Genomic signals of admixture and reinforcement between two closely related species of European sepsid flies

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ABSTRACT

Interspecific gene flow by hybridization may weaken species barriers and adaptive divergence, but can also initiate reinforcement of reproductive isolation trough natural and sexual selection. The extent of interspecific gene flow and its consequences for the initiation and maintenance of species barriers in natural systems remain poorly understood, however. To assess genome-wide patterns of gene flow between the two closely related European dung fly species *Sepsis cynipsea* and *Sepsis neocynipsea* (Diptera: Sepsidae), we tested for historical gene flow with the aid of ABBA-BABA test using whole-genome resequencing data from pooled DNA of male specimens originating from natural and laboratory populations. We contrasted genome-wide variation in DNA sequence differences between samples from sympatric populations of the two species in France and Switzerland with that of interspecific differences between pairs of samples involving allopatric populations from Estonia and Italy. In the French Cevennes, we detected a relative excess of DNA sequence identity, suggesting interspecific gene flow in sympatry. In contrast, at two sites in Switzerland, we observed a relative depletion of DNA sequence identity compatible with reinforcement of species boundaries in sympatry. Our results suggest that the species boundaries between *S. cynipsea* and *S. neocynipsea* in Europe depend on the eco-geographic context.

KEYWORDS: ABBA-BABA test, gene flow, hybridization, introgression, reproductive isolation, speciation, sepsid flies

Running title: Patterns of Gene flow in European Sepsid flies

38 **INTRODUCTION**

39 According to the biological species concept, species represent groups of individuals that
40 are reproductively isolated from other groups of individuals (Mayr 1942). Speciation entails
41 the evolution of reproductive isolation among lineages derived from a common ancestral
42 population and is considered completed if the diverged populations remain reproductively
43 isolated, for example, after coming into secondary contact (Coyne & Orr, 2004; Dobzhansky,
44 1951; Mayr, 1963). However, many animal and plant species remain distinct entities in nature
45 even if they occasionally hybridize and exchange genes in parapatry or sympatry (Anderson,
46 1949; Barton & Bengtsson, 1986; cf. DeMarais *et al*., 1992; Gante *et al*., 2016; Mallet, 2007;
47 Nolte & Tautz, 2010; Rieseberg *et al*., 2003; Trier *et al*., 2014). Research during the past
48 decades has indicated that hybridization not only has deleterious effects due to hybrid
49 inferiority and negative epistasis in admixed genomes, but that it may also fuel adaptive
50 diversification and speciation by facilitating novel combinations of alleles that become targets
51 of divergent selection (Arnold & Meyer, 2006; Berner & Salzburger, 2015; Fontaine *et al*.
52 2015; Seehausen, 2004; Saetre, 2013). Together, these findings corroborate the view that
53 genomes of recently diverged species may represent mosaics, consisting of genomic regions
54 with significant differentiation (i.e., low to no admixture) interspersed by genomic regions
55 that are more free to be exchanged (Nosil *et al*., 2009; Wu, 2001).

56 Sepsid flies (Diptera: Sepsidae) have become a model group for the study of sexual
57 selection and ecological adaptation (Baur *et al*., 2020; Blanckenhorn, 1999; Blanckenhorn *et*
58 *al*., 2000; Eberhard, 1999; Kraushaar & Blanckenhorn, 2002; Parker, 1972a,b; Puniamoorthy
59 *et al*., 2009; Pont & Meier, 2002; Rohner *et al*., 2015; Rohner, Blanckenhorn, &
60 Puniamoorthy, 2016; Ward, 1983; Ward, Hemmi, & Rösli, 1992). The phylogeny of sepsid
61 flies is well resolved (Su *et al*., 2008, 2016) and entails multiple pairs of closely related

species that occupy similar ecological niches. These species pairs provide excellent opportunities to study the genomic consequences of hybridization and introgression during early stages of speciation. One of these pairs comprises *Sepsis cynipsea* and *Sepsis neocynipsea*, sister species with a wide geographic distribution that occur in sympatry across major parts of their natural range. While *S. cynipsea* is the most abundant sepsid species in Central and Northern Europe and deposits its eggs into fresh cow dung, *S. neocynipsea* is common throughout North America, where it occupies a niche similar to that of *S. cynipsea* in Europe. While overall very rare in Europe, *S. neocynipsea* can be locally common at higher altitudes, such as the Alps, where the species occurs in sympatry with *S. cynipsea* (Ozerov, 2005; Pont & Meier, 2002; Rohner, Blanckenhorn, & Puniamoorthy, 2016). Despite strong similarities in morphology and behavior (Giesen, Blanckenhorn, & Schäfer, 2017), the two species are genetically distinct. Previous studies showed that *S. cynipsea* and *S. neocynipsea* produce fertile hybrid offspring under laboratory conditions despite strong pre- and post-mating isolating barriers. These barriers are mediated by assortative mating behaviors that are partly reinforced in areas where the two species occur in sympatry (Giesen *et al.*, 2017, 2019). While these findings imply that interspecific gene flow may occur and vary with eco-geographic context in nature, we know little about actual levels of historical and ongoing gene flow between the two species in areas where they occur in sympatry or parapatry. We therefore investigated the extent of gene flow between *S. cynipsea* and *S. neocynipsea* in nature by comparative population genomic analyses.

