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Supplementary Information for

Versatility of multivalent orientation, inverted meiosis and rescued fitness in holocentric chromosomal hybrids

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Other supplementary materials for this manuscript include the following:

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SI Methods

Experimental design. Given the existence of a triplet of externally virtually indistinguishable species (*L. reali, L. sinapis* and *L. juvernica*), the *Leptidea sinapis* specimens used as founders for the laboratory lines were identified based on genitalia examination and/or DNA barcoding. DNA barcodes were obtained using standard protocols (1, 2).

Two main laboratory lines were established based on wild-caught individuals. One was representative for *L. sinapis* populations with high chromosome number (2n=106, 108) (3) and included specimens originating from north-eastern Spain (Montseny area, Barcelona province, Catalonia). The other laboratory line was representative for *L. sinapis* populations with low chromosome number (2n=57, 58) (this study, Table S1) and included specimens originating from south central Sweden (two field sites in the vicinity of Stockholm). All mating experiments carried out in this study were performed under laboratory conditions at the Department of Zoology, Stockholm University, Sweden.

Pure Spanish and Swedish laboratory lines were maintained and used as controls with respect to crossed lines between male Spanish and female Swedish and female Spanish and male Swedish *L. sinapis*. All possible mating combinations between Spanish and Swedish *L. sinapis* were performed until F_2, and each mating combination was represented by at least five cases (i.e. at least five different pairs of specimens mated for each combination). The offspring of these pairs were bred to adults and used for further experiments (Fig. 1 in the main text, Fig. S1, Table S2). For generations F_3 and F_4, a subset of the potential hybrid mating combinations were performed, and the pure lines were stopped.

The laboratory mating protocol followed that described in (2, 4).

Each mating trial started with a virgin female being transferred to an empty cage (0.8 x 0.8 x 0.5 m) that was located underneath 400W metal halide lamps next to a window, letting in additional, natural daylight. The female was fed a 25% sugar solution for 10 minutes, before a male was released into the cage. Courtship initiation was mediated by manipulating males and females into sitting on different cotton tipped sticks. The female was thereafter presented to the male, which often responded by climbing over to the female cotton tip, uncoiling his proboscis an initiating the display by oscillating the proboscis (see the Supplementary Video, as well as (4, 5) for a detailed description of the courtship). After termination of copula females were transferred to individual egg-laying jars (0.5 L) covered with a fine net, that were placed in a quiet egg-laying room with a light

regime of 9:15 h light/dark and room temperature. Females were fed from soaked cotton tips a 25% sugar solution once a day throughout the experiment.

The number of eggs laid by each female on, or in close vicinity to the host plant (a fraction of eggs were sometimes laid on the walls of the pot or upper net), as well as the number of hatched larvae and emerged adults, were recorded. The offspring of parental generation females were reared in family groups of 3-5 individuals in 0.5 L jars with *ad libitum* access to one of their main larval host plants, *Lotus corniculatus* (6, 7). Larvae were reared at 23°C in a 22:2 light/dark cycle to induce direct development into adulthood. We considered as "adults" all individuals that reached the ultimate larval instar. This was done in order to include the last instar larvae that appeared perfectly healthy, but were sacrificed for karyological analyses. A number of (larval and adult) offspring from each mating combination was sacrificed for karyological studies (Table S1). Apart from the sacrificed larvae, the vast majority of last-instar larvae successfully pupated and adults emerged. Adult specimens were sexed and their dry weight measured using a Sauter Automatic Balance (AR 1014).

Emerging adults were sexed and individually marked with a marker pen on the ventral side of the right hind wing. Males were placed in species-specific flight cages at room temperature, whereas females were transferred into a cold room (10 °C) until they were used for further mating experiments. The above process was reiterated across forthcoming generations (until F_2 for within-population crosses, until larval generation F_4 for a subset of hybrid crosses, see Fig. 1 in the main text and Fig. S1).

Statistical analyses. All statistical analyses were performed in the statistical software R (R development core team), in a series of linear models, mixed-models and non-parametric tests aimed at exploring the impact of origin (hybrid/pure line) and generation (typically F_1-F_4) on a set of fitness variables.

The female mating propensity (yes/no) was tested in generalized linear models (binomial distribution), with logit as link function. First we included data only for the original cross and tested the effect of female population (C/S), male population (CS) and their interaction. Thereafter, we tested the effect of individual cross types across generations (parental - F_3; see Fig. 3 in the main text). The female time to acceptance (log transformed) was tested in a similar scheme using linear models (ANOVA II) (see Fig. 3 in the main text).

