *L. saligna* allele. Besides occurrence in BIL4A+8A, segment 4A was also present in BIL4A as a single homozygous *L. saligna* segment, whereas the homozygous *L. saligna* segment 8A was present only in combination with homozygous *L. saligna* segment 4A. Although we could not trace back if any effort had been taken to retrieve segment 8A singly, the observation that this and segment 4A—for which strong and opposite allele biases were observed in the interspecific F2 population—also appear together in a BIL, suggested selection against a heterospecific allelic combination at these locations.

To answer this question, we studied an F2 population named "F2_4A8A", which segregated for introgression segments 4A and 8A in an otherwise purely *L. sativa* background (*i.e.*, inbreds of F1-plant from cross: BIL4A+8A × *L. sativa* cv. Olof). Plants ($n = 691$) of "F2_4A8A" were genotyped with a pair of markers per introgression segment (see *Materials and Methods*). Individuals with crossovers between either pair of markers ($n = 56$) were excluded. The seed germination rate of "F2_4A8A" was normal (>95%) and no seedling lethality was observed.

The segregation ratio of the remaining 635 plants of "F2_4A8A" (Figure 3A) was significantly different from a Mendelian segregation of two independent genes (Hypothesis 1, Figure 3B). In a Mendelian segregation of two loci, four gametophyte genotypes are produced in equal frequencies (two conspecific genotypes "4c8c" and "4w8w" and two heterospecific genotypes "4c8w" and "4w8c"), leading to 16 gametophyte combinations (four male × four female) representing nine genotype classes. Genetic nomenclature: numbers refer to chromosome numbers of the introgressions, "c" is a cultivated parent (*L. sativa*) allele, "w" is a wild parent (*L. saligna*) allele. We observed only six genotypes instead of nine (Figure 3A), so three expected genotypes were absent. The nonobserved genotypes were "4cc8cw", "4cc8ww" and "4cw8ww". The three absent genotypes have in common that they are a product of at least one gametophyte with the heterospecific genotype "4c8w". Therefore, we postulated a second hypothesis stating that the heterospecific haploid genotype "4c8w" is not transmitted through either male or female gametophytes (Figure 3C). Segregation according to this hypothesis results in only six genotypes instead of nine, with expected genotype numbers close to the observed numbers in "F2_4A8A" (Figure 3C, $P = 0.8$). Furthermore,

Table 1 Comparison of transmission ratio distortion loci (TRDL) in the interspecific F2 population to hybrid incompatibility (HI) loci in BILs and to validated TRDL in BC1 populations

			TRDL characterization in F2													
						Genotype freq			Allele freq							
LG	Interval TRD (cM)	Representative marker	cM	Mb	Chi^2 Mendelian segregation	cc 0.25	cw/wc 0.50	ww 0.25	c 0.50	w 0.50	Reproductive barrier type	TRD F2 n = 126	TRD BC1cult n = 88	TRD BC1wild n = 33	TRD & HI BILs n = 31	HI segment
3	36–92	NL1187	66.9	134	38	0.28	0.71	0.02	0.63	0.37	Zygotic ****	✓	×	×	×	
4	0–26	Ls_v8_lg_4_020626270	5.6	21	41	0.05	0.50	0.46	0.30	0.70	Gametophytic		✓	×	n/a[a]	
4	136–157	Ls_v8_lg_4_348066968	147	348	15	0.39	0.47	0.14	0.62	0.38	Gametophytic	✓	×	×	×	
5	55–122	Ls_v8_lg_5_255102715	97.2	255	74	0.03	0.40	0.57	0.23	0.77	Zygotic *	✓	✓[b]	✓	n/a[a]	
7	0–33	Ls_v8_lg_7_021291267	9.3	21	102	0.64	0.34	0.03	0.81	0.19	Zygotic ***	✓	×	×	n/a[c]	
7	33–69	NL1205	58.9	145	16	0.39	0.47	0.14	0.63	0.37	Gametophytic	✓	×	×	✓	7B
7	69–84	LE9018	80.5	183	27	0.45	0.39	0.16	0.64	0.36	Zygotic ****	✓	×	×	×	
8	0–17	Ls_v8_lg_8_000481920	2.3	0.5	29	0.44	0.47	0.09	0.67	0.33	Gametophytic	✓	×	×	✓[d]	8A
8[e]	26–47	NL1117	38.4	109	ns	0.30	0.48	0.23	0.54	0.46	No TRD	×	×	×	✓	8B
9	0–50	RIN4	8	15	89	0.59	0.40	0.01	0.79	0.21	Zygotic *	✓	✓	✓	✓	9A
9	74–114	Ls_v8_lg_9_195009606	111.7	195	40	0.48	0.41	0.10	0.69	0.31	Gametophytic	✓	✓	✓	✓	9C

