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Exaggerated male forelegs are not more differentiated than wing morphology in two widespread sister species of black scavenger flies

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Abstract

Sexual selection represents a potent force that can drive rapid population differentiation in traits related to reproductive success. Hence, sexual traits are expected to show greater population divergence than non-sexual traits. We test this prediction by exploring patterns of morphological differentiation of the exaggerated fore femur (a male-specific sexual trait) and the wing (a non-sexual trait) among allopatric and sympatric populations of the widespread sister dung fly species *Sepsis neocynipsea* and *Sepsis cynipsea* (Diptera: Sepsidae). While both species occur in Eurasia, *S. neocynipsea* also abounds in North America, albeit previous studies suggest strong differentiation in morphology, behavior, and mating systems. To evaluate the degree of differentiation expected under neutrality between *S. cynipsea*, European *S. neocynipsea*, and North American *S. neocynipsea*, we genotyped 30 populations at nine microsatellite markers, revealing almost equal differentiation between and minor differentiation among geographic populations within the three lineages. Landmark-based analysis of 18 populations reared at constant 18 and 24°C in a laboratory common garden revealed moderate temperature-dependent phenotypic plasticity and significant heritable differentiation in size and shape of male forelegs and wings among isofemale lines of the three lineages. Following the biological species concept, there was weaker differentiation between cross-continental populations of *S. neocynipsea* relative to *S. cynipsea*, and more fore femur differentiation between the two species in sympatry versus allopatry (presumably due to character displacement). Contrary to expectation, wing morphology showed as much shape differentiation between evolutionary independent lineages as fore femora, providing no evidence for faster diversification of traits primarily engaged in mating.

KEYWORDS
character displacement, morphometrics, population differentiation, population genetics, Sepsidae, speciation

1 | INTRODUCTION

Due to high variance in mating and fertilization success, sexual selection can be much stronger than natural selection, leading to the diversification of phenotypic traits even beyond their fitness optima (Arnqvist & Rowe, 2005; Hosken & House, 2011; Ritchie, 2007). Hence, sexual traits are generally thought to evolve at higher rates relative to non-sexual traits. For example, arthropod genitalia are frequently cited to evolve extremely fast compared with many other types of characters due to intense pre- and/or postcopulatory sexual selection (Arnqvist, 1998; Arnqvist & Rowe, 2005; Eberhard, 2013; Hosken & Stockley, 2004; Puniamoorthy, Kotrba, & Meier, 2010). While sexual selection can drive speciation and corresponding phenotypic differentiation, it is less clear whether selection acts more or less continuously throughout the persistence of a lineage, or whether it primarily acts during early stages of speciation, thereby effectively minimizing costly hybridizations in geographic areas of co-existence (Ritchie, 2007). In the latter case, short periods of intense diversifying selection due to reproductive character displacement may be followed by periods of stabilizing selection, or even neutrality, as fitness gains diminish with decreasing hybridization frequency (Coyne & Orr, 2004). Because traits with sex-specific expression are exposed to selection half as often as sexually monomorphic traits, they are predicted to accumulate more mutations under selection–mutation balance. This explanation, for instance, has been proposed to account to some degree for the high amino-acid substitution rates of male seminal fluid proteins in insects (Haerty et al., 2007; Meiklejohn, Parsch, Ranz, & Hartl, 2003). Thus, under both adaptive and non-adaptive scenarios, one would expect sexually dimorphic traits to evolve at faster rates, that is, to show greater divergence among lineages, relative to non-sexual traits. Sexual traits evolving at increased rates have been shown by comparative phylogenetic studies (Arnqvist, 1998; Klaczko, Ingram, & Losos, 2015; Seddon et al., 2013), and empirically for traits subjected to female choice or antagonistic coevolution (Debelle, Ritchie, & Snook, 2014; Eberhard, 2013; Uy & Borgia, 2000).

We investigate the genetic differentiation of complex sexual versus non-sexual traits across populations of closely related, widespread sepsid fly species. Black scavenger flies (Diptera: Sepsidae) represent a clade of acalyptrates common around the globe that are typically associated with decaying organic matter (Ozerov, 2005; Pont & Meier, 2002). We here focus on genetic differentiation within and between the sister species *Sepsis cynipsea* and *Sepsis neocynipsea* (Figure 1). *Sepsis cynipsea* is the most common sepsid fly on cattle pastures throughout Eurasia, while *S. neocynipsea* is common in North America, there largely occupying the ecological niche that *S. cynipsea* has in Europe. However, *S. neocynipsea* can also be found in Europe, mainly in the Alps and other mountainous regions, where it usually co-occurs in sympatry with *S. cynipsea* (Rohner et al., 2015). As these two species do hybridize under laboratory conditions (though hybrid genotypes show reduced fertility: Giesen, Blanckenhorn, & Schäfer, 2017; Giesen, Schäfer, & Blanckenhorn, 2019), this system offers the opportunity to explore the evolutionary forces leading to morphological divergence during early stages of speciation in sympatry and allopatry. By considering neutral genetic variation as the baseline reflecting genetic drift, and by at the same time studying multivariate shape variation of sexual and non-sexual traits, we here evaluate the role of sexual versus non-sexual (natural) selection presumably driving speciation by reproductive versus ecological character displacement, respectively (Pfennig & Pfennig, 2009), a type of study surprisingly rare in the literature on speciation (Pfennig, 2008).

As most species of the genus *Sepsis*, *S. neocynipsea* and *S. cynipsea* show pronounced male-limited modifications of the fore femur, including protrusions and spines (see Figure 1), which are frequently used to delineate closely related species within the clade. Since males use their femur to hold on to the female's wing