The survival analysis included three separate response variables; survival from egg-larva identifying effects of larval origin (hybrid or pure-line) on larval hatch rate, survival from larva to adult identifying potential consequences of hybrid survival during larvalhood, and the total fitness (measured as survival from egg-adult). First, however, we made sure that larval survival was not affected by (i) the fact that 9 of 27 matings within the pure lines of the paternal and F_1 cross were sibling matings (Table S4) and (ii) that some of the F_1 females laid substantial numbers of eggs on the jar wall and on the net that capped the jar (linear regression hatch rate vs. proportion of eggs laid off host; R^2=0.021, $F_{1,30}$=0.64, P=0.43). Thereafter, we continued to analyze the fitness data using maternal family as the statistical unit, comparing larger-level effects of cross type and offspring generation. Because data on pure lines were available only for generation F_1 and F_2, whereas data on hybrid fitness were available also for generation F_3 and F_4, we applied two different sets of linear models (ANOVA II). First, we included data only from generation F_1 and F_2 and tested the effect of cross type (pure/hybrid) generation (F_1/F_2) and their interaction on three survival rates (egg-larva, larva-adult, egg-adult), sex ratio (proportion males) and on female fecundity. In a similar analysis, with the same response variables, we compared fitness among the four hybrid generations (F_1-F_4), to detect whether differences established between F_1 and F_2 crosses were maintained in the hybrid lineages. The proportional variables were arcsine-square root transformed, and the number of eggs was log-transformed prior to analysis to approach normality and homogeneity of variances. Post-hoc testing was done with Tukey's HSD tests.

We chose the linear modelling approach over the use of mixed model generalized linear modelling (glmm), because the latter approach could potentially be affected by the variation in the number of samples (e.g. eggs, larvae) coming from each different mother. Hence, when comparing hybrid and pure lines among generations the linear modelling could be seen as a more conservative approach. However, confirming analyses were performed also using the glmm-approach, and these provided qualitatively very similar results (Tables S7 and S8). In that set of models, we used the survival from egg to larva (dead=0, alive=1), survival from larva to adult, survival from egg to adult, and sex of the eclosing adult (female = 0, male =1) as binomially distributed response variables, respectively. In each model the cross type (pure line or hybrid), the offspring generation (F_1, F_2) and their interaction were used as fixed factors, the larval family was used as a random factor, and logit was used as link function. Thereafter, we compared the survival and sex across all four hybrid generations (F_1-F_4) using the same set of response variables in similar glmm models. Models were applied using the R package lme4 (8) and the resulting *P*-values were estimated through analysis of

deviance using the function Anova (model) in the R package car (9).Within the F_1 and F_2 generations, we also applied glmm-modelling to attempt a deeper analysis of the importance of cross direction for fitness. There were two hybrid lines in generation F_1 (crosses between Spanish females and Swedish males (C x S) or between Swedish females and Spanish males (S x C)), and four hybrid lines in generation F_2 (CS x CS, CS x SC; SC x CS and SC x SC), depending on the origin of the grandparents in each cross) (Fig. S1). The cross types and the pure lines (F_1: S x S, C X C; F_2: CC x CC, SS x SS) were used as fixed factors in glmm models with survival (from egg to larva, from larva to adult and from egg to adult, respectively) as the binomially distributed response variable, with sibling family as random factor and logit as link function. These models were run separately for each cross type (pure line/hybrid) and generation (F_1/F_2). By and large, these analyses revealed no or minor differences among hybrid lines (see results in the main manuscript, Table S5 and Fig. S2).

As an additional measure of fitness, we tested the adult dry-weights from a sample of adults from each generation. These data was not possible to transform to fit the linear model assumptions, and hence, we performed non-parametric Kruskal Wallis ANOVAs to evaluate differences among cross types (pure/hybrid) generations (F_1-F_3) and sexes (a total of 10 groups).

Chromosomal analysis. Only fresh adult males were used to analyse meiosis and to study meiotic karyotype. Adults were killed by a sharp pinch to the thorax and testes were immediately excised and placed into 0.5-mL vials with freshly prepared Carnoy fixative (ethanol and glacial acetic acid, 3:1). Gonads were stored in fixative for 2–6 months at 4 °C and then stained with 2% acetic orcein for 30 days at 20 °C. Cytogenetic analysis was conducted using a two-phase method as previously described (10). In the first phase, the stained testes were placed into a drop of 40% lactic acid on a slide, the gonad membranes were torn apart using fine needles and intact spermatocysts were removed and transferred into another drop of 40% lactic acid. Intact spermatocysts were studied and photographed. The first phase was most useful for counting the number of chromosome bivalents, multivalents and univalents. In the second phase, different stages of chromosome spreading were studied using a slight, gradually growing pressure on the coverslip. The second phase was most useful for studying the chromosome structure and distinguishing between uni-, bi- and multivalents.