In recent years, multiple approaches have been employed to explore the importance of interspecific gene flow in sympatry vs. allopatry (e.g. Nakazato, Warren, & Moyle, 2010; Nadeau *et al.,* 2013; Brandvain *et al.,* 2014; Bouchemousse *et al.,* 2016; Feulner & Seehausen, 2019; Kastally, Trasoletti, & Mardulyn, 2019; Moran *et al.,* 2019). The objective of our study is to assess the extent of genome-wide admixture between *S. cynipsea* and *S.*

87 *neocynipsea* in Europe by applying a version of the ABBA-BABA test for historical gene
88 flow (Green *et al*., 2010; Durand *et al*., 2011; Soraggi et al., 2018) that can exploit genome-
89 wide allele frequency data of single nucleotide polymorphisms (SNPs). To this end, we
90 sequenced the pooled genomic DNA of males from wild-caught populations of *S. cynipsea*
91 and *S. neocynipsea* collected at multiple sites ranging from Southern France to Estonia. Some
92 of these populations occur in sympatry (e.g., in the Swiss Alps and the French Cevennes),
93 others in allopatry (Pont & Meier, 2002). Based on hybridization opportunity alone, we
94 expected to find higher genome-wide levels of gene flow between *S. cynipsea* and *S.*
95 *neocynipsea* in geographic areas where both species occur in sympatry (e.g. Nadeau *et al.,*
96 2013, or Martin *et al.,* 2014, for *Heliconius* butterflies). In contrast, less gene flow in
97 sympatry than in allopatry would indicate selection against gene flow and thus suggest
98 reinforcement of reproductive barriers at sites of co-occurrence (Butlin, 1995; Noor, 1999;
99 Coyne & Orr, 2004; e.g. Kulathinal & Singh, 2000; Massie & Makow, 2005; Giesen *et al.*
100 2017, 2019).

101 **MATERIALS & METHODS**

102 **Sample collection and treatment of fly cultures**

103 We studied inter- and intraspecific gene flow in S. *cynipsea* and *S. neocynipsea* using
104 genomic data of sympatric *S. cynipsea* and *S. neocynipsea* populations from two high altitude
105 sampling sites in the Swiss Alps (Sörenberg) and the French Cevennes (Le Mourier) (Table
106 1). To complement these two pairs of sympatric populations with geographically distant
107 allopatric populations in Europe (Table 1), we further included *S. cynipsea* samples from the
108 Swiss lowlands (Zürich), from Tuscany, Italy (Petroia) and Estonia (Pehka), and from two
109 additional high-altitude *S. neocynipsea* samples collected in Switzerland (Geschinen and
110 Hospental) (Table 1). In line with the previous observation that *S. neocynipsea* tends to be

111 rare at low altitudes (Pont & Meier, 2002), we were not able to collect sufficient numbers of
112 flies of this species outside the Alps, except for the sample collected near Le Mourier. For
113 each of the wild-caught populations, we randomly selected 20 males for pooled DNA
114 resequencing (Pool-Seq).

115 In addition to wild-caught populations, we compiled genomic data from two laboratory
116 populations that were established from sympatric *S. cynipsea* and *S. neocynipsea* populations
117 collected near Zürich. We started each of these populations from a single gravid, wild-caught
118 female and subsequently propagated them in the laboratory for at least two years at census
119 population sizes ranging from 10 to 100. We then sampled and pooled the DNA of 50 males
120 from each population for whole-genome sequencing (see Supporting Information for more
121 details).

122 **DNA library preparation and next generation sequencing**

123 Genomic DNA was extracted from the pooled DNA of males using the UltraPure
124 Phenol:Chloroform:Isoamyl alcohol (25:24:1, v/v) extraction kit (Thermo Fischer Scientific,
125 Waltham, USA) according to the manufacturer's protocol. Quantification of genomic DNA
126 was performed with a Qubit Fluorometer (Thermo Fischer Scientific; Table 1). Library
127 preparation was carried out with the TruSeq DNA PCR-Free Library Preparation (Illumina,
128 San Diego, USA) kit according to the manufacturer's protocol. Fragment-size distributions of
129 all libraries were validated on a TapeStation 2200 (Agilent Technologies, Waldbronn,
130 Germany). Sequencing on Illumina HiSeq 2500 version 4 was conducted after labeling and
131 pooling the barcoded DNA representing the four laboratory populations onto one lane to
132 achieve a genome-wide coverage of ca. 60x per DNA pool library. All sequencing data are
133 available at the short-read archive (SRA; https://www.ncbi.nlm.nih.gov/sra) under the
134 accession number PRJNA612154.

135 **Table 1**: Sampling sites, sample IDs, percentages of DNA sequence reads mapping to the
136 reference genome and read-depths of pool-sequenced field-collected (a) and laboratory (b)
137 populations. Sites where *S. cynipsea* and *S. neocynipsea* occur in sympatry are in regular font.
138 Sites where *S. neocynipsea* does not occur to our knowledge are highlighted in italics.
139

Species	ID	Location	Country	Coordinates	Altitude (m)	Alignment rate to *S. thoracica* reference [%]	Average Read-depth
a) Wild-caught							
S. cynipsea	PhC	*Pehka*	*Estonia*	59°28'45.1''N,25°44'52.3''E	5	50.6%	19.8
	PtC	*Petroia*	*Italy*	43°15'06.4''N,12°32'41.2''E	570	48.1%	29.2
	SoC	Sörenberg	Switzerland	46°52'12''N, 8°16'12''E	1200	51.3%	35.1
	ZuC	Zürich	Switzerland	47°24'0.6''N, 8°34'24.0''E	450	51.3%	37.3
	MoC	Le Mourier	France	44° 3'30.5"N, 3°25'38.8"E	900	49.5%	62.5
S. neocynipsea	GeN	Geschinen	Switzerland	46°49'23.7''N, 8°1'54.6''E	1350	50.5%	32.1
	HoN	Hospenthal	Switzerland	46° 37'12''N, 8°34'12''E	1500	52.3%	25.4
	SoN	Sörenberg	Switzerland	46°24'8.2''N, 8°21'28.4''E	1200	50.7%	23.0
	MoN	Le Mourier	France	44° 3'30.5"N, 3°25'38.8"E	900	48.6%	74.2
b) Laboratory							
S. cynipsea	IZuC	Zürich	Switzerland	47°24'0.6''N, 8°34'24.0''E	402	50.1%	19.2
S. neocynipsea	IZuN	Zürich	Switzerland	47°24'0.6''N, 8°34'24.0''E	402	50.0%	67.2
c) *S. orthocnemis*		Lenzerheide	Switzerland	46°43'45''N, 9°33'25''E	1470	46.9%	13.0

140

141 **Raw read processing**

142 Qualitative validation of sequence data before and after trimming was done with FastQC
143 (v. 0.11.4; Andrews *et al*., 2011). After removal of Illumina-specific adapters and trimming
144 with *Trimmomatic* (v. 0.36; Bolger, Lohse & Usadel, 2014), the *S. cynipsea* and *S.*
145 *neocynipsea* reads were mapped to the draft genome of *S. thoracica* with *bwa mem* (v. 0.7.12;

146 Li & Durbin, 2009) using default parameters. The *S. thoracica* sample used for genome
147 sequencing was collected near Capriasca, Ticino, Switzerland. The draft genome, v. 0.1 was
148 built from Oxford Nanopore long reads (app. 25x read depth), assembled with Canu (Korner
149 *et al*., 2017), and polished with Illumina short reads (app. 20x read depth). The assembly used
150 in this study is available under the rules of the Fort Lauderdale agreement from
151 www.cgae.de/seto_01_genome.fasta and www.cgae.de/seto_01_genome_masked.fasta.