More details and unique TRDL of BC1 populations can be found in Table S4. HI: absolute hybrid incompatibility, *i.e.*, complete nontransmission of homozygous segment of *L. saligna* in a homozygous *L. sativa* background. In the F2 population, TRDL ($P < 0.003$) were designated as potentially zygotic or gametophytic barriers based on distortion or nondistortion of heterozygote frequency respectively, *$P < 0.05$, **$P < 0.01$, ***$P < 0.0005$, ****$P < 0.00001$. Three TRDL peaks were identified on LG7 based on a switch of the most prominent genotype. Allele codes: "c" is allele from cultivated parent *L. sativa* cv Olof, "w" is allele from wild parent *L. saligna* CGN05271. Genotype codes: "cc" is homozygous *L. sativa*, "cw" or "wc" means heterozygous, and "ww" is homozygous *L. saligna*. ✓: TRDL ×: no TRDL. ns: not significant ($P > 0.003$), n/a: not applicable.

[a] TRD with a bias toward *L. saligna* allele cannot be validated in BILs with *L. saligna* introgressions in *L. sativa* background.

[b] TRDL on LG5 in BC1cult with a slightly less strict *P*-value of 0.006 (instead of 0.003).

[c] Segment could not be studied due to loss in an early backcross generation.

[d] TRD for 8A segment was shown in the inbred progeny "F2_4A8A" which segregated distorted for segments 4A and 8A in an otherwise purely *L. sativa* background (details will follow in a later paragraph).

[e] No TRD in F2 and BC1, but absolute HI in BIL.

hypothesis 2 predicts that individuals with a homozygous *L. saligna* genotype for segment 8A are exclusively homozygous *L. saligna* for segment 4A, just as we observed in "F2 4A8A". These results indicate that our observation that segment 4A and 8A appeared together in one BIL was due to selection against a heterospecific allelic combination at these locations.

To validate the maternal and paternal effects, we analyzed segregation ratios of backcross progenies from a reciprocal cross between a double heterozygous genotype "4cw8cw" (four gametophyte genotypes: "4c8c", "4w8w", "4c8w" and "4w8c") and BIL4A with genotype "4ww8cc" (gametophyte genotype "4w8c" only). In both backcross progenies the double heterozygous genotype "4cw8cw" can result only from a fusion between the two heterospecific gametophytes "4c8w" and "4w8c". In both backcross progenies, the double heterozygous genotype "4cw8cw" was absent or observed in a very low frequency, probably due to some selfings. These results confirmed that the heterospecific haploid genotype "4c8w" was not transmitted through either male and female gametophytes (Figure S5j and k).

To exclude the possibility of an alternative explanation for the distorted segregation, we tested four alternative hypotheses, regarding genotype lethality, nontransmission of "4c8w" gametophytes by male or female gametophytes only, combined hypotheses, and genotype frequencies based on observed allele frequencies (Figure S5f–i). However, these hypotheses were all rejected as expected genotype numbers were very different from the observed numbers ($P < 0.001$).

Overall, our results suggest that the observed TRD for segments 4A and 8A are explained by a deleterious heterospecific haploid genotype resulting in nonparticipation of male and female "4c8w" gametophytes in reproduction. In the interspecific F2 population (cross *L. saligna* × *L. sativa*) we observed the same typical two-locus TRD, *i.e.*, absence of the same three genotypes. This interspecific F2 population had *L. saligna* cytoplasm, whereas the population "F2_4A8A" had *L. sativa* cytoplasm, suggesting that cytonuclear interactions do not play a role.

