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Secondary contact zones of closely-related *Erebia* butterflies overlap with narrow phenotypic and parasitic clines

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Abstract

Zones of secondary contact between closely related taxa are a common legacy of the Quaternary ice ages. Despite their abundance, the factors that keep species apart and prevent hybridisation are often unknown. Here we study a very narrow contact zone between three closely related butterfly species of the *Erebia tyndarus* species complex. Using genomic data, we first determined if gene flow occurs and then assessed whether it might be hampered by differences in chromosome number between some species. We found interspecific gene flow between sibling species that differ in karyotype by one chromosome. Conversely, only F1 hybrids occurred between two species that have the same karyotype, forming a steep genomic cline. In a second step, we fitted clines to phenotypic, ecological and parasitic data to identify the factors associated with the genetic cline. We found clines for phenotypic data and the prevalence of the endosymbiont parasite *Wolbachia* to overlap with the genetic cline, suggesting that they might be drivers for separating the two species. Overall our results highlight that some gene flow is possible between closely-related species despite different chromosome numbers, but that other barriers restrict such gene flow.

Key words: RADseq, Wolbachia, speciation, butterfly, clines

Introduction

During the Quaternary ice ages, the distribution ranges of many species became fragmented, and populations subsequently diverged from each other in allopatry. Following the glacial retreats, many of these isolated populations underwent range expansions, often leading to secondary contact zones between closely-related lineages or species (Hewitt, 2000). The evolutionary outcomes of such secondary contacts depend on the presence and strengths of pre- or postzygotic intrinsic and/or extrinsic barriers to gene flow (Marie Curie SPECIATION Network *et al.*, 2012; Seehausen *et al.*, 2014; Canestrelli *et al.*, 2016). The results can range from the extensive admixture of two lineages to rare hybridisation events, resulting in hybrid zones that are more or less wide depending on the degree of divergent selection or selection against hybrids (Harrison & Larson, 2014; Gompert *et al.*, 2017). Species may in particular form clines for traits experiencing divergent selection upon secondary contact, where the slope of a cline reflects the strength of divergent selection (Slatkin, 1973; Barton & Hewitt, 1985; Gompert *et al.*, 2017). Finally, secondary contact can lead to the coexistence of two isolated species if strong pre- and/or postzygotic barriers exist or evolve (Jiggins, 2000; Canestrelli *et al.*, 2016). The abundance of postglacial lineages or formerly allopatric species in secondary contact has attracted much interest, yet the isolating barriers separating such species often remain unclear (Ortiz-Barrientos *et al.*, 2009; Harrison & Larson, 2014; Gompert *et al.*, 2017).

Theory suggests that chromosomal rearrangements through fusion and fission of existing chromosomes could act as isolating mechanisms because hybrids are predicted to be at least partially sterile (White, 1978; King, 1995; Dion-Côté & Barbash, 2017). Fusion and fission events are often widespread in taxonomic groups with holocentric chromosomes, i.e. that lack a centromere, such as Lepidoptera (Melters *et al.*, 2012, de Vos *et al.*, 2020). However, the impact of karyotype evolution on rates of speciation has been challenged given the often widely observed hybridisation and introgression between closely related species despite varying chromosome numbers (Cohuet et al., 2005; Descimon & Mallet, 2009; Polyakov et al., 2011). Nevertheless, the potential for interspecific gene flow is expected to be less constrained between closely-related species sharing the same number of chromosomes than between species with different chromosome numbers. To prevent costly hybridization, further barriers may subsequently evolve. For example, closely related

species of *Agrodiaetus* butterflies evolved increased phenotypic differentiation through reinforcement, allowing for pre-zygotic isolation through mate choice and promoting their coexistence (Lukhtanov et al., 2005). However, other factors may similarly promote reproductive isolation between closely related species. These factors are often species-dependent and may include, among others, mate choice (Mérot *et al.*, 2017), differences in (micro-) habitat use (Kleckova *et al.*, 2014) or the infection by endosymbionts. The latter has been shown for the cytoplasmically inherited endosymbiotic bacteria *Wolbachia*, causing uni- or bidirectional cytoplasmatic incompatibility between insect species (Bordenstein *et al.*, 2001; Brucker & Bordenstein, 2012) or lineages (Zabal-Aguirre *et al.*, 2009). *Wolbachia* is common, occurring in ~20% of all insect species (Reuter *et al.*, 2004), yet the outcome of interspecific crosses that bring different *Wolbachia* lineages together, depends on the host species and may range from no effect to hybrid male sterility or the complete failure to produce viable offspring (Rokas, 2000; Brucker & Bordenstein, 2012).

With more than one hundred described species, *Erebia* is one of the most species-rich Palearctic butterfly genera. *Erebia* are cold-adapted and mountainous environments represent biodiversity hot-spots (Sonderegger, 2005; Peña et al., 2015). In Europe, *Erebia* diversified over the last ~15 million years, with species often differing in their chromosome numbers (Robinson, 1971; Lucek, 2018). This is particularly true for the *E. tyndarus* group, which diversified much more recently, i.e. over the last 0.15-1 million years (Martin et al., 2002; Albre et al., 2008a; Peña et al., 2015) into a total of 10 nominal species and 45 recognised subspecies, often representing different postglacial lineages whose taxonomic status has not been formally assessed (Albre et al., 2008b). The diversification of *Erebia* and the *E. tyndarus* group in particular has been attributed to changes in chromosome numbers (Lorkovic, 1958; Peña et al., 2015), where rates of speciation were found to be positively correlated with karyological diversity (de Vos et al. 2020). Importantly, changes in chromosome numbers were more frequently anagenetic, i.e. occurring along a branch of the phylogenetic tree than at a speciation event. Novel chromosomes may therefore help to buildup reproductive isolation over time, e.g. by suppressing recombination while some gene flow might still be possible (de Vos et al. 2020).

Species within the *E. tyndarus* group often form zones of secondary contact with reported cases of hybridisation and interspecific gene flow (Albre et al., 2008a; Descimon & Mallet, 2009). Three sister species of the *E. tyndarus* group provide the opportunity to study

the role of different barriers during secondary postglacial contact: *E. tyndarus, E. cassioides* and *E. nivalis*. While *E. cassioides* has many allopatric populations associated with different mountain ranges from the Cantabrian Mountains in Spain to the Balkans in Eastern Europe (Albre et al., 2008a), the distributions of *E. tyndarus* and *E. nivalis* are restricted to the Alps (Figure 1). *E. tyndarus* occurs in the central Alps excluding *E. cassioides*, while *E. nivalis* has its main distribution in the Eastern Alps occurring at higher elevations than *E. cassioides*. The exception is the narrow contact zone in the Grindelwald region in Switzerland, between an isolated population of *E. nivalis* that persists on the topmost parts of a mountain range, and *E. cassioides* that occurs in the west, and *E. tyndarus* in the east (Figure 1; Sonderegger, 2005).

Whereas *E. cassioides* and *E. tyndarus* have the same number of chromosomes (haploid n=10), *E. nivalis* has one chromosome more (n=11). Classic crossing experiments showed that *E. tyndarus* and *E. cassioides* hybridise readily in the lab but hybrids are only rarely observed in the wild (Lorkovic, 1958; Descimon & Mallet, 2009), despite the absence of apparent hybrid inviability or sterility (Presgraves, 2002). This is consistent with extrinsic barriers maintaining the species boundaries, but such barriers have not been assessed so far. Crosses between *E. tyndarus* and *E. nivalis* have not yet been performed, but intermediates can be found rarely in the wild, while crosses between *E. cassioides* and *E. nivalis* produced only a few and sterile F1 offspring (Lorkovic, 1958). Based on restriction site associated sequence (RADseq) data, a former study concluded that interspecific gene flow is absent between the three *Erebia* species (Gratton *et al.*, 2016). However, this conclusion was based on only a few (N=46) allopatric individuals, sampled from the entire ranges of the three species, excluding secondary contact zones. The study of secondary contact zones is required to understand interspecific gene flow and the evolutionary mechanisms restricting it (Seehausen *et al.*, 2014).