FISH with 18S rDNA probe. Testes of F_1 hybrids were dissected and placed into freshly prepared Carnoy fixative (ethanol: glacial acetic acid, 3:1) overnight. Then the testes were squashed on slides

using the two-phase method as stated above, the coverslips were flicked off with a razor blade, and the slides were passed through a graded ethanol series (70%, 80%, and 100%, 1 min each).

Unlabelled 18S rDNA probe was generated by PCR from *Cydia pomonella* (Tortricidae) genomic DNA as described previously (11). The probe was labelled with biotin-16 dUTP (Roche Diagnostics GmbH, Mannheim, Germany) by nick translation using the Nick Translation Kit (Abbott Molecular Inc., Des Plaines, IL, USA) with 1 hour and 45 minutes incubation at 15 °C. FISH with the 18S rDNA probe was carried out using a routine protocol (11). Biotin was detected with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs. Inc., West Grove, PA, USA), amplified with biotinylated anti-streptavidin (Vector Labs. Inc., Burlingame, CA, USA) and again detected with Cy3-conjugated streptavidin. The preparations were counterstained with 0.5 mg/mL DAPI and mounted in antifade based on DABCO (Sigma-Aldrich, St. Louis, MO, USA). Immediately thereafter, the FISH preparations were observed under a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss Jena, Germany) to minimize background noise caused by the presence of excessive cytoplasm. Black-and-white images were captured separately for each fluorescent dye using an Olympus CCD monochrome camera XM10 equipped with cellSens 1.9 digital imaging software (Olympus Europa Holding, Hamburg, Germany), pseudocolored (light blue for DAPI, red for Cy3) and superimposed with Adobe Photoshop, version 7.0.

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SI Text 1

The reduced viability of F_2, F_3 and F_4 generations in hybrid lines may be caused by mal-segregation of trivalents in spermatogenesis of F_1, F_2 and F_3 males, respectively, as well as by mal-segregation of trivalents in oogenesis of F_1, F_2 and F_3 females. These relative effects of spermatogenesis and oogenesis on reduced fitness in hybrid lines could be tested by comparing the viability of offspring from [F_1 male] x [pure-control-line female] backcrosses with the viability of offspring from [F_1 female] x [pure-control-line male] backcrosses (and similarly for F_2 males *vs* F_2 females, etc.). We have not carried out these backcrosses because this would require a different crossing scheme that is outside the main scope of this study. Nevertheless, they can be done in the future. In addition, despite the absence of backcross data, it is highly probable that mal-segregation in spermatogenesis (but not in oogenesis) is the main reason for reduced viability because in Lepidoptera, females have achiasmatic meiosis and a special organization of bi- and multivalents that reduces unbalanced segregation (explained in the main text).

SI Text 2

The equatorial orientation at metaphase I with the subsequent equational division (inverted meiosis) is not an indispensable condition for proper segregation of a holocentric trivalent. If the trivalent can bend sufficiently at metaphase I, it can form the U-shaped structure with a larger chromosome towards one spindle pole and with two smaller chromosomes towards the opposite pole. In that case, the axial orientation at metaphase I followed by reductional division (i.e. standard sequence of the main meiotic events) is the best option for proper segregation, and exactly this pattern was predominantly observed in the U-shaped sex chromosome trivalents of a holocentric homopteran species, *Cacopsylla mali* (see the reference 38 in the main text).

The comparison between trivalents of *L. sinapis* and *C. mali* indicated that sometimes axial orientation is better at metaphase I and sometimes equatorial alignment is better for subsequent segregation. It likely depends on the "bendability" of the trivalent, i.e., whether it can bend sufficiently at metaphase I to form the "U" shaped structure required for proper segregation of two small chromosomes in one direction and one large chromosome in the opposite direction, or whether this bendability is achieved at metaphase II instead.

Thus, there is a clear flexibility of holocentric organisms in terms of equatorial or axial orientation at metaphase I, which suggests an intrinsic versatility of holocentric chromosomes in dealing with chromosomal rearrangements at meiosis. This is not an option available to monocentric organisms.