Phenotypic evidence for the female side of the gametophytic barrier

Nontransmission of the heterospecific "4c8w" gametophytes may be due to disturbance of processes in reproduction. This heterospecific gametophyte may not be formed in meiosis, or formed but dysfunctional anywhere in the process to double fertilization. On the male side, nontransmission of one out of four gametophytes may result in 25% nonvital pollen. However, no phenotypic abnormality was observed at the tetrad stage and pollen vitality in the double heterozygote was similar to *L. sativa* cv Olof (>95%).

The ratio of aborted and nonaborted achenes (seeds) per capitulum in case of self-fertilization could indicate whether reproduction is disturbed before or after fertilization. If "4c8w" gametophyte transmission is disturbed by a process after fertilization (*e.g.*, zygote lethality), we would expect 75% (transmitted female gametophytes) * 75% (transmitted male gametophytes) = 56% nonaborted seeds and 44% aborted seeds from the selfed double heterozygous plant.

If "4c8w" gametophyte transmission is disturbed before self-fertilization, we would expect only the female gametophytes

to influence seed set, although a quarter of male as well as female gametophytes will not be transmitted to the next generation. The defect or absent "4c8w" pollen are not expected to influence seed set as they can be compensated by an abundance of vital pollen of the three other genotypes. In other words, the 75% vital female gametophytes have more than one chance to be fertilized by a pollen grain, and, therefore, all these ovules will be fertilized by a vital pollen grain in the end. Therefore, this scenario of TRD before self-fertilization would result in 75% nonaborted seeds and 25% aborted seeds for the selfed double heterozygous genotype "4cw8cw".

Twenty-nine percent of the seeds were aborted in this selfed double heterozygote, compared with 4–7% of aborted seeds for selfings on *L. saligna* and *L. sativa*, respectively. The 71% nonaborted seeds in the selfed double heterozygote indeed only consisted of six of the nine genotypes expected under Mendelian segregation, *i.e.*, the three genotypes "4cc8ww", "4cw8ww" and "4cc8cw" were absent. These results are consistent with a disturbance of gametophyte transmission before fertilization.

Mapping the HI between 4A and 8A

The typical two-locus TRD at the top of LG4 and LG8 is observed in the population "F2_4A8A" as well as in the interspecific F2 population (cross *L. saligna* × *L. sativa*). Only in recombinant F2 genotypes we did observe the three genotypes "4cc8ww", "4cw8ww" and "4cc8cw", which are not transmitted in the typical TRD. The presence of one of these three genotypes in recombinant plants is directly informative for narrowing the 26- and 27-cM HI map intervals, as it indicates the absence of the typical TRD of the HI (Figure S6a). We searched for these informative recombinant F2 genotypes within the HI regions on LG4 and LG8 to more precisely locate the genes responsible for this TDR (Figure S7). This resulted in an HI interval from 4.4 to 5.6 cM on LG4 and 0.0–9.5 cM on LG8. The 1.2 cM interval on LG4 has a physical length of 8 Mb and contains 165 genes according to the *L. sativa* genome v8 (Reyes-Chin-Wo *et al.* 2017). The HI interval on LG8 was further reduced by another HI phenotyping approach (Figure S6b). Here, recombinant plants are directly informative not by their genotype, but by their next inbred generation. These recombinants have a heterozygous genotype at one locus and a recombinant genotype at the other locus, in which the recombinant genotype at LG4 is switching from heterozygous to homozygous *L. saligna*, or the recombinant genotype at LG8 is switching from heterozygous to homozygous *L. sativa* (Figure S6b). If the inbred progeny of the recombinant plant shows 25% reduced seed set and the typical TRD, the HI must map within the interval of the double heterozygous genotype "4cw8cw". If the inbred progeny displays normal seed set and Mendelian segregation, the HI must map outside of the interval of the double heterozygous genotype. The most informative recombinant plant for the 8A segment showed normal seed set and Mendelian segregation in its offspring, indicating a non-HI phenotype. This narrowed down the HI locus on LG8 to an interval of 4.7 cM, from 0 to 4.7 cM (Figure S7). This 5.2 Mb interval contains 138 genes according to the *L. sativa* genome v8 (Reyes-Chin-Wo *et al.* 2017).