To test for interspecific gene flow and potential barriers during secondary contact, we studied the only known secondary contact zone between all three species in the Grindelwald region of Switzerland (Figure 1). Given classic crossing experiments (Lorkovic, 1958; Descimon & Mallet, 2009), we expected interspecific gene flow to be more common between the two sibling species *E. tyndarus* and *E. cassioides* that have the same karyotype. Conversely, if changes in chromosome numbers act as a barrier to gene flow, we predicted less gene flow between *E. nivalis* and its sibling species. In the absence of differing

chromosome numbers and under the assumption that hybridisation is at least to some degree maladaptive and costly, we predicted that other barriers prevent interspecific gene flow between *E. tyndarus* and *E. cassioides*. The latter may, for example, manifest as phenotypic differentiation, potentially aiding mate recognition or through differences in the utilised habitat to reduce interspecific encounters. Both phenotypic and ecological differentiation may reinforce interspecific differentiation (Gompert et al., 2017; Garner et al., 2018; Butlin & Smadja, 2018). Lastly, the presence of *Wolbachia* has been confirmed for *Erebia* (Gratton *et al.*, 2016). If different *Erebia* species are infected by different *Wolbachia* strains, the latter could select against interspecific gene flow by reducing hybrid fitness or even causing hybrid sterility (Rokas, 2000; Brucker & Bordenstein, 2012). Consequently, we also assessed the prevalence and diversity of *Wolbachia*, predicting different *Wolbachia* lineages to be associated with different host species.

Material and Methods

We collected 95 imagoes belonging to the *E. tyndarus* species complex during summer 2017 across the previously described contact zone in the Grindelwald area in the Swiss Alps (Sonderegger, 2005). Individuals were *a priori* assigned by eye to a species based on the location where they were collected and based on phenotypic traits that are commonly used to differentiate between taxa, being mainly the shape and extent of the orange spot on the forewings (Sonderegger, 2005; Figure 1). While clearly belonging to the three focal species, several individuals showed intermediate phenotypes in the contact zone. The sampling of *E. nivalis* was restricted, being a top-priority species on the national red list. All individuals were caught by hand-netting and immediately euthanised with an overdose of ethyl acetate. For each specimen, we recorded its place-of-catch (GPS). The wings of each specimen were clipped and stored in paper bags for further morphological analyses and the body stored in a -20°C freezer prior to DNA extraction. DNA for each specimen was extracted from thorax tissue with the Qiagen Blood & Tissue Kit (Qiagen, Zug, Switzerland) following the manufacturer's protocol. Individuals were genotyped employing a restriction site associated DNA (RAD) single-end sequencing at *Sbf*I restriction cut-sites (Baird *et al.*, 2008) on one Illumina HiSeq2500 lane. To increase complexity at the first 10 sequenced base pairs, 10% bacteriophage PhiX genomic DNA (Illumina Inc., San Diego, CA, USA) was

added to the run. Library preparation and sequencing was outsourced to Floragenex (Portland, OR, USA).

Data Filtering & Assembly

We filtered all sequencing reads to have an intact *Sbf*I restriction site, followed by de-multiplexing and barcode-trimming with PROCESS_RADTAGS 1.48 (Catchen *et al.*, 2013). Reads without the complete *Sbf*I recognition sequence were subsequently discarded. Using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), we removed reads containing bases with a Phred quality score <10 or more than 5% of base pairs with quality <30. This approach yielded 319'072'377 million high quality reads for analysis.

Given the lack of an *Erebia* reference genome, we generated a *de novo* assembly of RAD-tags using all filtered reads for all individuals with USTACKS 1.48 (Catchen *et al.*, 2011) with the following settings: minimum stack size of 100 reads, a maximum of two base pairs of difference for stacks to be merged, excluding loci with unusually high coverage to avoid repetitive regions. The initial *de novo* assembly consisted of 42'517 contigs. To further identify and remove exogenous contigs from the assembly, we compared the assembly against the NCBI GenBank nucleotide collection with the BLASTN function from BLAST+ 2.7.1 (Camacho *et al.*, 2009). A total of 3188 or 7.0 % of all contigs were of exogenous origin and removed from the initial assembly. We extracted contigs related to *Wolbachia* (157 contigs) as a separate *de novo* assembly to further map the prevalence of this parasite across all *Erebia* specimens (see below). We then mapped the reads for each individual against the cleaned assembly with BWA MEM 0.7.17 (Li, 2013) and used SAMTOOLS 1.7.20 (Li *et al.*, 2009) to filter reads with a mapping quality of <20. On average, 2.03 million reads mapped per individual (SD: ±921k reads; Table S1). We also aligned the raw sequencing reads against the PhiX 174 reference genome (accession: NC_001422; (Sanger *et al.*, 1977)), masking known variants. We then used the PhiX-alignments to create a base-quality score recalibration table with BASERECALIBRATOR from GATK v. 3.7-0 (McKenna *et al.*, 2010). We subsequently recalibrated the base quality scores of each individual to remove potential library effects with the GATK tool PRINTREADS. Lastly, we incorporated a recently published dataset comprising *Sbf*I based RADseq data for 46 individuals from across the entire range of the *E. tyndarus* complex in the Alps, as well as samples from the Pyrenees and the Apennines for *E. cassioides* (NCBI Sequence Read Archive – SRA, accession SRP065834 from (Gratton *et al.*,

2016); see Table S1). The dataset by Gratton *et al.* (2016) also included five individuals from the Grindelwald region (two *E. tyndarus* and three *E. nivalis*) as well as three individuals from *E. calcaria*, which we used as outgroup. We similarly aligned these individuals against the cleaned assembly, applying the same quality filtering settings.

We employed the GATK tool UNIFIEDGENOTYPER to call variants and genotypes in a combined fashion for all individuals, using the following parameters: minimal Phred-scaled base quality score threshold of 20, a genotype likelihood model calling both SNPs and insertions/deletions (indels). Using VCFTOOLS v0.1.14 (Danecek *et al.*, 2011), genotypes with quality < 28 or depth < 6 were set to missing. Three individuals with more than 80% missing data were subsequently removed from the data set. Variants with > 10% missing genotypes per sampling site, monomorphic sites, SNPs with > 2 alleles, indels and SNPs 10 bp around indels were further removed from the dataset also applying a minor allele frequency cut-off of 0.03. This filtering step was performed using either all specimens or only for the specimens from the Grindelwald region resulting in 4362 and 4289 polymorphic SNPs, respectively.

Population structure & gene flow

To infer the phylogenetic structure across all available specimens, we used RAxML 8.2.11 (Stamatakis, 2014) implementing a generalised time-reversible (GTR) model with optimised substitution rates and a gamma model of rate heterogeneity. We further applied an ascertainment bias correction to account for the fact that we only used polymorphic SNP positions with the ASC_GTRGAMMA function implemented in RAxML. Significance was assessed using 1000 bootstrap replicates followed by a thorough maximum likelihood search. Because hybrid individuals may induce homoplasies in a phylogeny (Seehausen, 2004), we repeated the analysis omitting two identified F1 hybrid individuals (see results) from the analysis.

We inferred population structure in the zone of secondary contact with ADMIXTURE 1.3.0, which uses a likelihood approach to estimate ancestry (Alexander *et al.*, 2009). We ran ADMIXTURE multiple times, varying the values for K assumed populations from 2 to 10. We then performed a cross-validation test to determine the optimal value of K. We further calculated the pairwise genetic differentiation (G_{ST}) among the resulting genetic clusters in

GENODIVE v. 2.0b27 (Meirmans & Van Tienderen, 2004). Significances of G_{ST}s were assessed with 1000 permutations.