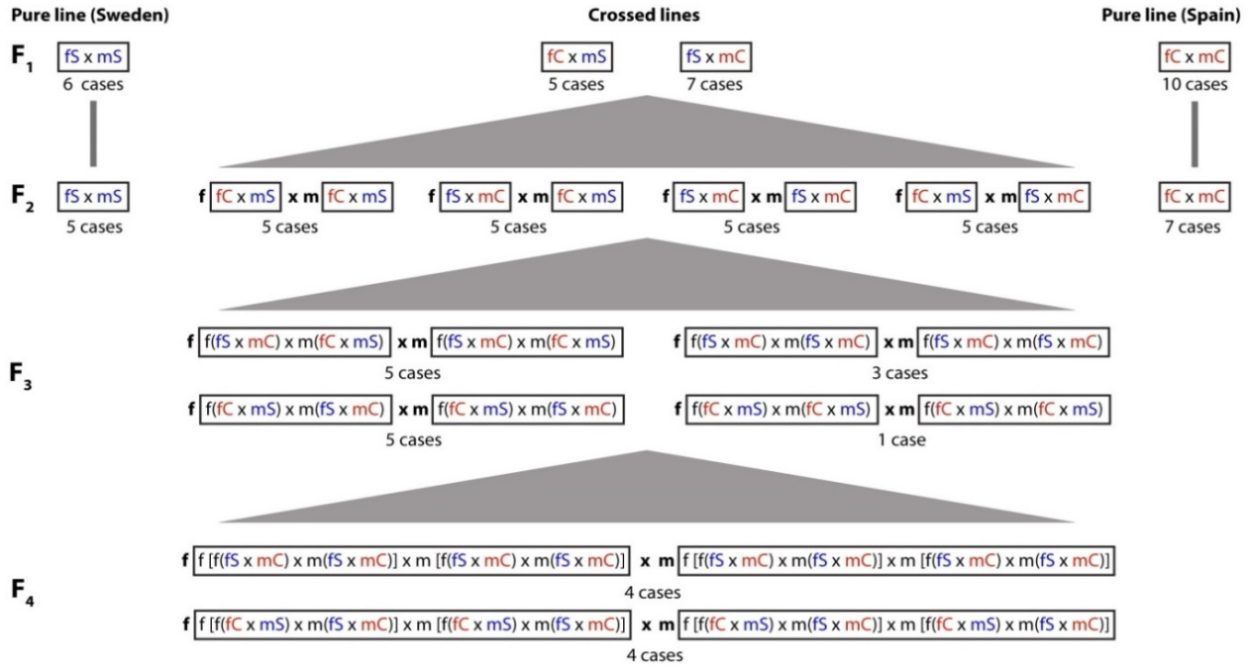

Fig. S1. Mating combinations between chromosomal races of *Leptidea sinapis*: Spain (C, 2n=106,108) and Sweden (S, 2n=57, 58). All mating combinations were performed until F_2 with pure lines serving as controls for F_1 and F_2 hybrid fitness. Several hybrid combinations were maintained through generation F_3, and F_4. The number of successful matings is indicated for each case.

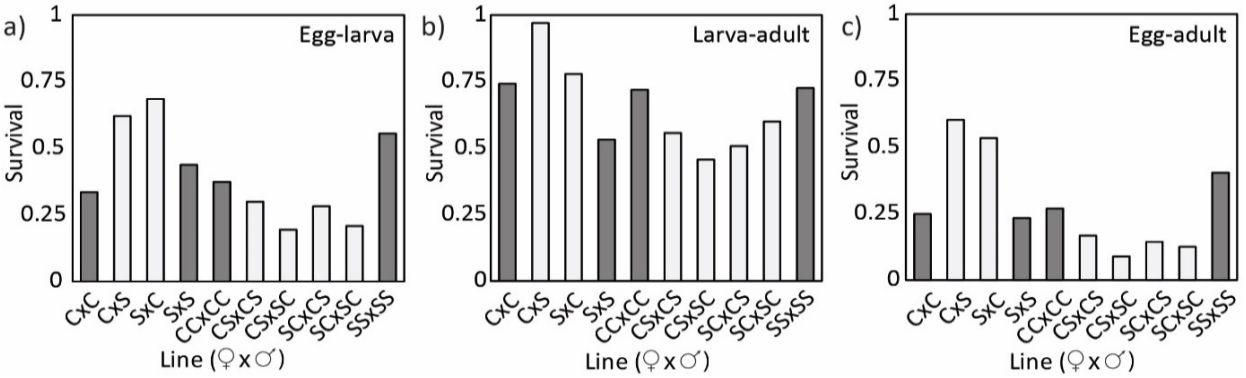

Fig. S2. Proportional survival of the progeny of *Leptidea sinapis* crosses between Spanish (C) and Swedish (S) chromosomal races from (**a**) egg to larva, (**b**) larva to adult, and (**c**) egg to adult for each of the crosses applied in F_1 and F_2. Dark grey bars indicate pure lines, and light grey bars indicate hybrid lines.