In summary, segregation ratios in inbred and backcross progenies of the double heterozygote "4cw8cw" indicated that the distorted segregation of 4A and 8A segments can be explained by nontransmission of male and female gametophytes with the heterospecific allele combination "4c8w", *i.e.*, a sex-independent gametophytic barrier. Phenotypic evidence for this specific nontransmission was obtained from the female side. The HI was fine mapped to 1.2 and 4.7 cM intervals on LG4 and LG8, respectively.

Discussion

The genome-wide analysis of TRD and HI in interspecific crosses may shed light on postzygotic reproductive barriers, *i.e.*, barriers after formation of an F1 hybrid plant. Domesticated lettuce, *L. sativa*, is closely related to, and cross-fertile with, its ancestor species *L. serriola*. *L. sativa* is distantly related to, and less crossable with, the species *L. saligna*. The most recent common ancestor (MRCA) for *L. saligna* and *L. serriola* has been dated, by a phylogenetic study with nuclear (ITS) and chloroplast sequences, to between 3.4 and 2 MYA (Kilian *et al.* 2017). Phenotypically, the divergence between *L. saligna* and *L. sativa* is clearly demonstrated by the observation of nearly complete sterility in the F1 hybrid and hybrid breakdown in the F2 population. Therefore, the cross between *L. saligna* and *L. sativa* can be defined as a wide cross. From a fundamental point of view, we were interested in the amount, mechanism, and genetic basis of reproductive barriers in this wide interspecific cross. For crop breeding purposes, we were interested in loci that have an increased or decreased frequency of wild relative introgressions.

HI in single introgression lines (BILs)

While developing the set of BILs, we observed that five introgression regions segregated abnormally, showing complete absence of the homozygous *L. saligna* genotype class, indicating HI. Such introgression regions were eventually only represented in BILs in a heterozygous state. TRD analysis in the interspecific F2 population revealed seven TRDL with a bias toward the *L. sativa* allele, of which four were related to an HI in the BILs. A fifth HI interval in the BILs, segment 8B, displayed a Mendelian segregation in the F2 and BC1 populations. A candidate HI is the top of LG7, because a TRDL with a bias in favor of the *L. sativa* allele was found in the F2 population and a *L. saligna* introgression at this locus was lost in an early backcross generation during BIL development. Lack of backcross plants with wild parent alleles at this LG7 locus prevented the further in-depth studies on this region needed to ascertain a true HI. In the F2 population, TRDL associated with absolute HI in BILs showed Chi^2 values of 16, 29, 40, and 89, whereas TRDL not associated with absolute

A Observed segregation in inbred progeny 'F2_4A8A'

Observed plant numbers

LG4 \ **LG8**	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	75	0	0	75	0.12	c=0.32
cw/wc	126	130	0	256	0.40	
ww	80	151	73	304	0.48	w=0.68
sum	281	281	73	**635**		
geno freq	0.44	0.44	0.11			
allele freq	c=0.66		w= 0.34			

Genotype frequencies

LG4 \ **LG8**	**cc**	**cw/wc**	**ww**
cc	0.12	0.00	0.00
cw/wc	0.20	0.20	0.00
ww	0.13	0.24	0.11

B Hypothesis 1 : Mendelian segregation

Chi²= 395, p=0, rejected

Expected plant numbers

LG4 \ **LG8**	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	40	79	40	159	0.25	c=0.50
cw/wc	79	159	79	317	0.50	
ww	40	79	40	159	0.25	w=0.50
sum	159	317	159	**635**		
geno freq	0.25	0.50	0.25			
allele freq	c=0.50		w=0.50			