Finally, we tested for patterns of introgression between different *Erebia* species within the studied contact zone by calculating Patterson's four-taxon D-statistics (Patterson *et al.*, 2012), which compare the frequencies of discordant SNP genealogies. The estimates are based on the so-called ABBA-BABA test, which compares counts of discordant site patterns along a phylogenetic tree. The latter consists of three ingroups and an outgroup, i.e. (((P1,P2),P3),O) with P1 and P2 being more closely related and P3 being more distantly-related. In the absence of interspecific gene flow, the number of ABBA and BABA trees should be unbiased and the expected value of Patterson's D-statistic close to zero. The values of D-statistic that are above zero correspond to a higher number of ABBA patterns and thus introgression between P2 and P3, whereas negative values indicate a higher frequency of BABA patterns and introgression between P1 and P3. We performed ABBA/BABA tests using *E. tyndarus* and *E. nivalis* as most closely related sister taxa (i.e. P1 and P2), more distantly related to *E. cassioides* (P3) and with *E. calcaria* as outgroup. This scenario reflects the mitochondrial phylogeny (Lucek, 2018) as well as the nuclear relationship when hybrids were excluded (Figure 2). We calculated average *D* across the genome, its variation across the genome using a jackknife approach and a Z score test to assess if *D* deviates significantly from zero, following (Martin *et al.*, 2015).

Morphological analyses

The forewings of *Erebia* butterflies often differ phenotypically between species in terms of wing shape, the size and extent of the orange spot and the number and position of eyespots (Sonderegger, 2005; Gratton *et al.*, 2016; Figure 1). To capture this phenotypic variation we selected a total of nineteen geometric morphometric landmarks that cover wing shape based on vein intersections or terminations as well as the extent and position of the white, black and orange spots of the right dorsal forewing (Figure S1). Individuals with damaged wings and/or damaged scale patterns were omitted from the analysis. Wings for a total of 72 specimens were digitised using a flatbed scanner. Landmarks were set using TPSDIG2 (Rohlf, 2006), with individuals in random order. We performed Procrustes fits on the obtained landmarks in MORPHOJ 1.06b (Klingenberg, 2011) and extracted the major axis

of phenotypic variation using a principal component (PC) analysis. We then tested for phenotypic differentiation along PC1 between the genetic groups assigned by ADEGENET using ANOVAs and TukeyHSD *post hoc* tests.

Testing for niche differentiation

Several distantly related and coexisting *Erebia* species have been shown to differ in their microhabitat use (Kleckova *et al.*, 2014), however, to which degree this may also apply to our closely related focal species is unknown. To test for niche differentiation, we extracted the values of a set of environmental variables with a spatial resolution of 25m (Guisan *et al.*, 2006) from the GPS recorded locations where each specimen was captured in the field. The variables included were: monthly mean cloudiness (%), monthly moisture index (1/10mm / month), monthly mean precipitation between 1961-1990 (mm), the monthly mean of average temperature between 1961-1990 (all temperatures in °C), annual growing degree days for plants (i.e. the number of days with temperature above a threshold of 3°C which is critical to growth), maximal temperature in summer and winter, minimal temperature in spring and autumn, annual average site water balance (mm / year), number of precipitation days per growing season, the annual average number of frost days during the growing season and lastly, the slope of the terrain (°). For monthly-based variables, we used only data for July-September, being the months where the studied *Erebia* species fly (Sonderegger, 2005). To reduce correlation among variables, we reduced the dataset to three independent principal component (PC) axes accounting for 92.5% of the total variation (80.4%, 8.0% and 4.2% for PC1-3 respectively, Table S2). We then used NICHEROVER (Swanson *et al.*, 2015) to assess potential multivariate niche differentiation among species based on the retained PC scores. Given the sample sizes, we only compared *E. cassioides* and *E. tyndarus*. Each variable was further tested for a difference between the two species using a MANOVA for all traits together and single ANOVAs separately for each trait. All statistical analyses were performed in R 3.5.1 (R Core Team, 2018).

Prevalence of Wolbachia

Sequences for all individuals from this study and the ones from Gratton *et al.* (2016) were aligned with BWA MEM against the 157 contigs belonging to *Wolbachia*, a parasitic

microbial endosymbiont common to arthropods (Hilgenboecker *et al.*, 2008). Using VCFTOOLS, genotypes with quality < 28 or depth < 6 were set to missing and only biallelic SNPs were retained while applying a minor allele frequency cut-off of 0.01. Only sites with less than 40% missing data were retained. This approach resulted in a total of 23 polymorphic sites. Unique genotypes were identified, and a haplotype network was constructed using POPART (Leigh & Bryant, 2015). Individuals with incomplete genotypes were subsequently assigned to the most likely haplotype (see Table S4).

Cline analyses

We fitted a simple sigmoid cline for a genetic hybrid index between *E. cassioides* and *E. tyndarus* based on ADMIXTURE assignment probabilities for K=2 following Westram *et al.* (2018), applying the equations in Derryberry *et al.* (2013), using maximum likelihood estimation (BBMLE package in R, Bolker 2017). The cline was fitted using either the individual geographic distances or the least cost distances from the westernmost specimen in Figure 3. Further clines were fitted to phenotypic data using PC1 scores, scaled monthly mean precipitation in August and the prevalence of *Wolbachia*.

Results

Population genetic structure & gene flow

Species were well resolved in the RAxML tree, where *E. tyndarus* and *E. nivalis* cluster as sister species (Figure 2), consistent with the mitochondrial phylogeny (Peña *et al.*, 2015; Gratton *et al.*, 2016; Lucek, 2018). Due to homoplasy, *E. tyndarus* and *E. cassioides* cluster as sister species when the two F1 hybrids between the two species were included (Figure S2).

Three genetic clusters (K=3), as inferred with ADMIXTURE and representing the three *Erebia* species, were the best fitting number when using individuals from the contact zone (Figures 3 & S3). Consistent with former reported extents of the contact zone (Sonderegger, 2005, Figure 1) the three species were spatially not randomly distributed, with *E. tyndarus* occurring more in the East and *E. cassioides* more in the West of the Grindelwald valley, while *E. nivalis* showed a more limited distribution (Figure 3). ADMIXTURE further suggested interspecific gene flow with two hybrid individuals that were almost equally assigned to *E. tyndarus* and *E. cassioides* (Figure 3). These two individuals occurred moreover only in a

narrow zone of secondary contact between the two species. In contrast, three specimens from very distinct parts of the distribution showed evidence consistent with gene flow between *E. nivalis* and the two other species. Levels of genetic differentiation between species as measured with G_{ST} were substantial: *E. tyndarus* vs. *E. cassioides*: G_{ST} =0.550 (95% confidence interval: 0.538-0.563); *E. tyndarus* vs. *E. nivalis*: G_{ST} = 0.454 (0.439-0.469); *E. cassioides* vs. *E. nivalis*: G_{ST} = 0.592 (0.579-0.605) (all p<0.001).

Average genome-wide *D* statistics were positive (*D* = 0.218; SE: 0.073), indicating an excess of ABBA over BABA sites. This is consistent with *E. nivalis* sharing more genetic variation with *E. cassioides* than with *E. tyndarus*, suggesting interspecific gene flow despite different chromosome numbers. The *Z* score was ≥3 and thus above the 99% probability of the underlying standard normal distribution, suggesting a significant deviation of *D* from zero.

Phenotypic differentiation of wing shapes

The first PC axis captured 62.3% of the phenotypic variation (Figure 4, Table S3), where PC scores differed among species ($F_{2,71}$ = 76.4, p < 0.001) with *E. cassioides* individuals being mostly distinct from individuals of the two other species (Tukey *post hoc* test: both p < 0.001). In contrast, *E. nivalis* did not differ from *E. tyndarus* (p = 0.546), but sample size was limited. Individuals differed by the shape and extent of the orange spot, both along the horizontal and vertical wing axis and wing shape, with individuals having more or less condensed wings (Figure 4).