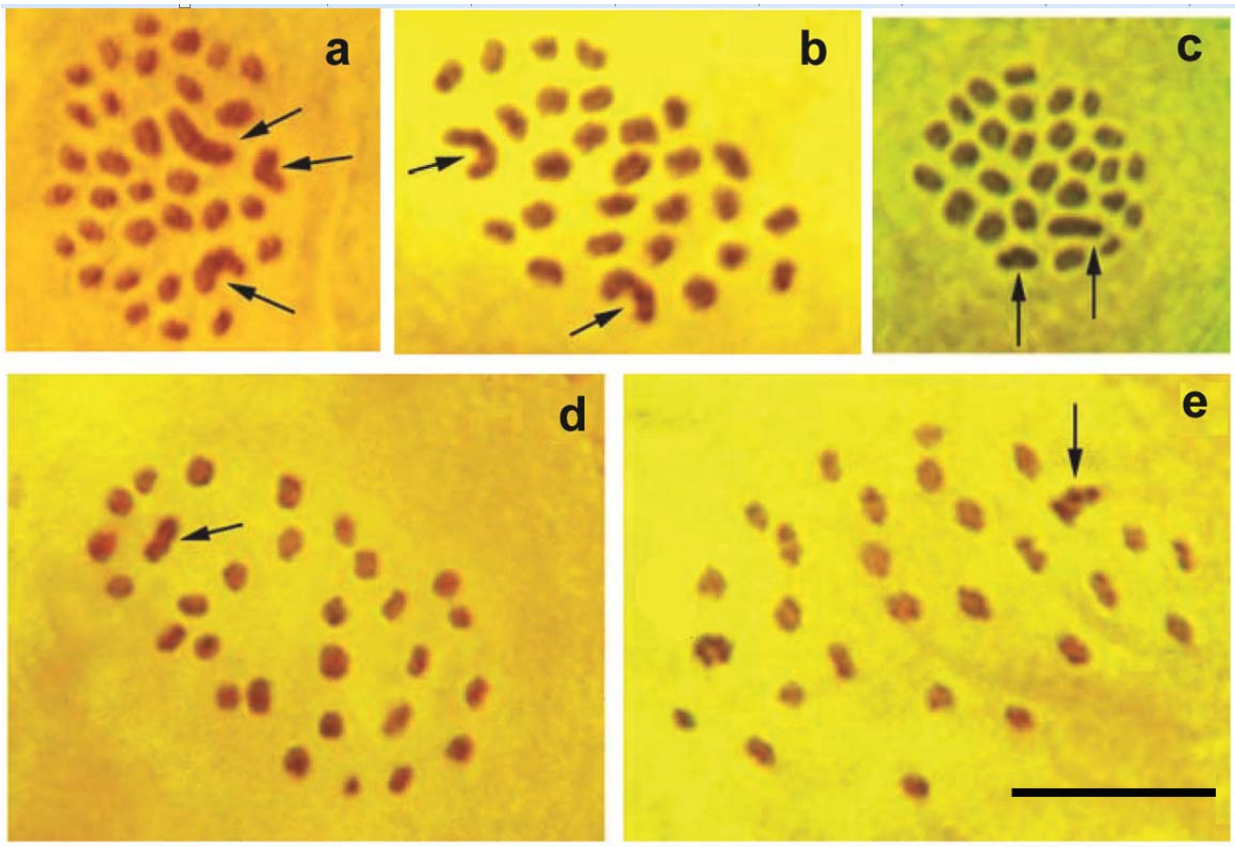

Fig. S3. Metaphase I plates of *Leptidea sinapis* with bivalents and trivalents. Arrows indicate trivalents. Scale bar corresponds to 10 µm in all figures. (a-d) Intact or weakly squashed metaphase plates, view from pole. All bivalents are oriented parallel to the spindle fibers, while trivalents lies in the equatorial plane and oriented transversely to the spindle fibers. (e) Squashed metaphase plate. The bivalents are oriented parallel to the spindle fibers, while the trivalent is oriented transversely to the spindle fibers. (a) Romania, RVcoll07E366, n=34. (b) Kazakhstan, RVcoll06H638, n=28. (c) Kazakhstan, RVcoll07Z236, n=28. (d) Kazakhstan, RVcoll06H640, n=29. (e) Kazakhstan, RVcoll07Z236, n=28.

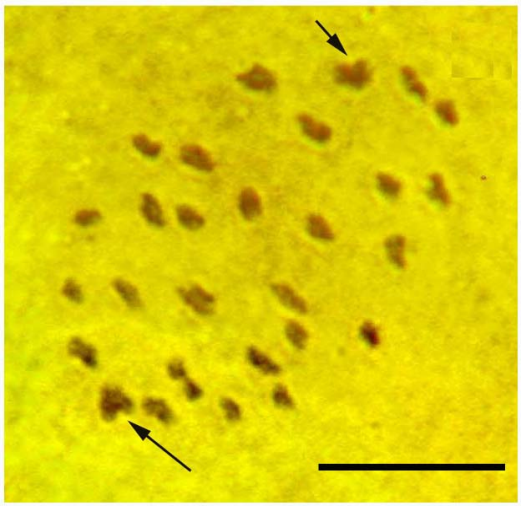

Fig. S4. Metaphase II plates of *Leptidea sinapis* with bivalents and trivalents. Arrows indicate trivalents. The trivalents are U-shaped and co-oriented, i.e. the larger homolog is oriented towards one pole, while two smaller homologs are oriented towards the opposite pole. Scale bar corresponds to 10 µm. Kazakhstan, RVcoll06H631, MII cell showing 28 chromosome elements.