Genotype frequencies

LG4 \ **LG8**	**cc**	**cw/wc**	**ww**
cc	0.06	0.13	0.06
cw/wc	0.13	0.25	0.13
ww	0.06	0.13	0.06

C Hypothesis 2 : Non-transmission of male and female '4c8w' gametophytes

Chi²= 4.6, p=0.8, not rejected

Expected plant numbers

LG4 \ **LG8**	**cc**	**cw/wc**	**ww**	sum	geno freq	allele freq
cc	71	0	0	71	0.11	c=0.33
cw/wc	141	141	0	282	0.44	
ww	71	141	71	282	0.44	w=0.67
sum	282	282	71	**635**		
geno freq	0.44	0.44	0.11			
allele freq	c=0.67		w=0.33			

Punnett square: expected gamete segregation

Bold underlined = non-transmitted

female \ **male**	4c8c	**<u>4c8w</u>**	4w8c	4w8w
4c8c	4cc8cc	**<u>4cc8cw</u>**	4cw8cc	4cw8cw
<u>4c8w</u>	**<u>4cc8wc</u>**	4cc8ww	**<u>4cw8wc</u>**	**<u>4cw8ww</u>**
4w8c	4wc8cc	**<u>4wc8cw</u>**	4ww8cc	4ww8cw
4w8w	4wc8wc	**<u>4wc8ww</u>**	4ww8wc	4ww8ww

Figure 3 Comparison of observed segregation of "F2_4A8A" to Mendelian segregation (H1) and to segregation biased by a sex-independent gametophytic barrier (H2). Genotype plant numbers and genotype frequencies are color-shaded from low (red) to high (green) numbers. (A) Observed segregation of inbred progeny "F2_4A8A" ($n = 635$) of the double heterozygous genotype,"4cw8cw", which is an F1 from cross BIL4A+8A × *L. sativa* cv Olof (B) Expected segregation according to hypothesis 1 (H1): Mendelian 1:2:1 segregation (both loci allele frequencies of 0.5). (C) Expected segregation according to hypothesis 2 (H2): nontransmission of male and female "4c8w" gametophytes, *i.e.*, sex-independent gametophytic barrier. In the lower panel: expected gametophyte segregation, bold underlined: nontransmitted gametophytes. Cells with the same color indicate gametophyte combinations that result in the same zygote genotype. Genetic nomenclature: "c"= *L. sativa* allele, "w"= *L. saligna* allele; "cc"= homozygous *L. sativa* genotype, "cw" or "wc"= heterozygous genotype, "ww"= homozygous *L. saligna* genotype. geno freq = genotype frequency, allele freq = allele frequency.

HI showed Chi² values of 15, 27, and 38 (Table 1). These values seem nondistinctive between the groups. Therefore, the strength of segregation distortion in the F2 population was not predictive for the degree of nontransmission of heterospecific allele combinations.

To summarize, five regions with absolute HI were detected in the set BILs, and four of these genomic regions display a similar, but less severe, TRD in a multi-locus segregating F2 population. So, four of seven TRDL with bias toward *L. sativa* alleles in the F2 population were predictive for an absolute HI in BILs

TRDL in segregating populations

Genome-wide TRD analysis in the F2 population resulted in 10 loci with severe TRD, in most cases with an almost complete lack of one of the homozygous genotypes. Eight of the 10 TRDL showed a bias in favor of *L. sativa* alleles, which was not significantly different from an equal distribution of wild and cultivated allele biases over TRDL (8:2 is not significantly different from 5:5). In recombinant inbred lines from the cross *L. sativa* x *L. serriola*, five regions were distorted ($P < 0.01$) (Truco *et al.* 2013). The number of TRDL was higher in our wide cross than in that of Truco *et al.* (2013). This suggests that the number of TRDL reflects the genetic distance between populations.