Niche differentiation

The multivariate niche assessed by NICHEROVER differed between *E. cassioides* and *E. tyndarus,* where *E. cassioides* showed a 47.1% overlap with *E. tyndarus* and *E. tyndarus* a niche overlap of 56.4% with *E. cassioides*. Despite great overlap (Figure 5a), the two species differed statistically along the ecological PC1 axis ($F_{1,81}$ = 4.3, p = 0.041), which was driven by differences in temperature and humidity (Table S2). The species similarly differed along PC2 ($F_{1,81}$ = 16.6, p < 0.001), which was driven by slope and annual average site water balance, and along PC3 ($F_{1,81}$ = 18.2, p < 0.001), explaining variation in humidity. A MANOVA using all environmental variables similarly supported an overall multivariate differentiation between

the two species ($F_{1,81}$ = 11.0, p < 0.001). Testing all variables independently with an ANOVA, while applying a Benjamini-Yekutieli correction for multiple testing, suggested that the environment where the two species occur differs mainly in monthly mean precipitation for months July-September (Figure 5b).

Wolbachia *prevalence*

Following filtering, the *Wolbachia* haplotypes for 59 individuals could be identified (Table S4). Within our studied contact zone, *Wolbachia* was common in *E. cassioides* and *E. nivalis* with a prevalence of 94.4% and 100% respectively (Figure 6). In contrast, *Wolbachia* was absent from *E. tyndarus* in the contact zone. Three distinct *Wolbachia* haplotypes occurred (h1-h3), which were differentially distributed among species (Figure 6; Table S4): One *Wolbachia* haplotype (h1) was exclusively found in an *E. calcaria* specimen. Haplotype h3 occurred in *E. cassioides* from the Grindelwald region and the Pyrenees as well as in one *E. nivalis* specimen from Grindelwald. Conversely, we found a widespread *Wolbachia* haplotype (h2), occurring in *E. cassioides* from Italy and Austria, in all but one *E. nivalis* and in the one *E. tyndarus* with an identifiable *Wolbachia* haplotype that was sampled by Gratton *et al.* (2016) from the Eastern part of Switzerland. Removing the missing data threshold for filtering suggested that *Wolbachia* occurred in three additional *E. tyndarus* individuals from the Italian Alps (Table S4), but given the high amount of missing data, *Wolbachia* could not be unambiguously assigned to a given haplotype. Lastly, the two F1 hybrids between *E. cassioides* and *E. tyndarus* (Figures 2 & 3) had the *Wolbachia* haplotype found in *E. cassioides* from Grindelwald (h2).

Cline analyses

Using geographic distances, the segregation between *E. cassioides* and *E. tyndarus* fell along a steep genetic cline where the cline centre was close to the position of the two F1 hybrids (centre position: 4.64 km [95% CI: 4.63-4.64], Figure 7a). The estimates for the cline centres for both the *Wolbachia* prevalence and wing morphology overlapped with the genetic cline (*Wolbachia*: 4.74 km [95% CI: 4.35-4.97]; wing morphology: 4.57 km [95% CI: 3.90-4.99], Figure 7b&c). Precipitation did show a clinal variation across the transect; however, its centre was shifted to the east (6.15 km [95% CI: 6.10-6.19], Figure 7d). The cline analyses based on least-cost distances similarly suggested an overlap between the cline centre for

the genetic data (6.64 km [95% CI: 6.63-6.65]; Figure S4) and the centres for *Wolbachia* (6.77 km [95% CI: 6.27-7.16]) and morphology (6.50 km [95% CI: 5.49-7.04]), while a shift occurs for precipitation (9.25 km [95% CI: 9.03-9.48]).

Discussion

Zones of secondary contact have gathered much theoretical and empirical interest to study the process of speciation (Barton & Hewitt, 1985; Kirkpatrick, 2001; Servedio & Noor, 2003; Ortiz-Barrientos *et al.*, 2009; Butlin & Smadja, 2018). However, the multivariate intrinsic and extrinsic factors that shape such contact zones, and that may prevent interspecific gene flow are often unknown (Harrison & Larson, 2014; Gompert *et al.*, 2017). Here, we studied such a secondary contact zone, combining population genomic with phenotypic and ecological data. We show that gene flow can occur across the species boundaries despite varying chromosome numbers, but that the potential for gene flow likely differs between species comparisons (Figure 3). The latter likely reflects different evolutionary mechanisms being at play. While some interspecific gene flow between the three *Erebia* species is consistent with former phenotypic and observational data from the wild (Sonderegger, 2005; Descimon & Mallet, 2009), our findings suggest that the species boundaries are more dynamic than found in a recent genomic study on the same species complex and whose data have been incorporated in this study (Gratton *et al.*, 2016). This is because Gratton *et al.* (2016) studied only a few, mostly allopatric individuals from across the entire ranges of the studied *Erebia* species where interspecific gene flow could not be detected.

While hybrids between species with different karyotypes are often predicted to yield offspring that are somewhat sterile (White, 1978; King, 1995; Dion-Côté & Barbash, 2017), we observed introgression despite such differences, i.e. current gene flow between *E. nivalis* and its two sibling species (Figure 3). The excess of ABBA over BABA sites is further consistent with past gene flow, where gene flow was higher from *E. cassioides* to *E. nivalis* than to *E. tyndarus*. Meiosis may still be possible between closely related species despite varying chromosome number (Searle, 1998; Lukhtanov *et al.*, 2018). However, the extent of introgression between *E. nivalis* and its sibling species is limited as indicated by the Admixture analysis (Figure 3), suggesting the presence of strong isolating mechanisms. Gene flow between *E. nivalis* and *E. cassioides* contrasts with evidence from classic crossing

experiments, where interspecific crosses were only possible in one direction, producing very few and sterile offspring in the lab (Lorkovic, 1958). Conversely, crosses between the closely-related *E. tyndarus* and *E. cassioides* that have the same karyotype, were found to readily produce offspring in the same study, consistent with the absence of inviability or sterility (Lorkovic, 1958; Presgraves, 2002). Contrary to these experimental crosses, we found interspecific hybridisation between *E. tyndarus* and *E. cassioides* to manifest solely as few F1 hybrids in our studied contact zone, in the absence of further introgression (Figures 3), resulting in a steep genomic cline separating the two species (Figure 7a). This suggests the presence of postzygotic incompatibilities or strong selection against hybrids. Although only few admixed individuals were found, the estimated cline may differ only little from an actual cline given the already relatively dense sampling and the small geographic range of this contact zone.

Hybrid adults may be absent due to pre-zygotic barriers, intrinsic incompatibilities or natural selection against hybrids imposed by the environment. Ecological differences between co-occurring *Erebia* species that could lead to habitat-dependent divergent selection and thus selection against hybrids, are often subtle in *Erebia* and linked to different microhabitat use even at a very small geographic scale (Kleckova *et al.*, 2014). The multivariate ecological niches of *E. tyndarus* and *E. cassioides* overlap to a large extent (Figure 5), differing mainly in mean monthly precipitation. However, this difference was consistent among the months of the main flight period of imagoes in the *E. tyndarus* complex (Sonderegger, 2005). Moreover, the observed difference in precipitation is consistent with the average ecological differentiation between the studied *Erebia* species across the entire species range (Schweiger *et al.*, 2014). Natural selection against hybrids could also act during the larval stage, which takes two years in the three *Erebia* species studied here (Lorkovic, 1958; Sonderegger, 2005). However, unlike for imagoes, little is known about the ecological niche of the different larval stages, where the very few existing observational data does not allow to determine if *Erebia* species differ, e.g., in their larval microhabitat or their host plant preference (Sonderegger, 2005; Albre *et al.*, 2008b). Although precipitation fell along a geographic cline, the latter did not coincide with the genomic cline but was shifted to the east (Figure 7d), suggesting that habitat-dependent selection is less likely to be the key driver of differentiation in this system.