Table S1. Male specimens of *Leptidea sinapis* used for karyological analyses.

Sample ID	Studied as	Line type	Chromosome number
L1	larva	pure lab line Spain	2n=106
L2	larva	pure lab line Spain	2n=108
L3	larva	pure lab line Sweden	2n=57
L4	larva	pure lab line Sweden	2n=58
12M060	adult	pure line Sweden	n=27
12M058	adult	pure line Sweden	n=27+trivalent
12M049	adult	pure line Sweden	n=29
12Z085	adult	pure line Sweden	n=29
10-B467	adult	pure line Sweden	n=29, 2n=ca59
12-M050	adult	pure line Sweden	no cell divisions
12-M052	adult	pure line Sweden	no cell divisions
12-M053	adult	pure line Sweden	no cell divisions
12-Z091	adult	pure line Sweden	no cell divisions
12-Z093	adult	pure line Sweden	no cell divisions
12M054	adult	pure line Sweden	no cell divisions
12M055	adult	pure line Sweden	no cell divisions
12M056	adult	pure line Sweden	no cell divisions
12M057	adult	pure line Sweden	no cell divisions
12M059	adult	pure line Sweden	no cell divisions
12M061	adult	pure line Sweden	no cell divisions
12M062	adult	pure line Sweden	no cell divisions
12Z086	adult	pure line Sweden	no cell divisions
12Z087	adult	pure line Sweden	no cell divisions
12Z088	adult	pure line Sweden	no cell divisions
12Z089	adult	pure line Sweden	no cell divisions
12Z090	adult	pure line Sweden	no cell divisions
12Z092	adult	pure line Sweden	no cell divisions
10-B466	adult	pure line Sweden	no result
13Y025	adult	F_1 hybrid, Spain x Sweden	2n=82
13Y037	adult	F_1 hybrid, Spain x Sweden	2n=82
13Y050	adult	F_1 hybrid, Spain x Sweden	2n=82
13Y039	adult	F_1 hybrid, Spain x Sweden	2n=82
13Y058	adult	F_1 hybrid, Spain x Sweden	n=28
12-Z065	adult	F_1 hybrid, Spain x Sweden	n=28-31
13Y045	adult	F_1 hybrid, Spain x Sweden	n=29
13Y057	adult	F_1 hybrid, Spain x Sweden	n=29
13Y063	adult	F_1 hybrid, Spain x Sweden	n=29
12-Z066	adult	F_1 hybrid, Spain x Sweden	n=29-32
12-Z051	adult	F_1 hybrid, Spain x Sweden	n=30-32
12-Z054	adult	F_1 hybrid, Spain x Sweden	n=30-33
12-Z048	adult	F_1 hybrid, Spain x Sweden	no cell divisions
12-Z055	adult	F_1 hybrid, Spain x Sweden	no cell divisions
12-Z057	adult	F_1 hybrid, Spain x Sweden	no cell divisions
12-Z067	adult	F_1 hybrid, Spain x Sweden	no cell divisions
12-Z073	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y023	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y024	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y029	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y030	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y036	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y043	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y047	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y048	adult	F_1 hybrid, Spain x Sweden	no cell divisions