152 PCR duplicates were removed with Picard (v. 1.109; http://broadinstitute.github.io/picard/)
153 and reads were realigned around indels in each raw alignment file using
154 RealignerTargetCreator and IndelRealigner from GATK (v. 3.4-46; McKenna et al. 2010).
155 Only reads with a PHRED-scaled mapping quality of 20 and more were retained. We applied
156 the same procedure to map reads of a pool of ten inbred *S. orthocnemis* males from near
157 Lenzerheide, Switzerland, to the reference genome (Table 1). We compiled the aligned reads
158 from all population samples into a single *mpileup* file using *samtools* (v. 1.3.1; Li *et al*.,
159 2009).

160 Using the variant-caller Pool-SNP (Kapun *et al*., 2018), we identified high-confidence
161 SNPs with the following combination of heuristic SNP-calling parameters: coverage of each
162 Pool-Seq sample ≥ 10x; coverage of each Pool-Seq sample ≤ 95 % percentile of coverage
163 distribution across contigs and samples; minimum allele count of a minor allele at a SNP
164 across all combined Pool-Seq samples > 20x; minor allele frequency at a SNP across all
165 combined Pool-Seq samples > 0.01. We only retained SNPs for which all samples fulfilled
166 the above coverage threshold criteria. To avoid paralogous SNPs due to mis-mapping around
167 indel polymorphisms, we removed SNPs located within a 5-bp distance to indels that
168 occurred in more than 20 copies at a given site across all pooled samples. In addition, we
169 ignored SNPs located within repetitive regions. The resulting VCF file was converted to the

170 SYNC file format (Kofler *et al*., 2011), and a custom Python script was used to calculate

171 sample-specific allele frequencies for major alleles at each SNP (*sync2AF.py*;

172 https://github.com/capoony/DrosEU_pipeline).

173

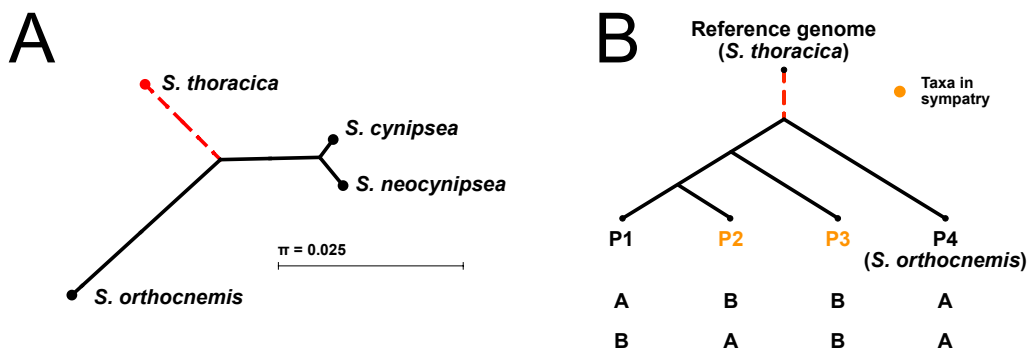
174 **Testing for introgression**

175 To test for introgression, we used the ABBA-BABA test for historical gene flow in the
176 presence of incomplete lineage sorting (Green *et al*., 2010; Durand *et al*., 2011). We provide a
177 brief motivation of the approach in the following and refer to the Supporting Information for
178 further details. Imagine a rooted phylogeny with four species (P1 to P4) and a topology of
179 (((P1, P2), P3), P4) as illustrated in Fig. 1B. In an alignment of one haploid genome from
180 each species, incomplete lineage sorting under neutral evolution leads to two mutational
181 configurations of the ancestral (A) and derived (B) allele, ABBA and BABA, that are
182 incompatible with the species topology (Fig. 1B) under the infinite-sites model of mutation
183 (Kimura, 1969). In the absence of gene flow between P2 and P3, ABBA and BABA
184 configurations occur with equal probability (Hudson, 1983; Tajima, 1983). In contrast,
185 historical gene flow between P2 and P3 causes an excess of ABBA configurations (Green *et*
186 *al*., 2010; Durand *et al*., 2011). This logic is captured by the *D*-statistic, a scaled difference
187 across a set of bases between counts of ABBA and BABA configurations bounded by –1 and
188 1 (Green *et al*., 2010). The ABBA-BABA test examines significant deviations of *D* from 0. A
189 significant excess of ABBA suggests evidence for gene flow between P2 and P3. A
190 significant depletion of ABBA is equivalent to an excess of ABBA if the positions of P1 and
191 P2 in the species topology are swapped, and hence either suggests gene flow between P1 and
192 P3, or a reduction in gene flow between P2 and P3 relative to gene flow between P1 and P3.

193 The original version of the ABBA-BABA test and modifications of it have been applied to
194 different types of genome-scale polymorphism data obtained with various sequencing
195 strategies, including whole genome sequencing (e.g. Green *et al*., 2010), RAD sequencing
196 (e.g., Eaton & Ree, 2013; Meier *et al*., 2017; Streicher *et al*., 2014), and exon capture data
197 (e.g., Heliconius Genome Consortium, 2012). More recently, Durand *et al*. (2011) and

198 Soraggi *et al*. (2018) extended the original test to allele frequency data, hence to unphased
199 sequencing data (including Pool-Seq data; Schlötterer *et al*. 2014). We implemented the
200 extensions by Durand *et al*. (2011) and Soraggi *et al*. (2018) in a Python script
201 (https://github.com/capoony/ABBABABA-4AF) and refer to the respective test statistics as
202 D_D and D_S. Our script also computes jack-knifed z-scores based on a matrix of allele
203 frequencies for previously defined high-confidence SNPs (see Supporting Information for
204 details). We adopted the commonly used significance threshold of $|z| > 3$ (Reich *et al*., 2011;
205 Jeong *et al*., 2016; Novikova *et al*., 2016) to identify significant deviations of D_S and D_D from
206 zero.