Differences in wing shape and colour patterns are common targets for mate choice in butterflies and thus a target for selection and the evolution of pre-zygotic isolation (Kemp, 2007; Finkbeiner *et al.*, 2014). *E. tyndarus* and *E. cassioides* are known to differ in both traits (Sonderegger, 2005; Gratton *et al.*, 2016) and consistently we found *E. cassioides* to show more elongated wings and reduced orange spots than *E. tyndarus* (Figure 4). The variation along the leading multivariate phenotypic axis fell along a cline for which the 95% confidence interval of the centre estimate overlapped with the genetic cline (Figure 7c). While the observed phenotypic differentiation could primarily reflect divergence in allopatry, reinforcement, whereby differentiation would be increased in zones of secondary contact compared to allopatric populations, may also be at play (Gompert *et al.*, 2017; Butlin & Smadja, 2018). Sonderegger (2005) indeed showed that *E. cassioides* becomes more *tyndarus*-like with increasing distance from the studied contact zone, i.e., exhibiting an increased orange coloration on the forewing. If, and to what degree reinforcement may be at play requires further investigation incorporating more allopatric populations.

Two different strains of the endosymbiotic bacteria *Wolbachia* were found among individuals of the *E. tyndarus* complex in the Grindelwald region – occurring predominantly in *E. cassioides* (haplotype *h3*) and *E. nivalis* (*h2*), while *E. tyndarus* showed no infection in the contact zone (Figure 6, Table S4). Because different *Wolbachia* infections can reduce hybrid fitness and even cause sterility in other species through cytoplasmic incompatibility (Rokas, 2000; Brucker & Bordenstein, 2012), the observed prevalence of *Wolbachia* may account for the observed lack of interspecific gene flow between *E. tyndarus* and *E. cassioides* in the contact zone. Indeed, both F1 hybrids between the aforementioned species carry the *Wolbachia* haplotype found in *E. cassioides*, and as a result, the *Wolbachia* prevalence falls along a cline that overlaps with the genomic cline (Figure 7b). The two *Wolbachia* strains are likely old, i.e. diverged in allopatry in different glacial refugia. This is because the haplotype *h3* is restricted to *E. cassioides* from the western part of the species distribution, including the Grindelwald region and the Pyrenees. whereas *h2* occurs in *E. cassioides* from a south-eastern refugium (Italy) as well as in the sister species *E. nivalis* and *E. tyndarus* from the same glacial refugium (Schmitt *et al.*, 2016). The crossing experiments conducted by Lorkovic (1958) were performed using *E. cassioides* from the Eastern Alps and thus between individuals that were exposed to the same *Wolbachia* strain, which could explain why *E. tyndarus* and *E. cassioides* could be crossed. *E. tyndarus* can be infected by

Wolbachia but the prevalence is much lower than in the two other species. It seems therefore as if *E. tyndarus* mostly lost *Wolbachia*, which can occur during the colonisation of a new environment through drift or altered selective regimes (Reuter *et al.*, 2004), such as during a postglacial range expansion. Further experimental work is thus needed to elucidate the role of *Wolbachia* as a barrier to gene flow.

Conclusions

Using population genomic data, we showed that some interspecific gene flow may still be possible between closely-related species despite karyological variation in the recently-evolved *E. tyndarus* complex. Changes in chromosome numbers may therefore represent a strong, but incomplete and only one of several barriers to gene flow in zones of secondary contact (Figure 3). Surprisingly though, the species form very narrow contact zones where hybridisation seems to be limited, i.e. resulting solely in F1 hybrids between the two species having the same karyotype, which contradicts classic crossing experiments (Lorkovic, 1958). The lack of subsequent later-generation introgression likely reflects the interplay of additional barriers to gene flow. Based on cline analyses, we identified two potential targets of divergent selection: phenotypic differentiation that could lead to pre-zygotic isolation and differences in the rates of infection of the endosymbiont *Wolbachia*. These results highlight that there are many evolutionary mechanisms at play in zones of secondary contact, whose strengths and sequence may vary and need to be assessed in detail (Lackey & Boughman, 2017; Mérot *et al.*, 2017).

Acknowledgement

Data Accessibility Statement

Genomic data will be archived on the NCBI Short Read Archive. Phenotypic and ecological data will be archived on DRYAD.

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Figure Legends

Figure 1: Elevation map of the Alps in central Europe (modified from Wikimedia). Following Sonderegger (2005), the ranges of the three focal species *E. cassioides* (green), *E. tyndarus* (blue) and *E. nivalis* (orange) are indicated. The centre of the black square further indicates the location of the studied contact zone in the Grindelwald area. For each species, the picture of a mounted specimen collected in the contact zone by Peter Sonderegger is shown.

Figure 2: Phylogram artificially rooted on the node that separates *E. calcaria* from the other species of the *E. tyndarus* complex. Red dots represent nodes with >95% bootstrap support. Circles represent samples from the Grindelwald area, triangles samples from elsewhere, i.e. AT – Austria, CH – Switzerland, FR – France, IT - Italy (from Gratton et al. 2016). Colours indicate species.

Figure 3: Individual-based assignments using Admixture for samples from the Grindelwald region. The case for three genetic clusters (*K* =3) is shown as a bar diagram (top) and as pies plotted on the map (bottom; © OpenStreetMap Project 2018).

Figure 4: Bar plot summarising phenotypic variation for wing shape and the shape of the orange spot along the leading principal component (PC) axis explaining 62.3% of all phenotypic variation.

Figure 5: Niche variation between *E. cassioides* (green) and *E. tyndarus* (blue). a) Principal component (PC) scores based on all ecological data. b) Boxplots summarising monthly mean precipitation (in mm) for July-September, i.e. the months where imagoes occur. Significance levels are based on ANOVAs.

Figure 6: *Wolbachia* strains and prevalence among *Erebia*. a) median joining network for the three *Wolbachia* haplotypes (*h1, h2, h3*) found in 59 *Erebia* specimens. Indicated are the countries where haplotypes were found, i.e. AT – Austria, CH – Switzerland, FR – France, IT – Italy, SLO – Slovenia. Dashed lines indicate the number of substitution differences. b)

Wolbachia prevalence across the sampled range in Grindelwald. Two *Wolbachia* genotypes are indicated (*h2*: square, *h3*: circle). Colours indicate species assignments from Admixture (see Figure 3). For *h3*, the black area in a) represent the two F1 hybrids. *Wolbachia* was absent in the *E. tyndarus* samples (see Table S4).