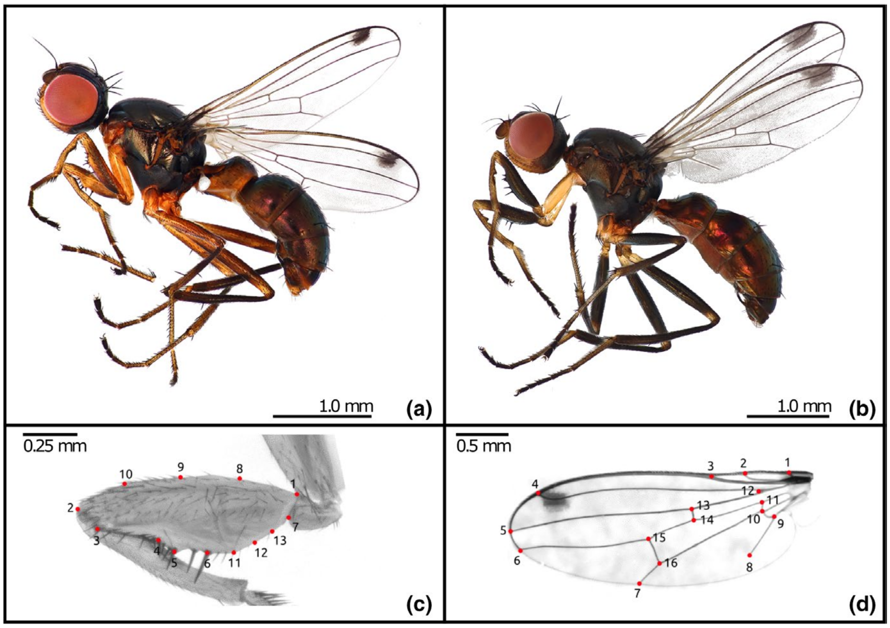


FIGURE 1 Male *Sepsis neocynipsea* (a), male *Sepsis cynipsea* (b), location of the 7 landmarks (1–7) and the 6 sliding semi-landmarks (8–13) on the femur (c), and location of the 16 landmarks on the wing (pictures a & b with kind permission by Dr. Yuchen Ang via sepsidnet: Ang et al., 2013)

base during copulation, these structures have received considerable attention in the context of highly variable pre- and postcopulatory sexual selection (Ang, Puniamoorthy, & Meier, 2008; Blanckenhorn, Kraushaar, Teuschl, & Reim, 2004; Eberhard, 2013; Puniamoorthy, Ismail, Tan, & Meier, 2009; Puniamoorthy, Su, & Meier, 2008), encompassing female choice and resistance (Blanckenhorn, Mühlhäuser, Morf, Reusch, & Reuter, 2000; Dmitriew & Blanckenhorn, 2012, Puniamoorthy, Blanckenhorn, & Schäfer, 2012; but see Ingram, Laamanen, Puniamoorthy, & Meier, 2008) as well as male–male competition (Busso & Blanckenhorn, 2018; Rohner & Blanckenhorn, 2018; Rohner, Blanckenhorn, & Puniamoorthy, 2016). The mating system of *S. cynipsea* has been described in detail and can be characterized by male scramble competition, female choice, and occasionally intense sexual conflict (Blanckenhorn et al., 2004, 2000; Martin & Hosken, 2003; Parker, 1972a, 1972b; Puniamoorthy et al., 2009), while only little is known about *S. neocynipsea* in this regard (Eberhard, 1999; Puniamoorthy et al., 2009; Rohner et al., 2016).

In contrast to strongly sexually dimorphic armaments or ornaments, insect wings are typically seen as targets of mainly natural (i.e., viability) selection. Flight is the prime agent of dispersal in most pterygotes and thereby contributes crucially to foraging, predator avoidance, and thermoregulatory behavior, although wings can sometimes contribute to sexual signaling, courtship, or mate search (as in several *Drosophila* species: Hoikkala, Hoy, & Kaneshiro, 1998; Ritchie, 2007; Snook, Robertson, Crudgington, & Ritchie, 2005). Since males of the species studied here show no obvious courtship display with their wings, and wings are sexually monochromatic, we can assume that morphological divergence in wing shape is primarily shaped by natural selection. Comparative analysis of 40 million years of wing shape evolution in *Drosophila* revealed that interspecific shape variation is remarkably similar to that resulting from standing genetic variation or mutation, but that rates of evolution are 10,000 times slower than expected under mutation-drift equilibrium, implying strong and consistent stabilizing selection on wing shape (Houle, Bolstad, van der Linde, & Hansen, 2017; see also corresponding communication by Cheverud, 2017). Similar conclusions have been derived from intraspecific studies of drosophilids. Although wing shape typically exhibits a highly polygenic basis of largely additive effects (Mezey, Houle, & Nuzhdin, 2005; Weber et al., 2001; Zimmerman, Palsson, & Gibson, 2000), implying that shape variation can diverge quickly via selection or drift (Fragata et al., 2010; Kapun, Schmidt, Durmaz, Schmidt, & Flatt, 2016; Simões et al., 2015), shape variation between cross-continental *Drosophila melanogaster* populations is very weak, implying a role of stabilizing selection within species as well (Gilchrist, Azevedo, Partridge, & O´Higgins, 2000; Gilchrist & Partridge, 2001).

By genotyping 30 populations at nine polymorphic microsatellite markers, we first explored patterns of neutral molecular variation between the three lineages (*S. cynipsea*, North American [NA] *S. neocynipsea*, and European [EU] *S. neocynipsea*). We further used common garden laboratory rearing of multiple populations of all three lineages at two temperatures (18 and 24°C) to compare heritable geographic differentiation patterns of the exaggerated male fore femur and wing morphology, with neutral expectations derived from microsatellite analysis. As the general null hypothesis under neutrality, we expected morphological (i.e., quantitative genetic) differentiation among lineages to mirror patterns of neutral genetic divergence. Because geographic patterns of morphological differentiation can be environment-specific, we implemented two temperature regimes, thereby exploring thermal plasticity across lineages as well. If sexually selected traits generally diverge faster between species (lineages) than traits mainly subject to ecological viability or fecundity (i.e., natural) selection, we expected quantitative genetic differentiation between lineages relative to within-lineage (population) variation to be overall greater for fore femora than for wings. If sexual selection primarily acts during early stages of speciation to prevent costly interspecies hybridizations, we further expected stronger divergence between *S. cynipsea* and *S. neocynipsea* in Europe, where both species co-occur sympatrically, relative to the differentiation between *S. cynipsea* and North American *S. neocynipsea*.

2 | MATERIAL AND METHODS

2.1 | Study organisms

The sister species *S. cynipsea* (Linnaeus, 1758) and *S. neocynipsea* (Melander & Spuler, 1917) exhibit only little differentiation at the mitochondrial barcoding genes *Cytochrome c oxidase I* (*COI*) and *Cytochrome b* (*CyB*) (Su, Kutty, & Meier, 2008), but are differentiated in behavior, distribution, and ecology (Giesen et al., 2017; Pont & Meier, 2002; Puniamoorthy et al., 2009; Rohner et al., 2015). *Sepsis cynipsea* is the most abundant sepsid in north-central Europe, where it occurs in sympatry with the rare *S. neocynipsea* in some mountainous regions such as the Swiss Alps (Rohner & Bächli, 2016; Rohner et al., 2015). In North America, however, where *S. cynipsea* is absent, *S. neocynipsea* is common in lowland and highland habitats (Pont & Meier, 2002).