Sample ID	Studied as	Line type	Chromosome number
13Y060	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y026	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y027	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y028	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y031	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y032	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y033	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y034	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y035	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y038	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y040	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y041	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y042	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y044	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y046	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y049	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y056	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y059	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y061	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y062	adult	F_1 hybrid, Spain x Sweden	no cell divisions
13Y064	adult	F_1 hybrid, Spain x Sweden	no cell divisions
L5	larva	F_2 hybrid, Spain x Sweden	2n > 80, unknown exact number
L6	larva	F_2 hybrid, Spain x Sweden	2n > 80, unknown exact number
L7	larva	F_2 hybrid, Spain x Sweden	2n=73, approximate
L8	larva	F_2 hybrid, Spain x Sweden	2n=77, approximate
L9	larva	F_2 hybrid, Spain x Sweden	2n=77, approximate
L10	larva	F_2 hybrid, Spain x Sweden	2n=82, approximate
L11	larva	F_2 hybrid, Spain x Sweden	2n=82, approximate
L12	larva	F_2 hybrid, Spain x Sweden	2n=83
L13	larva	F_2 hybrid, Spain x Sweden	2n=83
L14	larva	F_2 hybrid, Spain x Sweden	2n=83
L15	larva	F_2 hybrid, Spain x Sweden	2n=85
L16	larva	F_2 hybrid, Spain x Sweden	2n=90
13Y080	larva	F_2 hybrid, Spain x Sweden	n=31
11-H440	adult	F_2 hybrid, Spain x Sweden	n=31-32 in MI
13Y079	larva	F_2 hybrid, Spain x Sweden	n=32
13Y082	larva	F_2 hybrid, Spain x Sweden	n=32
11-H437	adult	F_2 hybrid, Spain x Sweden	n=32 in MI
13Y084	larva	F_2 hybrid, Spain x Sweden	n=32, 33
13Y083	larva	F_2 hybrid, Spain x Sweden	n=33
11-H479	adult	F_2 hybrid, Spain x Sweden	n=35 in MI
13Y077	larva	F_2 hybrid, Spain x Sweden	n=35, 36
11-H467	adult	F_2 hybrid, Spain x Sweden	n=35?
13Y081	larva	F_2 hybrid, Spain x Sweden	n=36
11-H439	adult	F_2 hybrid, Spain x Sweden	n=37? or n=34
11-H447	adult	F_2 hybrid, Spain x Sweden	no divisions
11-H451	adult	F_2 hybrid, Spain x Sweden	no divisions
11-H460	adult	F_2 hybrid, Spain x Sweden	no divisions
11-H461	adult	F_2 hybrid, Spain x Sweden	no divisions
11-H475	adult	F_2 hybrid, Spain x Sweden	no divisions
13Y078	larva	F_2 hybrid, Spain x Sweden	no results
L17	larva	F_3 hybrid, Spain x Sweden	2n > 80, unknown exact number
L18	larva	F_3 hybrid, Spain x Sweden	2n= 95 or 96
L19	larva	F_3 hybrid, Spain x Sweden	2n= 96, approximate
L20	larva	F_3 hybrid, Spain x Sweden	2n=76
L21	larva	F_3 hybrid, Spain x Sweden	2n=76

Sample ID	Studied as	Line type	Chromosome number
L22	larva	F_3 hybrid, Spain x Sweden	2n=77
L23	larva	F_3 hybrid, Spain x Sweden	2n=86
14A002	larva	F_3 hybrid, Spain x Sweden	n=29
14A004	larva	F_3 hybrid, Spain x Sweden	n=29
14A003	larva	F_3 hybrid, Spain x Sweden	n=31
14A001	larva	F_3 hybrid, Spain x Sweden	n=32
14A000	larva	F_3 hybrid, Spain x Sweden	n=33
14A005	larva	F_3 hybrid, Spain x Sweden	n=35
14A006	larva	F_3 hybrid, Spain x Sweden	no meiotic divisions
14B000	larva	F_4 hybrid, Spain x Sweden	n=32
14B003	larva	F_4 hybrid, Spain x Sweden	n=33
14B004	larva	F_4 hybrid, Spain x Sweden	n=33
14B002	larva	F_4 hybrid, Spain x Sweden	n=34
14B005	larva	F_4 hybrid, Spain x Sweden	n=37
14B001	larva	F_4 hybrid, Spain x Sweden	n=38

Table S2. Sample sizes of *Leptidea sinapis* families used for survival analyses

		Sample size (families)		
Cross type	**Generation**	**eggs**	**larvae**	**adults**
Pure	**F_1**	373 (16)	143 (16)	87 (16)
Hybrid	**F_1**	286 (12)	189 (12)	160 (12)
Pure	**F_2**	767 (12)	362 (12)	262 (12)
Hybrid	**F_2**	908 (20)	225 (17)	121 (15)
Hybrid	**F_3**	371 (14)	110 (8)	69 (7)
Hybrid	**F_4**	204 (7)	70 (6)	43 (4)

Table S3. Statistical output of linear models (ANOVA II) testing the impact of cross type (pure-/hybrid) and offspring generation (F_1/F_2) in *Leptidea sinapis* on survival between life stages (fitness), on the offspring sex ratio and on the female fecundity. Significant results are indicated in bold.

	Survival egg-larva			**Survival larva-adult**			**Survival egg-adult**			**Sex ratio**			**Fecundity**		
	df	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Cross Type (CT)	1	0.56	0.45	1	0.18	0.68	1	0.10	0.75	1	0.24	0.63	1	0.36	0.63
Offspring Generation (OG)	1	19.4	**<0.001**	1	7.49	**0.008**	1	21.2	**<0.001**	1	1.18	0.28	1	18.5	**<0.001**
CT * OG	1	10.5	**<0.001**	1	14.9	**<0.001**	1	24.7	**<0.001**	1	0.003	0.96	1	2.19	0.14
Error	56			54			56			52			56		

Table S4. Impact of *Leptidea sinapis* sibling crosses on survival. There were no significant differences in survival between offspring from sibling crosses and offspring from non-related crosses (ANOVA II).