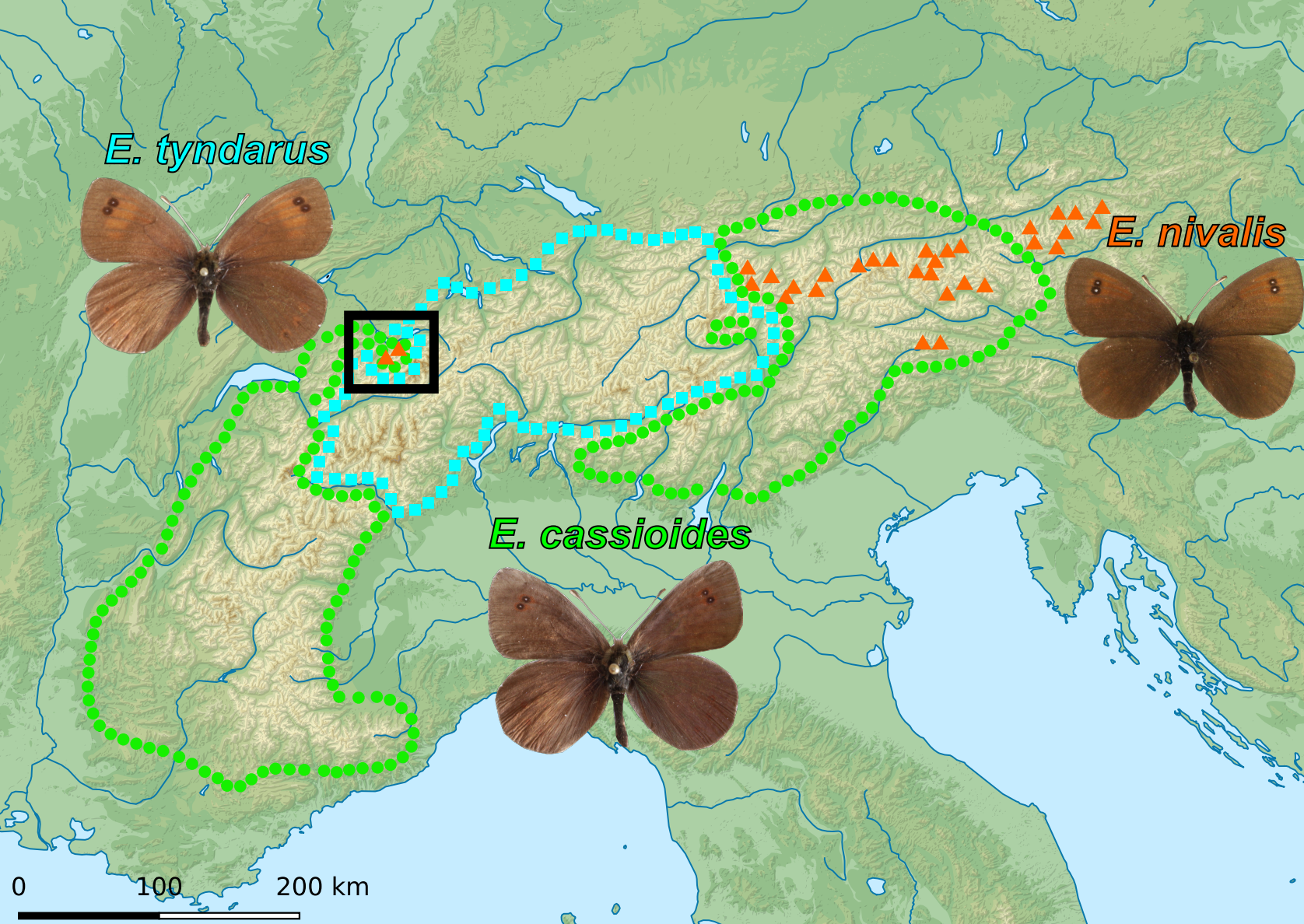
Figure 7: Clines between *E. cassioides* (green) and *E. tyndarus* (blue) along a west-east transect for a) genomic data, b) *Wolbachia* prevalence, c) PC1 scores of wing morphology, d) scaled precipitation in August. The black dots indicate the two F1 hybrids. The orange line represents the fitted cline, the black bar the cline centre with the respective 95% confidence interval in grey.

Figure S1: Upper forewing showing the landmarks used for the geometric morphometric analysis: LM1 – base of wing, LM2 – intersection of the cell and the second term, LM3 – intersection of the cell and the third term, LM4 – intersection of the cell and the fourth term, LM5-9: outer margins of the second to sixth termen, LM10 outer margin of the apex, LM11 upper extent of upper black spot, LM12 inner upper extent of upper black spot, LM13 centre of the white spot in the upper black spot, LM14 centre of the white spot in the lower black spot, LM15 inner lower extent of lower black spot, LM16 lower extent of lower black spot, LM17 lower extent of orange cell, LM18 basal extent of orange cell, LM19 upper extent of orange cell. LM11-16 were set along a line that is parallel to the extent of the orange cell.

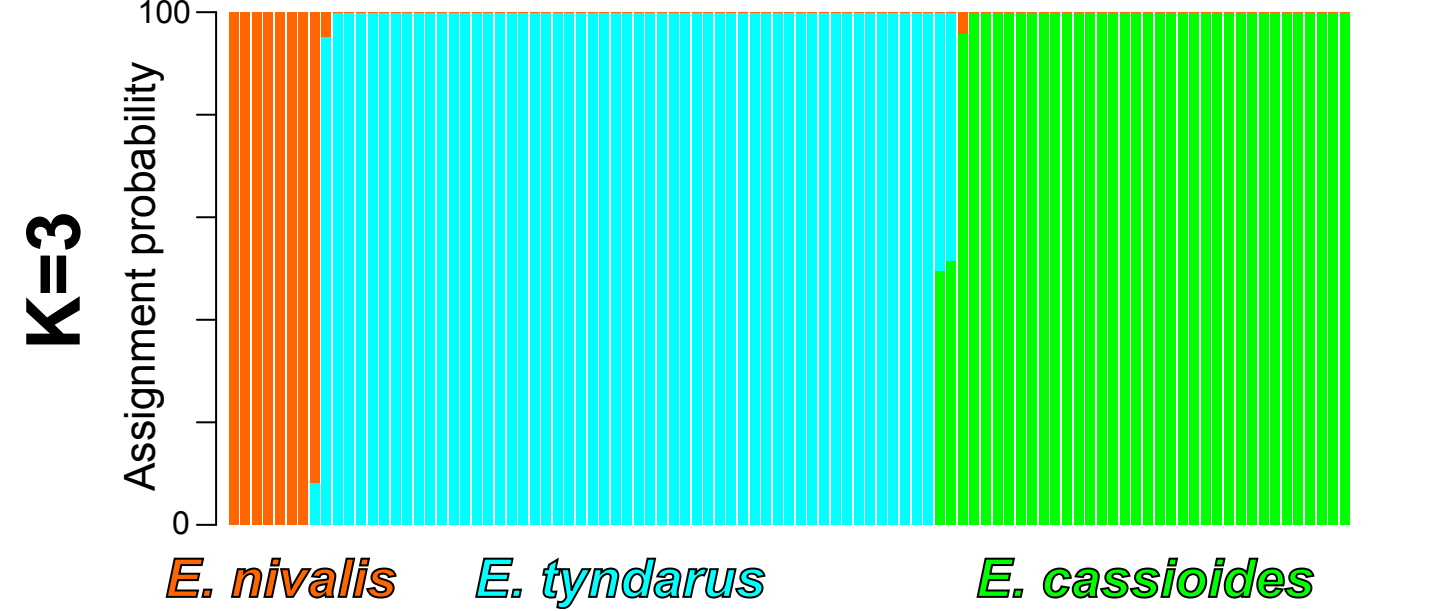
Figure S2: Phylogram artificially rooted on the node that separates *E. calcaria* from the other species of the *E. tyndarus* complex. The two F1 hybrids between *E. tyndarus* and *E. cassioides* were retained in this analysis (see Figure 1). Grey dots represent nodes with >95% bootstrap support. Circles represent samples from the Grindelwald area, triangles samples from elsewhere, i.e. AT – Austria, CH – Switzerland, FR – France, IT - Italy (from Gratton et al. 2016). Colours indicate species.