2.2 | Microsatellite genotyping and data analyses

A total of 338 specimens from 14 European *S. cynipsea* populations, 116 specimens from 10 North American, and 97 specimens from six European *S. neocynipsea* populations were collected in the field to represent the distributional range of both species on two continents (Appendix 1; Figure 2). DNA was isolated from entire flies using the DNeasy Blood and Tissue Kit (Qiagen AG) according to the manufacturer's protocol. Nine highly polymorphic microsatellite markers were genotyped following the M13-tail PCR method (Schuelke, 2000). Six of these markers had already been isolated for *S. cynipsea* (Greminger, Schäfer, Nater, Blanckenhorn, & Kruetzen, 2009), and we designed primers for three additional markers (J60, G53, E67) amplifying in both species (for primer sequences, see Table S1). PCR amplification and separation followed the protocol are described in Greminger et al. (2009).

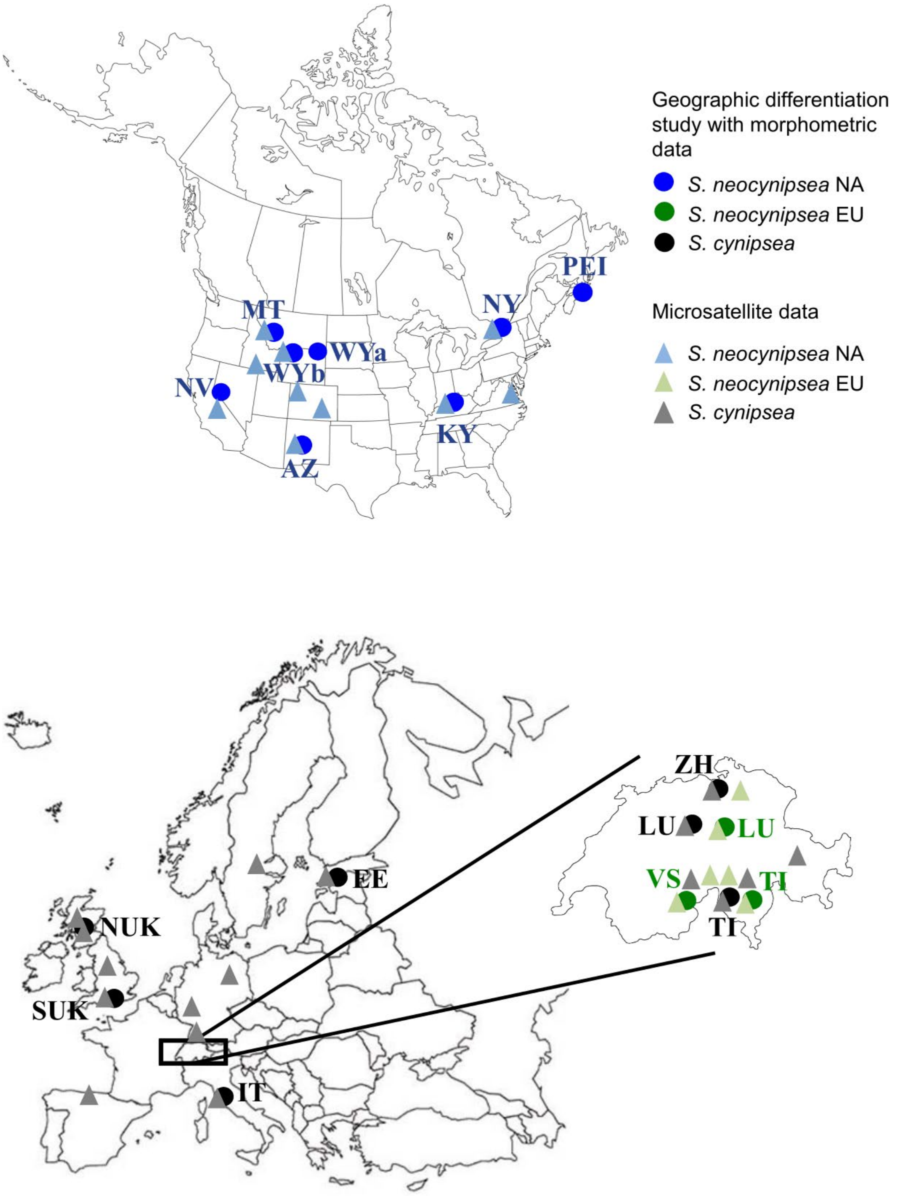


FIGURE 2 European *Sepsis cynipsea* (black), North American (blue; top), and European (green; bottom) *Sepsis neocynipsea* populations sampled for the microsatellite analysis (light triangles) and the morphological differentiation study (dark circles). Abbreviations correspond to the names used in Appendix 2 for sampling locations

To illustrate the relationships among lineages, we constructed a neighbor-joining (NJ) tree using the package *poppr* (Kamvar, Tabima, & Grünwald, 2014) in R (R Development Core Team, 2015) based on the proportion of shared alleles calculated with the *memgene* package in R (Galpern, Peres-Neto, Polfus, & Manseau, 2014). Node support was calculated with 10,000 bootstrap replicates resampled using *poppr*. Genetic differentiation among continents relative to that between populations within continents was investigated with the *ade4* (Dray & Dufour, 2007) AMOVA implementation in the *poppr* package. We further performed a Mantel test (Manly, 1991) using the *ade4* package to compare matrices of pairwise F_{ST}-values with matrices of pairwise geographical distances. Pairwise and global F_{ST}-values were estimated according to Weir and Cockerham (1984), while statistical significance was determined by permuting genotypes among populations 10,000 times. F_{ST} estimates were calculated using Microsatellite Analyzer version 3.12 (Dieringer & Schlötterer, 2003).

2.3 | Quantitative genetic differentiation: common garden rearing

To assess quantitative genetic differentiation in wing and fore femur size and shape between lineages and populations within lineages, a total of 591 individuals from 228 iso-female lines of *S. cynipsea* (seven populations) and *S. neocynipsea* (Europe: three populations; North America: seven populations) were reared in two common laboratory environments at constant 18 and 24°C with ad libitum cow dung as breeding substrate (Figure 2; see Appendix 2 for more information about the sampling locations and sample sizes). As originally field-caught flies had been held as iso-female lines (i.e., full-sib families) for multiple generations in the laboratory under identical environmental conditions, the morphological variation explained by population and lineage identity can be attributed to genetic, that is, evolved, differentiation (as opposed to mere phenotypic differentiation that also includes environmental variation: Lynch & Walsh, 1998). For each line and temperature regime, three adult males per line were chosen at random for morphometric analysis after having been stored in 70% EtOH at −18°C. Consider Rohner et al. (2016) for more details about general laboratory procedures.