207 We were concerned that using the same genome assembly both as the reference for read
208 mapping and as the outgroup (P4) in the ABBA-BABA tests could lead to biased estimates of
209 gene flow. We therefore mapped the three ingroup and the outgroup species against the
210 reference genome of yet another species, *S. thoracica*. A phylogenetic analysis based on CO-
211 II sequences by Su *et al*. (2008) indicated that *S. thoracica* is approximately equally related to
212 *S. cynipsea* and *S. neocynipsea* as it is from the outgroup species *S. orthocnemis* (Fig. 1A).
213 We therefore expected that reads of the ingroup and the outgroup species would map equally
214 well to the reference species, which is indeed what we observed (Table 1).



215

Fig. 1. Putative phylogeny of sepsid species studied. (A) Species phylogeny inferred from alignments of nucleotide sequences at the cytochrome oxidase subunit II (CO-II) mitochondrial locus (Genbank sequences from Su *et al*., 2016) with the Neighbor-Joining method implemented in CLC Main Workbench (v. 8.1.2; https:// https://www.qiagenbioinformatics.com/products/clc-main-workbench/). The tree topology is consistent with the phylogeny presented by Su *et al.'s* (2016) combined phylogenetic analysis of multiple nuclear and mitochondrial markers. *Sepsis orthocnemis* was used as the outgroup for the focal species *S. cynipsea* and *S. neocynipsea*. Whole-genome sequencing reads from all three species were aligned to the *S. thoracica* reference genome (dashed red branches). Branch lengths are proportional to the mean number of pairwise sequence differences. (B) Generic species tree assumed in all our ABBA-BABA tests for gene flow among triplets of *S. cynipsea* and *S. neocynipsea* populations from various sites in Europe.

We focused our analysis of historical interspecific gene flow on the three sampling sites Sörenberg, Zürich and Le Mourier (Table 1). At all these sites, *S. cynipsea* and *S. neocynipsea* occur in sympatry. In all ABBA-BABA tests, we positioned the two focal populations as P2 and P3 ingroups in the phylogeny (in both possible orders) and used various P1 ingroup populations (Fig. 1B) assumed to occur in allopatry with the P2 populations. We used various P1 ingroups, because Durand *et al*. (2011) showed that the choice of the ingroup P1 can influence the test results.

RESULTS

Signals of reinforcement in Swiss alpine and sub-alpine regions

The majority of our tests for gene flow at the Sörenberg site revealed a genome-wide deficiency of derived-allele sharing (i.e., ABBA patterns) among the local *S. cynipsea* and *S. neocynipsea* populations (Table 2), suggesting that interspecific gene flow in sympatry (among P2 and P3) is lower than interspecific gene flow among putatively allopatric populations (P1 and P3). This observation is robust to our choice of the P1 ingroup population, but sensitive to the version of *D*-statistic used (D_S vs. D_D). We first conducted tests involving two *S. neocynipsea* populations from the Swiss Alps (*S. neocynipsea* from Geschinen, GeN; *S. neocynipsea* from Hospental, HoN; Table 1) as putatively allopatric P1,

246 and the sympatric interspecific pair from Sörenberg as P2 (*S. neocynipsea*) and P3 (*S.*
247 *cynipsea*). We observed a significant deficiency of ABBA patterns in all configurations ($D_S >$
248 0; Table 2, upper part), suggesting overall evidence for higher gene flow among putatively
249 allopatric than sympatric interspecific population pairs. While GeN and HoN are both
250 separated by high mountains (altitudes ranging from ca. 600–3,200 m asl) from the Sörenberg
251 site, they are geographically close (ca. 67.5 km and ca. 46.7 km Euclidean distance for GeN
252 and HoN, respectively) and thy occur in sympatry with other sepsid species, including *S.*
253 *cynipsea*. We were therefore concerned that our assumption of no gene flow between P1 and
254 P3 was violated, because GeN and HoN are parapatric rather than allopatric to our focal
255 populations in Sörenberg.

256 To address this concern, we conducted additional ABBA-BABA tests involving P1
257 populations from geographically more remote sites in Europe. Specifically, we performed a
258 second series of tests with two alternative *S. cynipsea* populations as allopatric P1 ingroups
259 (Fig. 1B), one from Pehka, Estonia (PhC), and one from Petroia, Italy (PtC; Table 1).
260 Contrary to our expectation based on the much larger geographical distance between P1 and
261 P3 (ca. 1,850 km and ca. 550 km for PhC and PtC, respectively) than between P2 and P3 in
262 these configurations, we still found a significant deficiency of ABBA patterns in both tests
263 ($D_S > 0$; Table 2, lower part). In agreement with our previous tests involving GeN and HoN as
264 P1, these additional tests thus equally suggest higher interspecific gene flow among allopatric
265 (P1 and P3) than among sympatric populations (P2 and P3). As we are much more confident
266 that PhC and PtC represent truly allopatric P1 ingroups relative to P2 and P3, we think that
267 the deficiency of ABBA patterns at the Sörenberg site indicates a local reduction in
268 interspecific gene flow in sympatry, likely due to reinforcement.