The number of observed TRDL in the bidirectional BC1 populations was, in both cases, about half of the number observed in the F2 population. This is consistent with the notion that the more introgressed regions from one species are present in the other species' genetic background, the more deleterious interactions may occur (Moyle and Graham 2006). Variation in numbers of TRDL are observed in studies

of TRD in intraspecific and interspecific crosses of other organisms (Gadau *et al.* 1999; Fishman *et al.* 2001; Harushima *et al.* 2001; Myburg *et al.* 2004; Hall and Willis 2005; Nakazato *et al.* 2007; Wu *et al.* 2010; Leppälä *et al.* 2013; Reflinur *et al.* 2014). However, direct comparison between these and our study is difficult, as a diverse width of crosses were used, as well as different detection criteria for TRD.

Digenic interactions

Intrinsic postzygotic reproductive barriers between diploids are generally thought to be caused by two mechanisms: (1) large chromosomal rearrangements between parental species resulting in abnormal meiotic products and reduced fertility in hybrids, and (2) DM incompatibilities, *i.e.*, deleterious epistatic interactions between heterospecific alleles leading to hybrid sterility and/or inviability. There are no indications of large chromosomal rearrangements between *L. saligna* and *L. sativa*, as our interspecific F2 linkage map was collinear with the *L. sativa de novo* genome (Reyes-Chin-Wo *et al.* 2017). Therefore, we expected DM incompatibilities to explain HI between wild and cultivated lettuce.

The homozygous *L. saligna* genotype was absent in the five HI regions in BILs. However, the homozygous *L. saligna* genotype was present in these five regions in a few genotypes of the interspecific F2 population (cross *L. saligna* × *L. sativa*). This was probably explained by the presence of other conspecific regions in these F2 plants. HI might be nullified when two conspecific genetic components or genetic "dance partners" are present. For each HI case, we identified candidate conspecific interacting regions in the interspecific F2 population. Segregation analysis of five developed double-introgression progenies demonstrated nullification of HI by a Mendelian segregation at three HI regions, *i.e.*, segments 7B, 8A and 9A. This provided evidence that nuclear pairwise incompatibilities were responsible for these three cases of HI. The other two regions with HI symptoms (segment 8B and 9C) might also be due to simple pairwise incompatibilities and more candidate conspecific partners should be tested to verify this. However, they may also be explained by higher-order epistasis or by dysfunctional cytonuclear interactions. We cannot exclude cytonuclear interactions for these two specific cases of HIs, as we either detected similar TRDs in the F2 population and BILs both with *L. saligna* cytoplasm (segment 9C), or we detected TRD only in the BIL with a contrasting parental cytoplasm between the F2 population and this BIL (segment 8B).

In conclusion, this part of the study showed that pairwise nuclear incompatibilities explain three of the five detected HI in BILs. Two-locus incompatibilities may be very common between *L. saligna* and *L. sativa*. Furthermore, we show that BILs are ideal for empirical validation and fine mapping of genes causing HI, as they exclude effects of other loci. Our interspecific F2 population was most useful for identification of the interacting loci that can nullify HI, as well as confirming that there is no cytonuclear component involved.

A sex-independent gametophytic barrier

Our observation that segment 8A appeared together with segment 4A in one BIL, suggested selection against a heterospecific allelic combination at these loci. The pattern of TRD for these loci in "F2_4A8A" fitted the hypothesis that heterospecific "4c8w" male and female gametophytes were not transmitted, *i.e.*, a sex-independent gametophytic barrier. Phenotypic evidence was given from the female side by seed set analysis. Selfings of double heterozygous plants ("4cw8cw") showed close to 25% aborted seeds, which was expected if one out of four female gametophytes is nontransmitted, and if the effect of nontransmission is before fertilization. Since all pollen grains appeared vital in pollen viability tests, the "4c8w" pollen likely do not form. The HI loci were fine-mapped to intervals of 1.2 and 4.7 cM, each containing >100 genes in the *L. sativa* genome assembly. F1 plants of *L. saligna* × *L. sativa* showed 98% reduced seed set compared to the parental lines. A quarter of this reduced seed set in the F1 may be assigned to the digenic HI of segments 4A and 8A. Future analysis of seed set in BILs with heterozygous introgression segments may reveal HI loci that cause the remaining percentage of reduced seed set.