2.4 | Morphometric data acquisition

Both forelegs and wings were removed from the thorax in 70% EtOH and, after evaporation of the ethanol, embedded in Euparal (Carl Roth GmbH) on a glass slide. Slides were then placed on a 35°C heating plate for 5 min to liquefy the resin before samples were dried at room temperature. Wings and legs were photographed using a Leica DFC490 camera mounted on a Leica MZ12 microscope. tpsDig2 (Rohlf, 2006) was used to acquire landmark coordinates.

Seven landmarks were placed to describe shape variation of the male fore femur, marking distinct and most probably interspecifically homologous points (Figure 1c). In addition, three sliding semi-landmarks (Gunz & Mitteroecker, 2013) were placed between landmarks 1 and 2 as well as between landmarks 6 and 7 to measure the curvature of the leg between them. Sixteen landmarks were chosen to describe wing morphology, marking vein-node positions covering the wing base and the blade (Figure 1d). Landmark data were aligned by applying full Procrustes transformation (Rohlf & Slice, 1990) using *Past* (Hammer, Harper, & Ryan, 2001), and left and right forelegs and wings were averaged to account for potential fluctuating asymmetry.

2.5 | Geographic variation in shape

To investigate genetic shape variation among lineages and populations within lineages, allometry and plastic responses to temperature, we used Procrustes ANOVAs following Klingenberg, Barluenga, and Meyer (2002). To this end, we computed univariate ANOVAs for each Procrustes-transformed landmark coordinate (one *x*-coordinate and one *y*-coordinate per landmark). Iso-female line means were analyzed as a function of lineage, population nested within lineage, trait centroid size, and temperature. The interactions between temperature, lineage, and population within lineage were also included. We summed the (type III) sums of squares and degrees of freedom for all effects of all coordinates and computed the corresponding mean squares, *F*-, and *p*-values following Goodall (1991). Partial eta square ($\eta_p^2 = \frac{SS_{lineage}}{SS_{lineage}+SS_{population(lineage)}}$) was used to estimate effect size. We bootstrapped the data 999 times to acquire confidence limits for η_p^2. This procedure was performed separately for wing and fore femur shape data.

To visualize shape differentiation between lineages, we used two complementary ordination techniques: principal component analysis and canonical variate analysis (PCA and CVA). Because wing shape shows allometric scaling in sepsids (Rohner, Roy, Schäfer, Berger, & Blanckenhorn, 2019) and the lineages differ in body size, differentiation in shape could be driven by allometry alone. Therefore, prior to ordination we calculated the residuals of a multivariate regression of shape on centroid size to statistically remove the effect of static allometric scaling. By applying this procedure to each lineage separately, we thus also account for variation in allometric scaling relationships between lineages. We first subjected the allometry-corrected Procrustes-transformed landmark data to PCA. This analysis captures the main axes of the overall shape variation among individuals and allows qualitative assessment of the relative morphological similarity among groups. However, the main axes of morphological variation do not necessarily correspond to the main axes of differentiation between groups. We therefore also used a CVA, which finds the linear combinations of shape variables (canonical variates) that differentiate best between group means (Klingenberg, Duttke, Whelan, & Kim, 2012; McCune, Grace, & Urban, 2002). Because canonical variates are scaled by the within-group variance, distances in CVA space do not reflect the distance among groups in the original shape space (Zelditch, Swiderski, Sheets, & Fink, 2004). To obtain a quantitative estimate of how strongly the lineages differ in their mean shape, we calculated pairwise Procrustes distances between average shapes per lineage.

3 | RESULTS

3.1 | Phylogeographic patterns of microsatellite variation

A NJ tree based on the proportion of shared alleles illustrates that populations of *S. neocynipsea* and *S. cynipsea* form distinct clusters (Figure 3). The tree further shows a clear division between North American and European *S. neocynipsea* populations. Branch lengths within lineages were relatively short, however, and weak node support indicates that populations share a large proportion of alleles.

AMOVA revealed that 20.1% of the total genetic variance is explained by the differences among the three lineages, and only 0.9% could be attributed to differences among populations within lineages. The remaining 78.5% of the total molecular variance was localized among iso-female lines within populations. Further pairwise comparisons indicated strong genetic differentiation between *S. cynipsea* and North American populations of *S. neocynipsea* (F_{ST} = .22; $p < .001$), whereas the corresponding differentiation within Europe was somewhat lower (F_{ST} = .16; $p < .001$) and of similar magnitude as that between New and Old world populations of *S. neocynipsea* (F_{ST} = .16; $p < .001$).

The degree of genetic differentiation among populations within each of the three lineages was very low but nonetheless statistically significant (*S. cynipsea*: F_{ST} = .01, $p < .001$; *S. neocynipsea* Europe: F_{ST} = .01, p = .001; *S. neocynipsea* North America: F_{ST} = .03, $p < .001$). Mantel tests further yielded a significant correlation between pairwise F_{ST}-values and spherical geographic distances across Europe in *S. cynipsea* (r = .45, p = .011), though this correlation greatly depended on the Estonian population from Pehka, which was significantly differentiated from all other populations (Figure 3). When this population