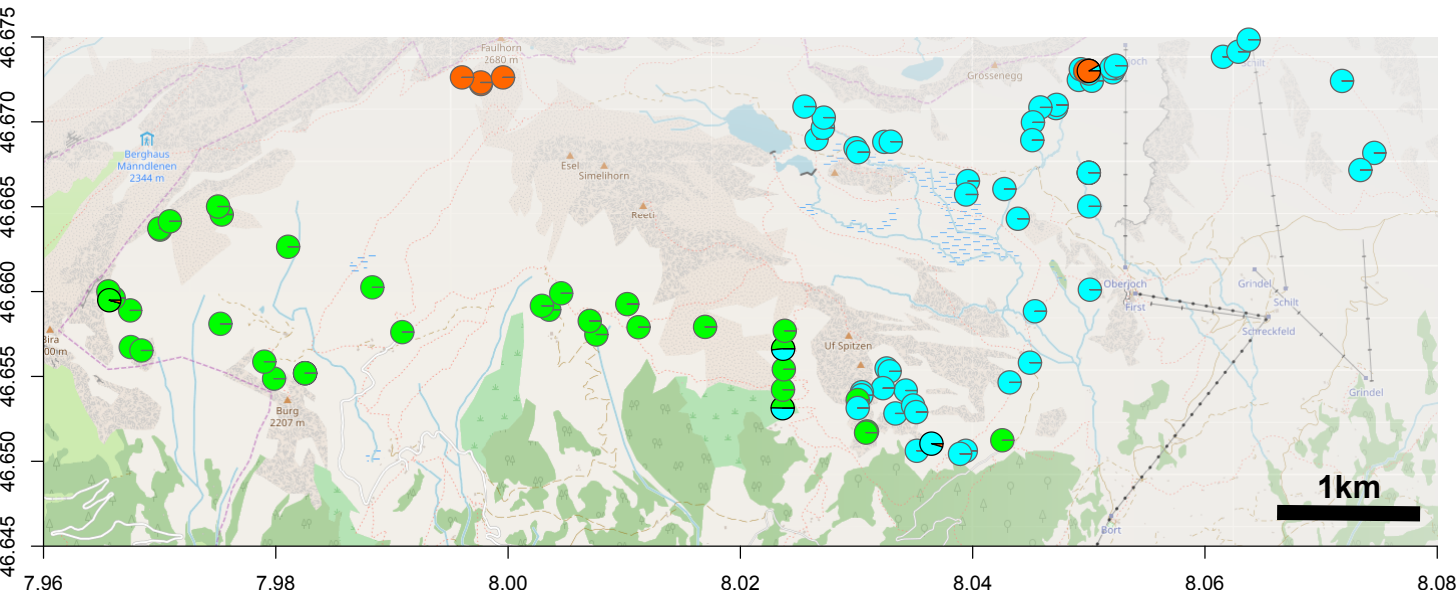
Figure S3: Cross validation error from Admixture when assuming 1-10 genetic clusters (K).

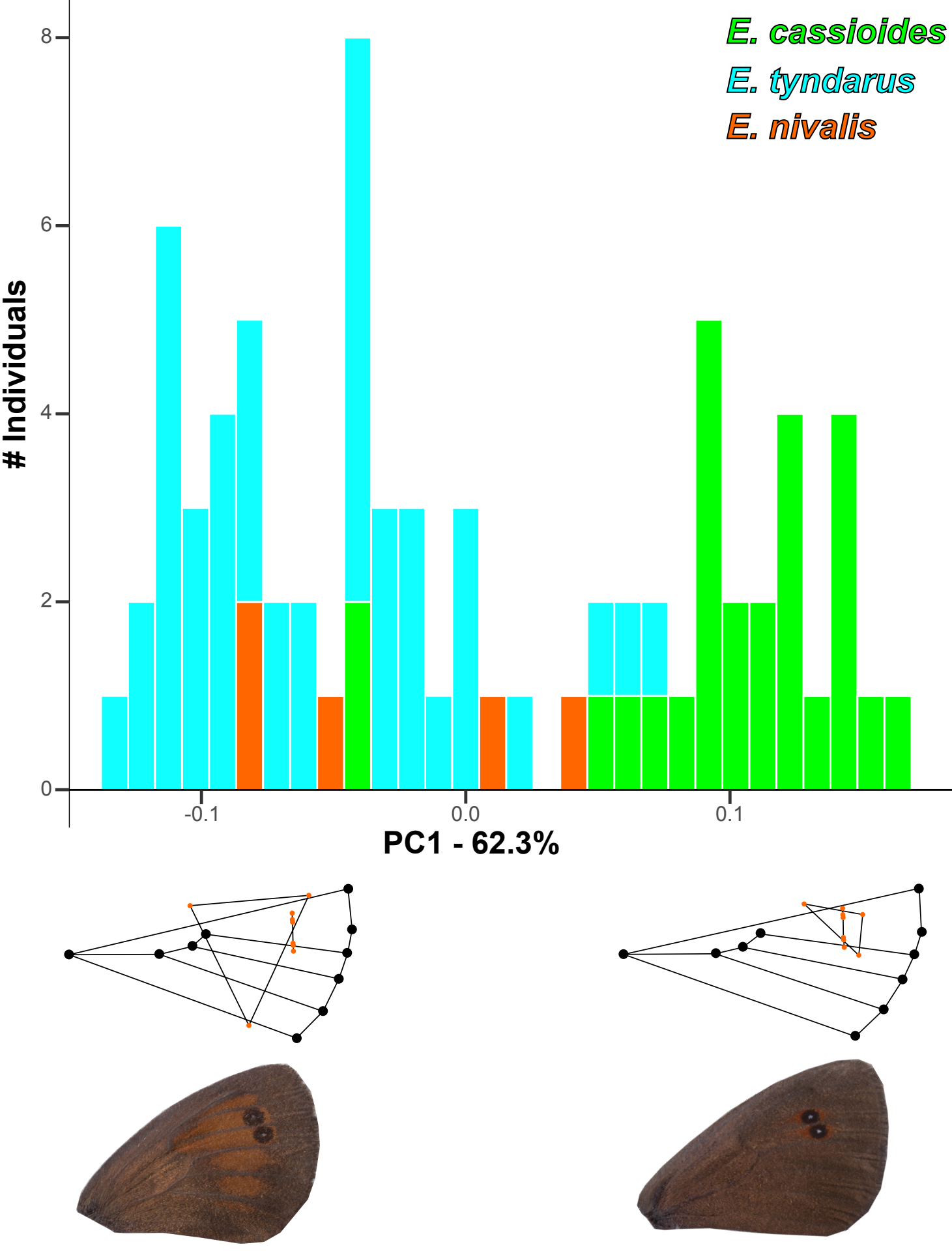
Figure S4: Clines fitted on least cost distances along a west-east transect for a) genomic data, b) *Wolbachia* prevalence, c) PC1 scores of wing morphology, d) scaled precipitation in August. The black dots indicate the two F1 hybrids. The orange line represents the fitted cline, the black bar the cline centre with the respective 95% confidence interval in grey.