269 When repeating the ABBA-BABA tests with the *D*-statistic (D_D) suggested by Durand *et*
270 *al*. (2011), we obtained results that qualitatively agree with those obtained with the D_S statistic
271 only when using PhC and PtC as P1 ($D_D < 0$; z scores -8.4 and -7.7, respectively; Table S1
272 lower part), but not with GeN and HoN as P1 (*z*-scores 1.4 and 1.9, respectively; Table S1,
273 upper part). Besides the above qualitative differences between tests based on D_S vs. D_D, we
274 observed that variances of D_D estimated by jackknifing were about one order of magnitude
275 larger than the corresponding variances of D_S, which might indicate differences in the
276 robustness of the two measures. Nonetheless, the qualitatively similar results obtained with
277 the two versions of *D*-statistics makes us confident that our findings are robust. We further
278 observed that windows of elevated D_S (Fig. 2, top) were not homogeneously distributed along
279 the genome, but aggregated in clusters in several contigs. Such clustering might suggest that
280 the respective genomic regions contain variation involved in reinforcement caused by
281 divergent selection against gene flow between the two species.

282 Since both species commonly co-occur in alpine and sub-alpine regions of Switzerland, we
283 were curious to see if a signal of reinforcement was also evident at other sites of sympatry in
284 this geographic area. We therefore investigated a nearby lowland sampling site in Zürich
285 where we also found *S. cynipsea* and *S. neocynipsea* in sympatry. In contrast to the
286 sequencing data from Sörenberg, which were generated from wild-caught specimens only, the
287 sequencing data from the Zürich site derive from a combination of samples from natural and
288 laboratory populations. Specifically, for *S. cynipsea*, we sequenced a pool of flies sampled
289 directly from the natural population as well as a pool of flies from a laboratory population that
290 was derived from the same natural population (see Material and Methods for details); for *S.*
291 *neocynipsea*, we only had a pool of flies sampled from a laboratory population derived from
292 the natural population (Table 1). We found that the ABBA-BABA tests were qualitatively
293 robust to whether we used *S. cynipsea* data from natural or laboratory populations. However,

294 results depended on the choice of the D statistic (D_S vs. D_D) and of the P1 ingroup
295 (Supplementary Information, Supplementary Table S2). All ABBA-BABA tests based on D_S
296 that included GeN, HoN, or PtC as P1 were significant and revealed a deficiency of ABBA
297 patterns, whereas tests with PhC as P1 were not significant (Supplementary Table S2). All
298 significant tests were therefore consistent with our tests for the Sörenberg site in that they
299 suggested reduced interspecific gene flow in sympatry relative to allopatry. In contrast, only
300 two out of eight ABBA-BABA tests based on D_S were significant, one suggesting an excess
301 and one a deficiency of ABBA patterns. Overall, our results for the Zurich site are compatible
302 with reinforcement, although the signal seems to be weaker compared to the Sörenberg site.

303 **Table 2:** Results of ABBA-BABA tests applied to samples originating from sympatric
304 populations of *S. cynipsea* and *S. neocynipsea* in Sörenberg as P2 and P3 (or vice versa) based
305 on D_S. The upper half of the table shows tests with two European *S. neocynipsea* populations
306 from Geschinen (GeN) and Hospental (HoN) as P1 ingroups, and the bottom half shows the
307 reciprocal approach with two *S. cynipsea* populations from Pehka, Estonia (PhC) and Petroia,
308 Italy (PtC) as P1 ingroups. The first three columns indicate the phylogeny assumed for the
309 test, with P4 always being *S. orthocnemis*; columns 4 to 6 give the genome-wide average D_S,
310 its jackknifed standard deviation SD(D_S), and the corresponding z-score. Significant tests are
311 shown in bold.
312

P1	P2	P3	D_S	SD(D_S)	z
GeN	**SoN**	**SoC**	**0.008**	**1.35E−03**	**5.8**
HoN	**SoN**	**SoC**	**0.006**	**1.34E−03**	**4.2**
PhC	**SoC**	**SoN**	**0.026**	**1.92E−03**	**13.4**
PtC	**SoC**	**SoN**	**0.039**	**1.97E−03**	**19.8**

313

314 **Signals of introgression in high altitude populations from Southern France**

315 Complementary to the alpine location in Sörenberg (Switzerland), we further investigated
316 a pair of sympatric *S. cynipsea* and *S. neocynipsea* populations at high altitude site around Le
317 Mourier in the Cevennes, Southern France (790 m asl), approximately 450 km southwest of
318 the sampling site in Switzerland. Interestingly, we found opposite patterns to those observed
319 at the Sörenberg site (Table 3). Specifically, all tests based on D_S showed a highly significant
320 excess of ABBA patterns, and hence more interspecific gene flow in sympatry than allopatry.
321 The corresponding tests based on D_D also indicated an excess of ABBA patterns (recall the
322 difference in sign between D_S and D_D), but were not significant (Supporting Table S3). In
323 contrast to the patterns in Sörenberg, we did not observe genomic clustering of extreme
324 values of D_S (Fig. 2, bottom).

325 **Table 3:** Results of ABBA-BABA tests applied to samples originating from sympatric
326 populations of *S. cynipsea* and *S. neocynipsea* in the French Cevennes as P2 and P3 (or *vice*
327 *versa*) based on D_S. As in Tables 2 and 3, the upper half of the table summarizes tests
328 involving two *S. neocynipsea* populations from Geschinen (GeN) and Hospental (HoN) as P1
329 ingroups, and the bottom half summarizes tests involving *S. cynipsea* populations from Pehka,
330 Estonia (PhC) and Petroia, Italy (PtC) as P1.

331

P1	P2	P3	D_S	SD(D_S)	z
GeN	**MoN**	**MoC**	**–0.014**	**1.30E–03**	**–11.0**
HoN	**MoN**	**MoC**	**–0.014**	**1.36E–03**	**–10.6**
PhC	**MoC**	**MoN**	**–0.030**	**1.84E–03**	**–16.2**
PtC	**MoC**	**MoN**	**–0.016**	**1.93E–03**	**–8.3**