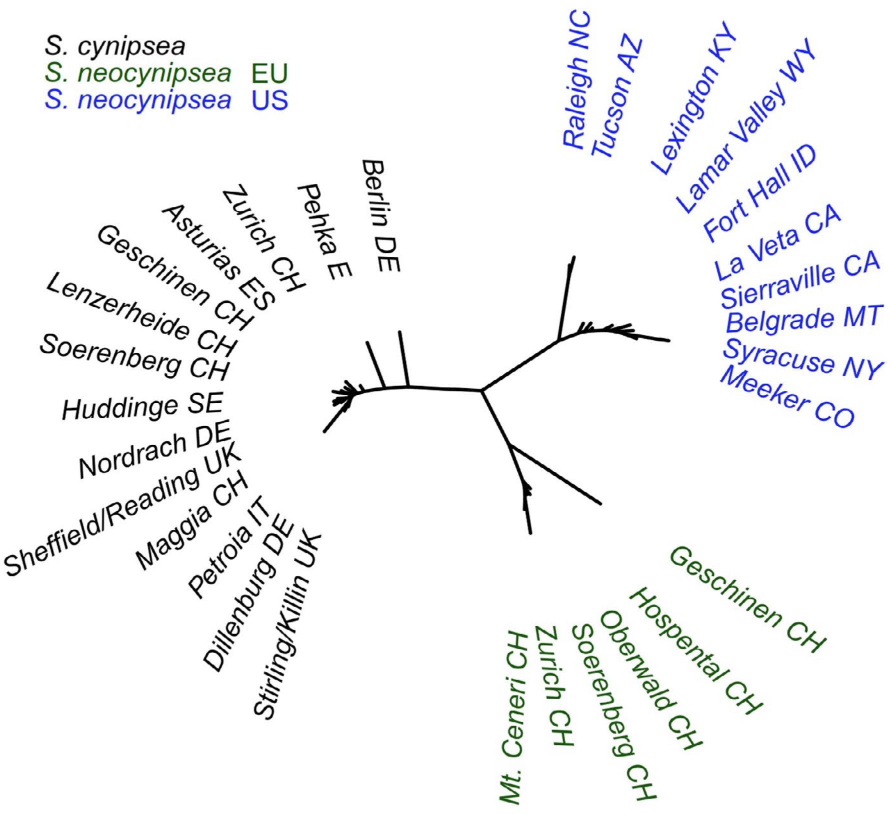


FIGURE 3 Neighbor-joining tree based on nine highly polymorphic, putatively neutral microsatellite markers for multiple populations of European *Sepsis cynipsea*, European, and North American *Sepsis neocynipsea*. The three lineages are strongly differentiated (node support for *S. cynipsea*–*S. neocynipsea*: 75%; *S. neocynipsea* EU–*S. neocynipsea* United States: 62%) with only minor genetic variation among populations within lineages. Branch lengths are proportional to the genetic distances of populations and lineages

was excluded from the analysis, the Mantel correlation turned non-significant ($r = .19$, $p = .13$). Perhaps surprisingly, no isolation by distance was evident across North American populations of *S. neocynipsea* ($r = -.15$, $p = .77$). Note that we did not test for isolation by distance among European *S. neocynipsea* populations due to the limited number of populations sampled over very short distances (Figures 2 and 3). Appendix 3 provides more information on the sample sizes, the number of alleles, mean observed (H_O), and expected (H_E) heterozygosity for each locus across the three study lineages.

3.2 | Geographic patterns of morphological differentiation

Sepsis cynipsea has smaller wings and fore femora than its sister species (wings: $F_{2,194} = 101.14$, $p < .001$; fore femur: $F_{2,192} = 167.7$, $p < .001$). Body size tended to increase with decreasing temperature, following the temperature–size–rule (Atkinson, 1994), although thermal plasticity was rather weak and not consistent across species (temperature effect on wings: $F_{1,194} = 36.54$, $p < .001$; fore femur: $F_{1,192} = 13.36$, $p < .001$; temperature*lineage interaction for wings: $F_{2,194} = 321.1$, $p < .001$; fore femur: $F_{2,192} = 1.47$, $p = .233$; Figure 4).

Even when controlling for shape differentiation caused by allometry (by adding centroid size as covariate), we recovered significant shape variation among continents as well as differentiation among populations within continents for both fore femur and wing morphology (Table 1). The main differences in fore femur shape between *S. cynipsea* and *S. neocynipsea* lie in the horizontal positioning of landmarks 8–10 relative to landmarks 6 and 11 (Figure 5a). *Sepsis neocynipsea* also shows a more pronounced notch (LM 4). Further, the fore femora of North American *S. neocynipsea* were broader than those of their European conspecifics (Figure 5a).

The wings of *S. cynipsea* are comparably slender and elongated. North American *S. neocynipsea* have the broadest, most roundish wings, while European *S. neocynipsea* fall in between *S. cynipsea* and its conspecific populations from North America (Figure 5b).

The proportion of variance attributable to genetic differentiation among lineages relative to that among populations amounts to $\eta_p^2 = 0.45$ (95% CI: 0.39, 0.52) for fore femur and $\eta_p^2 = 0.55$ (0.50, 0.68) for wing shape. The variation among lineages (scaled by the variation within lineages) for wing shape is therefore at least as strong as that for fore femur shape. Both structures are also phenotypically plastic in response to temperature, although this response had relatively little effect on the traits and varied between lineages and populations (Table 1).

When correcting for allometric shape variation, PCA reveals little differentiation in fore femur and wing shape among lineages based on the two major axes (Figure 5). By contrast, CVA clearly indicates quantitative genetic differentiation among the three evolutionary independent lineages (Figure 6). The main axes of morphological variation do therefore not correspond to the main axes of differentiation between lineages, as CVA demonstrates significant evolutionary divergence in both fore femur and wing shape, while PCA does not (compare Figures 5 and 6). The overall classification accuracy of CVA was high, irrespective of whether allometry was accounted for (fore femur: 90.00%; wing: 95.04%) or not (fore femur: 93.57%; wing: 93.62%). Similarly, pairwise Procrustes distances suggest differentiation among lineages

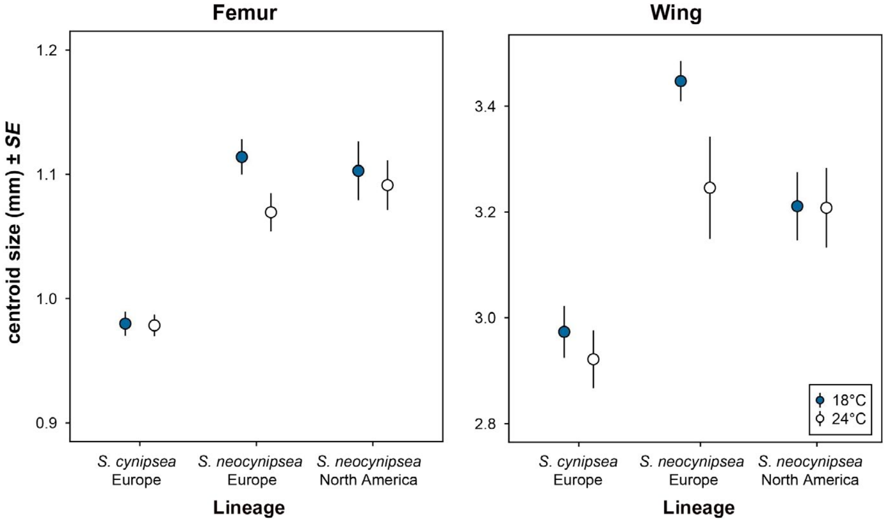


FIGURE 4 Lineage-specific temperature-dependent plasticity in (centroid) size of fore femora (left) and wings (right)