Almost all reported segregation distortion by gametophytic barriers act on the male or female side only (Ouyang and Zhang 2013). Sex-independent TRD appears to be much less common (Taylor and Ingvarsson 2003; Koide *et al.* 2008; Ouyang and Zhang 2013). Still, several of the mutations that affect female gametogenesis in *Arabidopsis* also affect male gametogenesis (Drews *et al.* 1998; Drews and Yadegari 2002; Wang *et al.* 2012). Apparently, some processes of gametogenesis are identical in gametophytes of both sexes (Christensen *et al.* 1998; Ding *et al.* 2012) and disruption of such a similar process may explain our case of sex-independent gametophytic HI.

An explanatory model for the digenic HI on LG4 and LG8 might be an internal genetic conflict in one of the two species. Selfish genes may negatively affect their own species, which can lead to the evolution of suppressor genes (Burt and Trivers 2006; Maheshwari and Barbash 2011). HI can arise if the selfish gene is uncoupled from its suppressor in certain individuals of the selfed hybrid. For *L. sativa*, a selfish allele on LG4 that is suppressed by an allele on LG8 could explain the HI, or, for *L. saligna*, a selfish allele on LG8 that is suppressed by an allele on LG4.

Another explanatory model for the digenic HI could be gene duplication, followed by loss of function in one of the redundant gene copies (Bikard *et al.* 2009; Zuellig and Sweigart 2018). In 1957, Oka proposed reciprocal loss of duplicated genes for hybrid sterility in rice (Oka 1957). Recently, several cases of reciprocal loss of duplicated genes have been demonstrated molecularly for male gametophytes in rice (Mizuta *et al.* 2010; Yamagata *et al.* 2010; Nguyen *et al.* 2017). The HI genes on LG4 and LG8 may be ancient duplicates, after which the gene on LG8 lost its functionality in *L. saligna*, and the gene on LG4 lost functionality in *L. sativa*.

This would explain the nontransmission of "4c8w" gametophytes, as both genes in this gametophyte would be nonfunctional. Lettuce has undergone an ancient whole-genome triplication (Reyes-Chin-Wo *et al.* 2017), which could make this type of incompatibility possible. However, the regions on 4A and 8A that are involved in HI have not been found to be each other's syntelogs (Reyes-Chin-Wo *et al.* 2017).

Until now, only one indication of digenic gametophytic hybrid lethality that acts in both male and female gametophytes has been reported, *viz*. in *Mimulus* (Kerwin and Sweigart 2017). However, in that case also other explanations were needed to fully explain the TRD and the heterospecific gametophytes were only undertransmitted in contrast to our observation of complete nontransmission. Our case of nontransmission of a heterospecific gametophyte in both males and females completely explains the TRD, and may be the first identified two-locus sex-independent gametophytic HI. As the incompatibility appears at the haploid stage in both gametophytes, it likely represents a recessive dysfunctional interaction essential for cell metabolism. The dysfunction may act at the level of methylation, microRNA, mRNA, or proteins. If it acts at the level of protein–protein interaction, the heterospecific allele combination may lead to changed protein binding interfaces, biochemically dysfunctional allosteric changes in proteins, protein mislocalization in the cell, or to a dysfunctional heterodimer.

Overall, our data confirm that two-locus incompatibility systems may be very common in *Lactuca*. We characterized two digenic HI: a zygotic barrier leading to hybrid necrosis and reduced viability (Jeuken *et al.* 2009), and a sex-independent gametophytic barrier leading to reduced fertility (this study). Phenotypic analysis of the remaining cases of HI in the BILs could further expand our knowledge of the mechanisms for *Lactuca* speciation. Here, we have laid the foundation for experiments that can reveal the identity of these HI genes, and, ultimately, the selective forces acting upon them.

Acknowledgments

We thank M.J.M. Smulders for critically reading the manuscript and two reviewers for valuable comments. This work was part of the research programme "Open Technology" with project number 12683, which is (partly) financed by the Netherlands Organization for Scientific Research (NWO).

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Communicating editor: L. Moyle