332

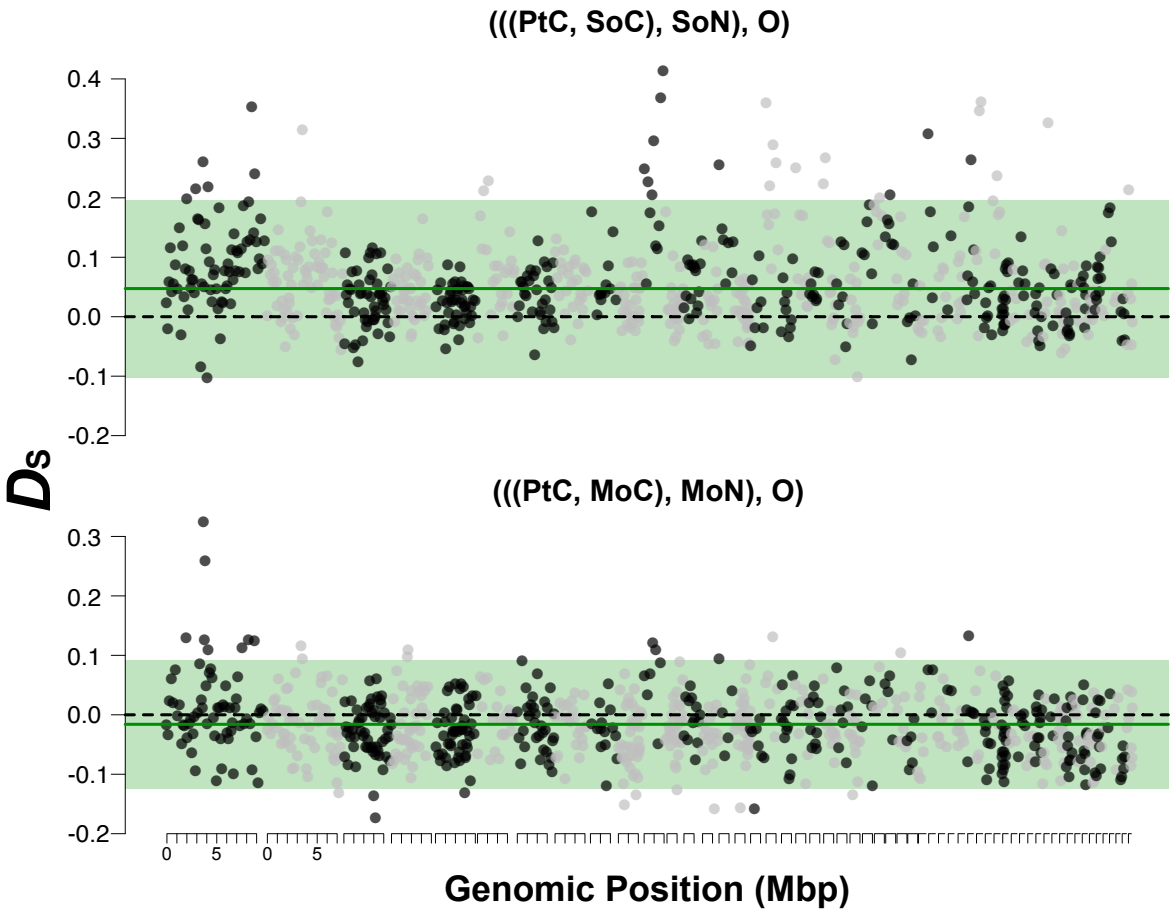


Fig. 2. Variation of D_S along the genome. Dots indicate D_S (Soraggi *et al*. 2018) for genomic windows of 500 consecutive SNPs, oriented by contigs of the *S. thoracica* reference genome with a length ≥ 50,000 bp (highlighted by alternating black and grey colors). The top and bottom panels show results for the population tree topologies used to test for interspecific gene flow at the focal sites in Sörenberg (SoC-SoN; deficiency of ABBA patterns) and Le Mourier (MoC-MoN; excess of ABBA patterns), respectively. The horizontal dashed black and green lines indicate the neutral expectation ($D_S = 0$) and the mean genome-wide jackknife estimate, respectively. Green shading delimits mean $D_S \pm 2$ standard deviations (based on windows of 500 SNPs).

DISCUSSION

We applied the ABBA-BABA approach (Green *et al*., 2010; Durand *et al*., 2011; Soraggi *et al*., 2018) to infer historical gene flow between a pair of closely related species of sepsid flies for which we have detailed information on ecology, morphology, life history, and behavior (Blanckenhorn, 1999; Blanckenhorn *et al*., 2000; Eberhard, 1999; Ozerov, 2005;

348 Parker, 1972a,b; Pont & Meier, 2002; Puniamoorthy *et al*., 2009; Rohner, Blanckenhorn &
349 Puniamoorthy, 2016; Ward, 1983; Ward, Hemmi & Rösli, 1992; Giesen *et al*., 2017, 2019).
350 Previous population genetic analyses of microsatellite markers (Baur *et al*., 2020) and
351 hybridization experiments in the laboratory (Giesen *et al*., 2017, 2019) suggested that *S*.
352 *cynipsea* and *S*. *neocynipsea* are genetically distinct, but may occasionally hybridize in nature
353 when occurring in sympatry. Here, we tested this hypothesis by analyzing patterns of DNA
354 sequence variation among pools of field-caught and laboratory specimens from multiple
355 sampling sites across Europe at which either both species occur in sympatry or only one
356 species (*S*. *cynipsea*) occurs. As discussed in detail below, depending on the focal site of
357 sympatry, we found two qualitatively opposite patterns of interspecific gene flow in sympatry
358 versus allopatry: a relative reduction of gene flow in sympatry at two sampling sites in
359 Switzerland, and a relative excess of gene flow in sympatry at a sampling site in Southern
360 France. Previous studies comparing levels of interspecific gene flow in sympatry and
361 allopatry in other taxa found the full spectrum of results that we here obtained for one species
362 pair. Martin *et al*. (2014; see also Nadeau *et al*., 2013) and Brandvain *et al*. (2014; see also
363 Grossenbacher & Whittall, 2011) found evidence for higher levels of interspecific gene flow
364 in sympatry than in allopatry when studying *Heliconius* butterflies and monkey flowers
365 (*Mimulus guttatus*/*nasutus*), respectively. In contrast, less gene flow in sympatry than
366 allopatry was observed in studies on *Drosophila arizonae*/*mojavensis* (Massie & Makow,
367 2005) and on two species of sea squirt (Tunicata: Bouchemousse *et al*., 2016), while no
368 geographic variation in levels of gene flow between sympatric and allopatric populations was
369 found in studies on wild tomatoes (Nakazato *et al*., 2010). A reduction in average gene flow
370 in sympatry compared to allopatry may suggest reinforcement by natural or sexual selection
371 (Butlin, 1995; Noor, 1999; Coyne & Orr, 2004; cf. Giesen *et al*., 2017, 2019). However, we
372 caution that comparing average levels of gene flow between sympatric and allopatric

373 population pairs may fall short of detecting selection against interspecific gene flow. In
374 monkey flowers, there is evidence for divergent selection against gene flow in sympatry (in
375 terms of a negative correlation between nucleotide divergence and recombination rate) even
376 though average levels of introgression are higher in sympatry than allopatry (Brandvain *et al*.
377 2014; Aeschbacher *et al*. 2017).