	Survival egg-larva			**Survival larva-adult**			**Survival egg-adult**		
	df	*F*	*P*	*df*	*F*	*P*	*df*	*F*	*P*
Cross (sibling/outcrossed)	1	0.15	0.70	1	1.39	0.25	1	0.14	0.71
Error	26			26			26		

Table S5. Statistical output (Analysis of deviance) of generalized mixed logistic regression models testing the impact of larval cross type within each generation on survival between life stages (fitness) of *Leptidea sinapis*. Analysis was made in separate models for pure lines and for hybrid lines. Significant effects are indicated in bold font. In the pure lines of generation F_1, 75% of the larvae from Spain x Spain crosses survived from larva to adult, as compared with 53% of the Sweden x Sweden crosses, and in the hybrid lines of the same generation 97% of the larvae of the Spain x Sweden cross survived until adulthood, whereas 78% of larvae in the Sweden x Spain cross eclosed as adults. These were the only significant differences (see also Fig. S3).

	Egg-larva			**Larva-adult**			**Egg-adult**		
Cross/Generation	χ^2	***df***	***P***	χ^2	***df***	***P***	χ^2	***df***	***P***
Pure/F_1	3.3	1	0.07	4.33	1	**0.037**	0.46	1	0.50
Hybrid/F_1	0.2	1	0.67	7.25	1	**0.007**	0.11	1	0.74
Pure/F_2	2.5	1	0.12	2.61*	1	0.27	2.30	1	0.13
Hybrid/F_2	1.2	3	0.75	1.58	3	0.66	5.90*	3	0.12

*these models either failed to converge with max|grad| or included non-uniquely determined parameters, indicating unsuitable data structure for the glmer analysis. Instead, these data were tested in generalized linear models (glm) not including family as random factor.

Table S6. Statistical output of general linear models (ANOVA II) testing the impact of hybrid offspring generation (F_1-F_4) on survival between life stages (fitness) of *Leptidea sinapis*, on the offspring sex ratio, and on female fecundity (parental crosses, and crosses in generation F_1-F_3). Significant effects are indicated in bold font. Post-hoc significant testing was obtained through Tukey's HSD test (see also Fig. 2).

	Egg-larva			**Larva-adult**			**Egg-adult**			**Sex ratio**			**Fecundity**		
	df	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***	***df***	***F***	***P***
Hybrid generation	3	11.2	**<0.001**	3	5.2	**0.004**	3	17.0	**<0.001**	1	0.41	0.75	3	2.31	0.088
Error	49			40			49			37			49		

Table S7. Statistical output (Analysis of deviance) of generalized linear mixed models testing the impact of cross type (within-/between populations) and offspring generation (F_1/F_2) of *Leptidea sinapis* on survival between life stages (fitness) and on the offspring sex ratio. All models have family as random factor. Significant results are indicated in bold.

	Survival egg-larva			Survival egg-adult			Survival larva-adult			Sex ratio		
	χ^2	*df*	*P*	χ^2	*df*	*P*	χ^2	*df*	*P*	χ^2	*df*	*P*
Cross Type (CT)	1.3	1	0.25	0.46	1	0.5	0.012	1	0.97	0.68	1	0.41
Offspring Generation (OG)	16.2	1	**<0.001**	18.8	1	**<0.001**	6.2	1	**0.013**	3.6	1	0.078
CT * OG	12.9	1	**<0.001**	25.8	1	**<0.001**	16.8	1	**<0.001**	0.22	1	0.64

Table S8. Statistical output (Analysis of deviance) of generalized mixed logistic regression models testing the impact of offspring generation (F_1-F_4) on survival between life stages (fitness) and on the offspring sex ratio in the hybrid lines of *Leptidea sinapis*. Bottom section shows the output of similar linear models for adult dry weight (separate for males and females; generation F_1-F_3) and female fecundity (Parental crosses, and crosses in generation F_1-F_3). All models have family as random factor. Significant effects are indicated in bold.

Hybrid fitness	χ^2	*df*	*P*
Egg-larva	25.1	3	**<0.001**
Egg-adult	37.6	3	**<0.001**
Larva-adult	21.9	3	**<0.001**
Sex ratio	3.46	3	0.33

SI Video. Courtship involving male and female *Leptidea sinapis* from north-eastern Spain (Catalonia).