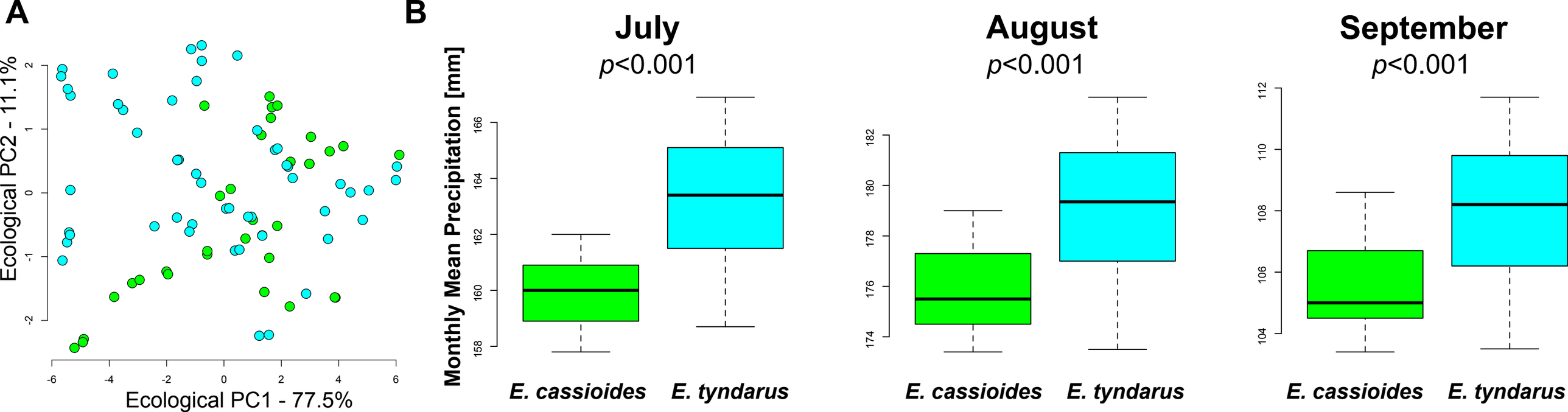

E. tyndarus
E. nivalis
E. cassioides
0
100
200 km

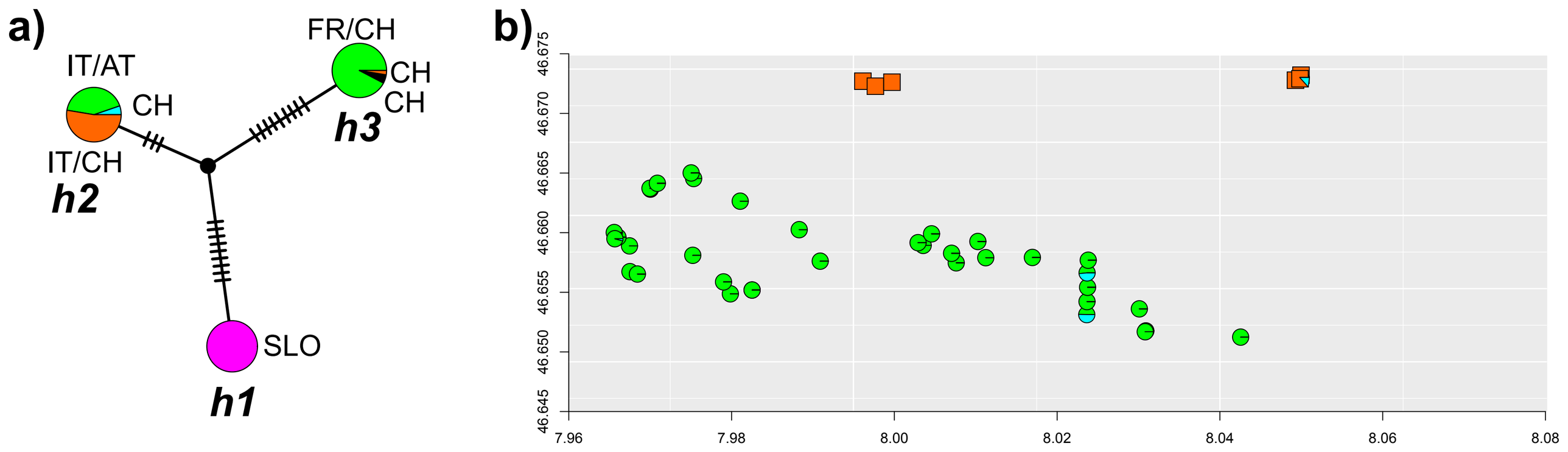
E. calcaria
IT
Grindelwald
IT/AT/CH
E. tyndarus
Grindelwald
IT
E. nivalis
AT
Grindelwald
FR/IT/AT
E. cassioides

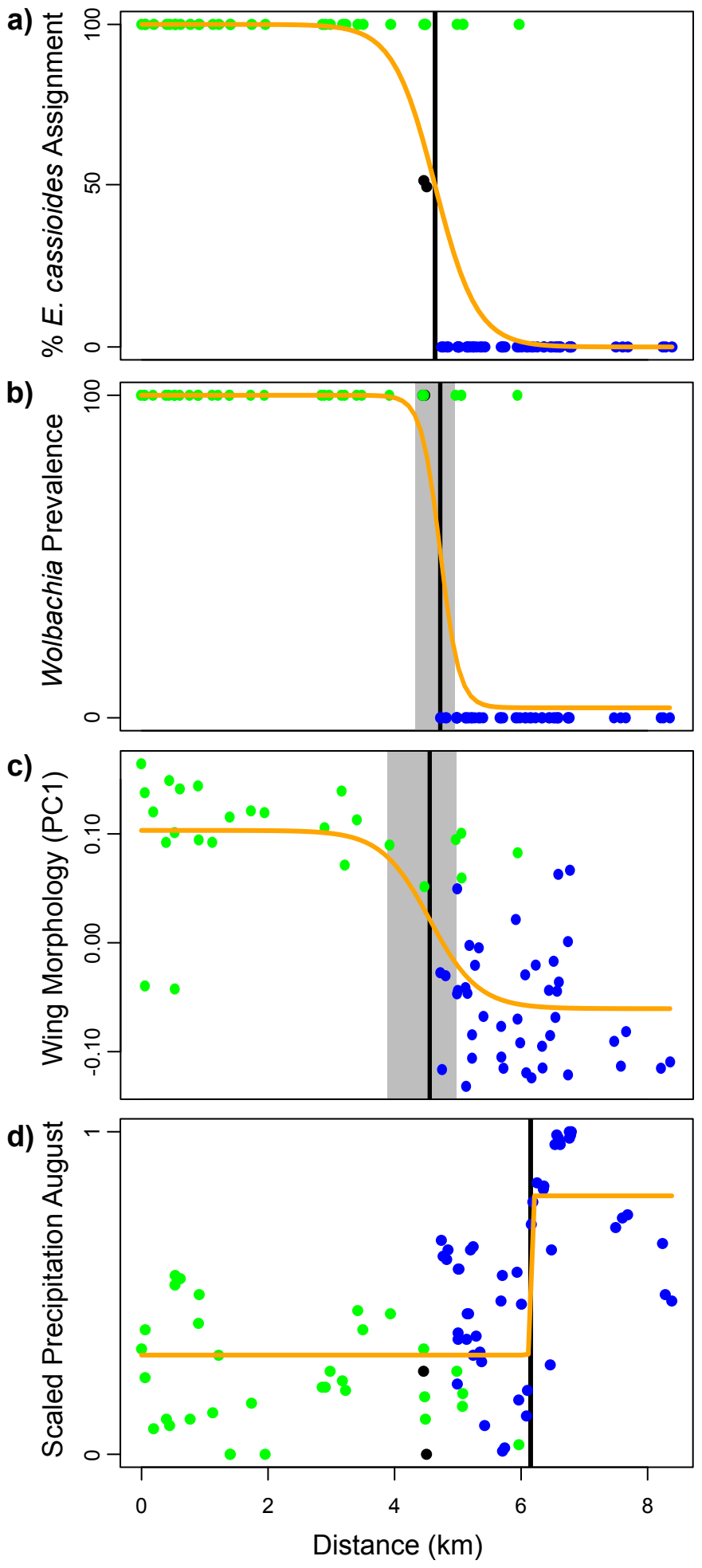

K=3
Assignment probability
100
0
E. nivalis
E. tyndarus
E. cassioides


46.675
46.670
46.665
46.660
46.655
46.650
46.645
7.96
7.98
8.00
8.02
8.04
8.06
8.08
Faulhorn
2680 m
Grossenegg
Berghaus
Männdlenen
2344 m
Esel
Simelihorn
Ufs Spitzen
Oberjoch
First
Grindel
Schilt
Schreckfeld
Burg
2207 m
1km


E. cassioides
E. tyndarus
E. nivalis
Individuals
0
2
4
6
8
-0.1
0.0
0.1
PC1 - 62.3%


A
Ecological PC2 - 11.1%
Ecological PC1 - 77.5%
B
July
p<0.001
August
p<0.001
September
p<0.001
Monthly Mean Precipitation [mm]
E. cassioides
E. tyndarus


a)
FR/CH
CH
CH
h3
IT/AT
CH
IT/CH
h2
SLO
h1
b)
46.675
46.670
46.665
46.660
46.655
46.650
46.645
7.96
7.98
8.00
8.02
8.04
8.06
8.08


a)
% E. cassioides Assignment
100
50
0
b)
Wolbachia Prevalence
100
0
c)
Wing Morphology (PC1)
0.10
0.00
-0.10
d)
Scaled Precipitation August
1
0
0
2
4
6
8
Distance (km)