378 Similar to research on *Heliconius* (Martin *et al*., 2014), we found evidence for an excess of
379 average levels of interspecific gene flow in sympatry compared to allopatry in flies from Le
380 Mourier based on pool-sequenced field-caught specimens (Table 3; Table S3). In contrast, at
381 two Swiss sites where the two species also occur in sympatry, we observed reduced average
382 levels of interspecific gene flow relative to interspecific pairs of parapatric and allopatric
383 populations, similar to patterns found in *Drosophila arizonae/mojavensis* (Massie & Makow,
384 2005) and sea squirts (Bouchemousse *et al*., 2016). Consistent with our previous study
385 showing behavioral reinforcement after enforced hybridization in the laboratory (Giesen *et*
386 *al*., 2017), the genomic data analyzed here suggest that selection against interspecific gene
387 flow (Noor, 1999) might also occur in nature in the two sympatric Swiss populations. The
388 reduction of interspecific gene flow was more pronounced at the Sörenberg site in the Swiss
389 Alps (Table 2; Table S1) than at the lowland site in Zürich (Tables 3; Table S2), but
390 qualitatively consistent. However, as evidenced by our contrasting findings for the site in Le
391 Mourier, reinforcement may not be a necessary outcome whenever *S. cynipsea* and *S.*
392 *neocynipsea* occur in sympatry. The extent of gene flow between these two dung fly sister
393 species thus appears to covary with local environmental conditions. Well-documented
394 differences in climate between Southern France and the Swiss Alps, and between the Swiss
395 Alps and the Swiss lowlands, might provide an ecological explanation for the qualitative and
396 quantitative differences in interspecific gene flow we observed at the different locations
397 (Doebeli & Dieckmann, 2003; Rohner *et al*., 2015). While average minimum temperatures are

398 similar in the French Cevennes and the Swiss Alps, average maximum temperatures are up to
399 5 °C higher at the French site compared to Sörenberg (Figure 3). In addition, both sampling
400 sites are characterized by very different precipitation regimes. During the main growing
401 season from May until October, Sörenberg (a peat bog site) has on average twice as much
402 rainfall than the location in Le Mourier. However, the mechanisms by which climate affects
403 behavior and/or fitness remain to be understood (Giesen *et al*., 2017, 2019).

404 We found a weaker signal of reinforcement between *S. cynipsea* and *S. neocynipsea* at the
405 lowland Zürich site than at the high altitude Sörenberg site in the Swiss Alps. Differences in
406 the strength of reinforcement may also result from climatic variation between these two sites
407 in Switzerland to ultimately influence the strength of selection on natural hybrids. While
408 precipitation patterns of both locations are comparable, mean temperature is approximately
409 4 °C lower in Sörenberg than in Zürich (Supplementary Figure 1). Alternatively, differences
410 between the two Swiss sites might relate to *S. neocynipsea* being rare around Zürich, in fact in
411 European lowlands in general (Pont & Meier, 2002), while this species is common at higher
412 alpine altitudes. If based primarily on behavioral mate choice, reinforcement is expected to be
413 stronger, and hence its evolution more likely, wherever interspecific encounter frequencies
414 are higher. Moreover, given that the evidence for the Zürich site is based on both wild-caught
415 and laboratory specimens, the weaker patterns of reinforcement observed in Zürich might be
416 partially explained by purifying selection in the laboratory against introgressed variants.
417 Nevertheless, the results from our analyses performed with both wild-caught and laboratory *S.*
418 *cynipsea* samples from Zürich yielded consistent results (Supplementary Table 2), suggesting
419 that whether flies came from a natural or laboratory population did not affect the outcome.
420 Overall, we therefore do not think that purifying selection in the laboratory confounded our
421 results.

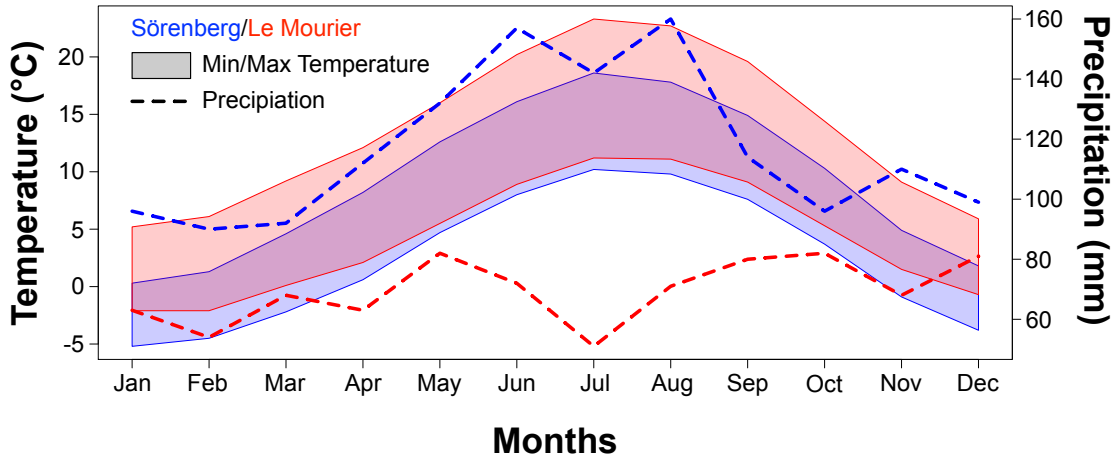


Fig. 3. Climatic differences between the Sörenberg (Swiss Alps; blue) and Le Mourier (French Cevennes; red); polygons and dashed lines show monthly average temperature ranges and precipitation at the two sampling sites, respectively. The data from the WorldClim dataset (Hijmans *et al*. 2011) represent 50-year averages of observations in quadratic grid cells with and edge of length 2.5' (~ 5 km^2 in size) around the coordinates of the two sites (Table1). The two locations are characterized by strong differences in precipitation, especially during the main reproductive season of sepsid flies from May to October.