TABLE 1 Procrustes ANOVAs for (a) fore femora and (b) wings

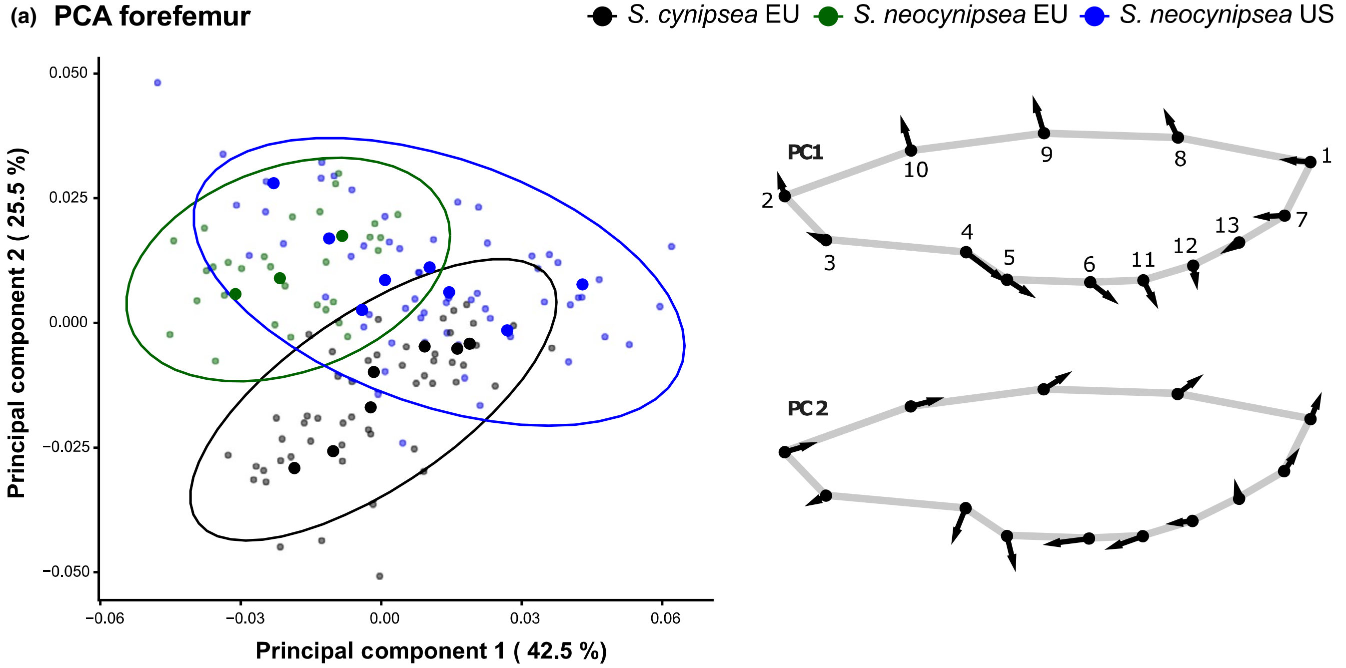
	SS	*df*	MS	*F*	*p*	η_p^2
(a) Fore femur						
Centroid size	3.05E-02	26	1.17E-03	48.50	<.001	.20
Lineage	8.91E-02	52	1.71E-03	6.12	<.001	.45
Temperature	1.92E-03	26	7.39E-05	3.06	<.001	.02
Population (lineage)	1.09E-01	390	2.80E-04	11.59	<.001	.48
Lineage × temperature	2.78E-03	52	5.35E-05	1.18	.200	.14
Population(lineage) × temperature	1.66E-02	364	4.55E-05	1.88	<.001	.12
Error (iso-female line means)	1.20E-01	4,966	2.42E-05			
(b) Wing						
Centroid size	1.03E-02	32	3.23E-04	24.29	<.001	.11
Lineage	3.25E-02	64	5.09E-04	9.02	<.001	.55
Temperature	1.18E-03	32	3.68E-05	2.76	<.001	.01
Population (lineage)	2.71E-02	480	5.64E-05	4.24	<.001	.25
Lineage × temperature	1.50E-03	64	2.34E-05	0.79	.875	.10
Population(lineage) × temperature	1.33E-02	448	2.96E-05	2.23	<.001	.14
Error (iso-female line means)	8.22E-02	6,176	1.33E-05			

following their phylogenetic history with little differences between wings and femora (Figure 6c).

4 | DISCUSSION

Our analysis of morphological and molecular variation in two closely related sepsid fly species from two continents yielded three main results. First, microsatellite analyses showed clear, almost equal molecular genetic differentiation between European *S. cynipsea*, European *S. neocynipsea*, and North American *S. neocynipsea*, with very little differentiation among geographic populations within the three lineages. Second, CVA revealed significant quantitative genetic differentiation in male foreleg and wing morphology among the three molecularly distinct lineages, though the main patterns of lineage differentiation do not necessarily follow the main axes of variation among individuals (PC1 and PC2). Third, even though sexual traits are generally expected to show greater evolutionary divergence between lineages than non-sexual traits, the variance among lineages relative to the variation within lineages (η_p^2) and the cross-continental relative to the interspecific Procrustes distances of *S. neocynipsea* were similar for wings and fore femora. Wing shape is therefore as lineage-specific as the shape of the male fore femur. In the following, we first discuss phylogeographic and demographic scenarios that might explain the patterns of molecular variation, and then consider the role of adaptive and non-adaptive evolutionary processes that might have contributed to morphological diversification of forelegs and wings among these three closely related lineages.