Laboratory hybridization experiments have shown that interspecific mating between *S. cynipsea* and *S. neocynipsea* can result in viable and fertile F_1 hybrid females and offspring in backcrosses with the parental species (Giesen *et al*., 2017, 2019). Together with morphological and behavioral similarities among the two species, these laboratory experiments suggest that hybridization may well take place in areas of co-occurrence, such as the French Cevennes. In other areas, including our sampling site in the Swiss Alps, subtle premating behavioral barriers and the reductions in fecundity and fertility also previously documented in the laboratory by Giesen *et al*. (2017, 2019) may combine with micro-ecological niche differences to mediate spatio-temporally divergent reproductive timing. Such a divergence may effectively prevent hybridization in nature and ultimately reinforce species boundaries. This interpretation is strengthened by a study on Central American *Archisepsis diversiformis* flies showing that mating between two disjunct populations is only evident under forced laboratory conditions, while under conditions of free mate choice flies from the

443 different populations do not interbreed (Puniamoorthy 2014). Behavioral mating barriers
444 therefore seem to evolve comparatively fast (Gleason & Ritchie, 1998; Puniamoorthy *et al*.,
445 2009; Puniamoorthy, 2014), especially if species occur in sympatry and reinforcement by
446 natural or sexual selection can operate (Coyne & Orr, 2004; Seehausen, 2004; Ritchie, 2007).

447 Our study of introgression patterns among sympatric *S. cynipsea* and *S. neocynipsea*
448 populations relied on the ABBA-BABA test, which was initially designed for the analysis of
449 haploid genotypes of one individual from each of four lineages (Green *et al*., 2010). Recent
450 extensions of this original approach to allele frequency data by Durand *et al*. (2011) and
451 Soraggi *et al*. (2018), which incidentally were here shown to not be equally sensitive in
452 picking up introgression patterns, enabled us to apply the method equivalently to pool-
453 sequenced laboratory as well as to field-caught specimens (see also Deitz *et al*. 2016). As a
454 technical innovation, we have here provided a Python implementation of the ABBA-BABA
455 approaches by Durand *et al*. (2011) and Soraggi *et al*. (2018) that takes a simple 2D matrix of
456 allele frequencies as input. This implementation allows restricting the ABBA-BABA test to
457 high-confidence SNPs that pass a set of user-defined filters. Such filtering can reduce
458 artefacts of pool-sequencing (Schlötterer *et al*. 2014) that may produce false polymorphisms
459 due to sequencing errors (Futschik & Schlötterer 2011; Cutler & Jensen 2012). In addition,
460 and in contrast to the software package ANGSD (Korneliussen *et al*. 2014), our
461 implementation accommodates an outgroup (P4) taxon different from the one used as a
462 reference to call the SNPs. This feature reduces a potential reference bias if the ingroup taxa
463 (P1, P2, P3) strongly differ from an outgroup that also serves as reference (Ballouz *et al*.
464 2019). Our script and detailed instructions for how to use it are available on GitHub
465 (https://github.com/capoony/ABBA-BABA-4-AF; see also Supporting Information).

466 In summary, we found evidence that the extent of interspecific gene flow between two
467 closely related sepsid fly species in sympatry relative to allopatry varies with the geographic
468 location of the site. Interspecific gene flow in sympatry appears to be reduced at two Swiss
469 sites, where the two species have likely locally adapted to different (micro-)ecological niches,
470 such as different breeding times or phenologies, and/or where pre- or postmating reproductive
471 barriers (Giesen *et al*., 2017) have been and are subject to stronger reinforcement. Future
472 studies are needed to determine if ecological and/or sexual selection are indeed driving the
473 gene flow patterns we identified, and future genomic research should analyze patterns of
474 genomic differentiation across allopatric populations from Europe as well as North America
475 (Baur *et al*., 2020). It remains unclear why *S. neocynipsea* is rare in Europe, but common in
476 North America, and why *S. cynipsea* abounds around fresh dung all over Europe north of the
477 Alps and apparently outcompetes and ultimately relegates *S. neocynipsea* towards presumably
478 marginal, high altitude habitats (Pont & Meier, 2002). Such analyses would provide further
479 insights into introgression patterns across the species' entire natural range, as well as into the
480 role of local (e.g., longitudinal or latitudinal) adaptation in shaping genome-wide patterns of
481 differentiation.

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489 Biodiversity Research (GBR), in which context the reference draft genomes of several *Sepsis*

species explored in this study were sequenced and assembled. We particularly thank the GBR members Jeanne Wilbrandt, Jan Philip Øyen, and Malte Petersen for their contribution to the assembly pipeline. The University Research Priority Program 'Evolution in Action' of the University of Zürich, the Swiss National Science Foundation, and the Georges and Antoine Claraz-Donation funded the work of A.G., the Austrian Science Foundation (grant no. FWF P32275) the work of M.K.

DATA ACCESSIBILITY

All sequencing data have been deposited at the short-read archive (SRA; https://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA612154. Novel software is available at GitHub (https://github.com/capoony/ABBA-BABA-4-AF).

AUTHORS CONTRIBUTION

AG, WUB, KKS, HELL, SA and MK conceived and designed the study. MAS and AG gathered and prepared the samples and generated the genomic data. BM, ON and LP sequenced and constructed the reference genome. MK, AG, SA and HELL analyzed the genomic data. MK developed new software. AG, MK, SA and WUB wrote the manuscript, with ON, KKS, RSI, LP, BM, MAS and HELL contributing to the interpretation of the data, and editing and commenting on various versions of the manuscript. WUB, KKS, RSI, and HELL were members of AG's PhD committee, providing guidance and input at all stages.

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