Phylogeographic studies provide insights into the evolutionary history of the studied species, but at the same time reveal


(a) PCA forefemur

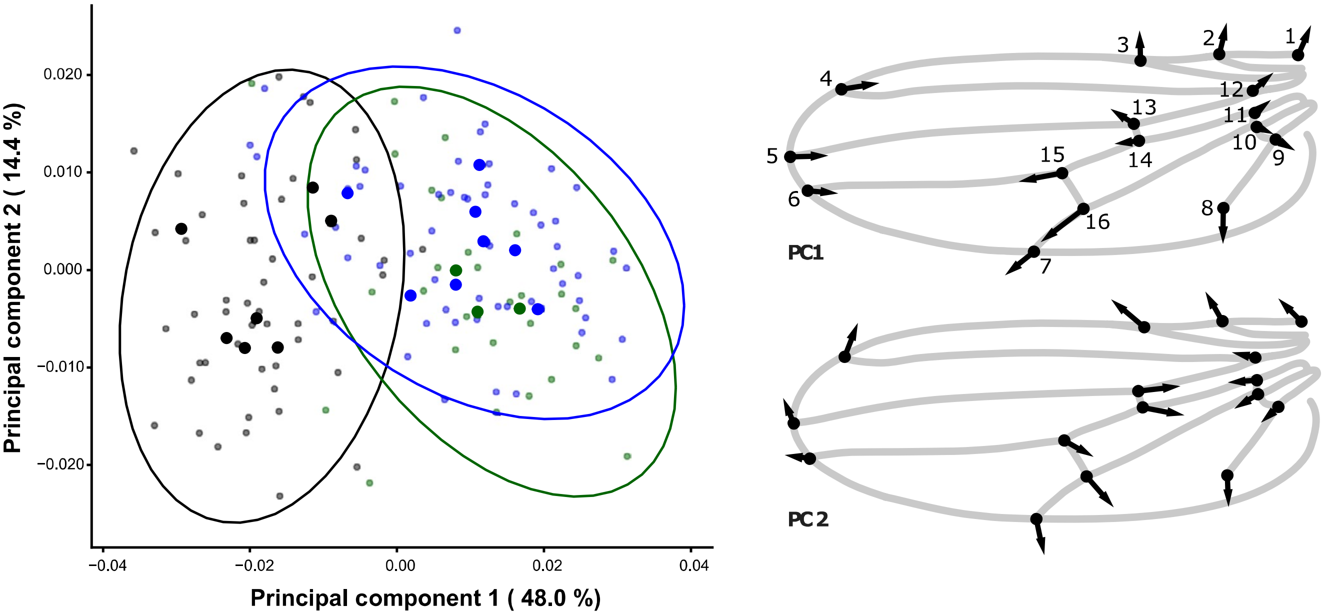
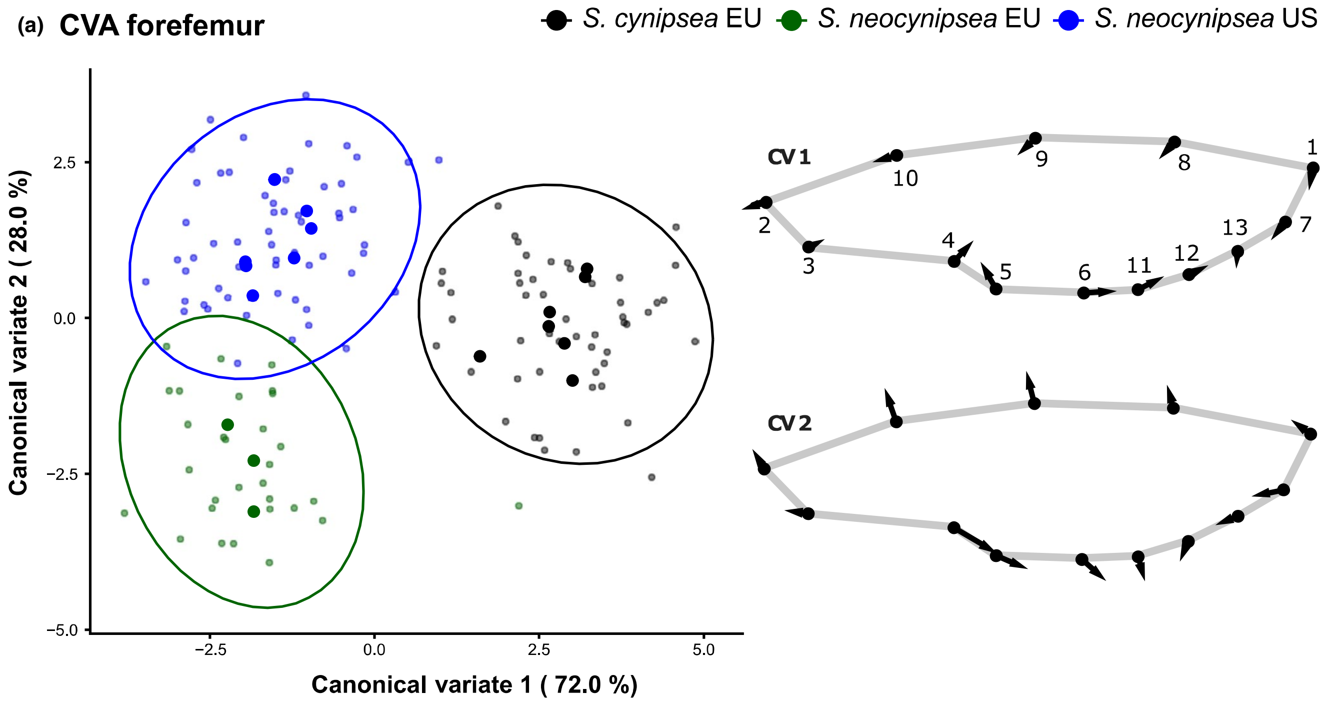

(b) PCA wing

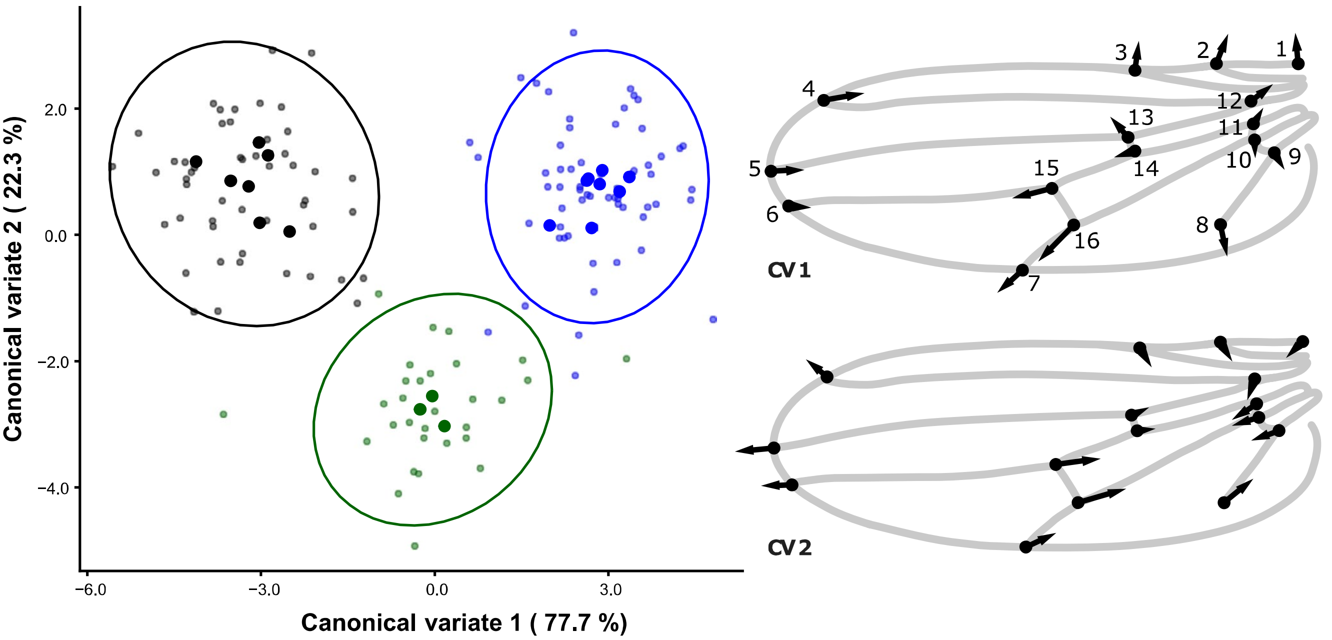
FIGURE 5 Principal components for (a) fore femora and (b) wings of the three lineages. Shape data were corrected for allometric scaling. Large solid dots represent population means, and small dots represent individual male flies. Shape changes associated with the two major axes are given in the right

concurrent patterns of gene flow and genetic drift, both of which have different implications for magnitudes and rates of local adaption (Hewitt, 2001). While populations can respond rapidly to divergent selective pressures when gene flow is restricted and population sizes are sufficiently large, high rates of dispersal counterbalance local adaptation and instead may favor the evolution of phenotypic plasticity. So far, molecular studies of *S. cynipsea* and *S. neocynipsea* focused on interspecific differences based on sequence data of the barcording genes *COI* and *CyB*, leaving the species' geographic and demographic history unresolved (Pont & Meier, 2002; Puniamoorthy et al., 2009; Su et al., 2008). Our analysis confirms that the two species indeed differ at the molecular level (Figure 3), but additionally shows clear and strong genetic separation between continental populations of *S. neocynipsea* of roughly similar extent to that between European *S. cynipsea* and *S. neocynipsea*. The most plausible explanation for the observed pattern is a bottleneck followed by relatively low effective population size and drift during, and for some time after, the putative

(a) **CVA forefemur**



(b) **CVA wing**



(c) **Procrustes distance**

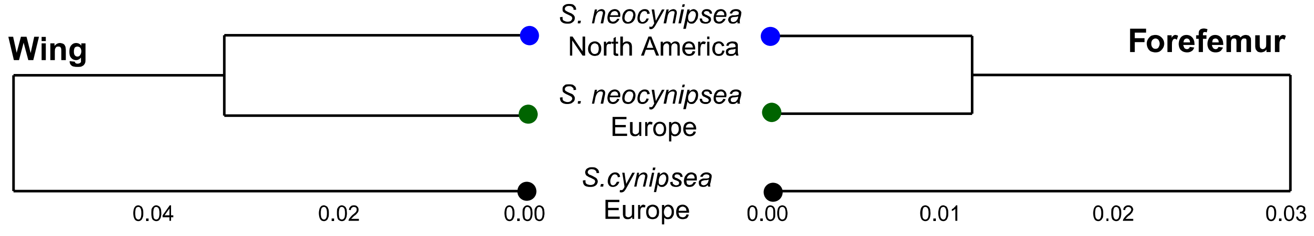


FIGURE 6 Canonical variates and pairwise procrustes distances for (a) fore femora and (b) wings of the three lineages. Shape data were corrected for allometric scaling. Large solid dots represent population means, and small dots represent individual male flies. Shape changes associated with the two canonical variates are given in the right. Mean Procrustes distances among the three lineages are plotted in (c)

secondary colonization of the American continent by *S. neocynipsea*. This hypothesis is supported by lower allelic richness at all but one locus (E67) in American populations (Appendix 3). By contrast, within lineages there was very little molecular differentiation among populations. This disagrees with earlier microsatellite findings for the related *Sepsis punctum* (Puniamoorthy, Meier, Blanckenhorn, & Schäfer, 2013), which show strong continental (European and North American) differentiation but also substantial population differentiation within lineages; particularly, populations from northern and southern Europe were quite distinct. Also, *S. punctum* exhibits significant isolation by distance across central and northern Europe as well as across North America, which is not the case for the two sister species studied here. Since all these species are likely very similar in dispersal ability and ecology, but differ in thermal preferences, such variation in the population structure may best be explained by differences in their ancient colonization history rather than by differences in concurrent gene flow and drift. While *S. punctum* appears to prefer warmer habitats and is rather rare at high altitudes (Rohner et al., 2015), *S. cynipsea* and *S. neocynipsea* occur in great numbers at higher elevations. Recolonization from multiple northern refugees and large effective population sizes minimizing genetic drift thus might explain the low degree of population differentiation across European *S. cynipsea* and North American *S. neocynipsea* compared to *S. punctum* despite restricted gene flow (Bhagwat & Willis, 2008; Hewitt, 2004), providing opportunity for local adaptation in response to spatially varying selection regimes.

Sexual selection is considered an important evolutionary force that may not only lead to rapid trait diversification, but also promotes population differentiation and speciation (Arnqvist & Rowe, 2005; Hosken & Stockley, 2004). Our quantitative genetic breeding design uncovered significant differentiation in fore femur shape among the two closely related sister species, as well as between the continental populations of *S. neocynipsea*, which are likely undergoing incipient speciation in allopatry (Giesen et al., 2017, 2019). However, the differentiation of femur morphology was of similar magnitude as that of wing vein positioning, which refutes our original prediction that male ornaments evolve at a higher rate than wings. Since patterns of shape differentiation of forelegs and wings follow the traditional species concept, with stronger differentiation between species than between cross-continental populations within species, our results are more in line with a major role of neutral diversification. Nevertheless, our results also support some role of reproductive character displacement shaping the fore femur in geographic areas of sympatry. Under character displacement, we would expect stronger morphological differentiation between sympatric *S. cynipsea* and *S. neocynipsea* in Europe, which indeed seems to be evident, albeit merely slightly, in the CVA results for fore femur but not wing morphology (Figure 6).

Direct evidence that male femur shape might be subject to sexual selection comes from comparative studies showing two evolutionary independent losses of male fore femur modifications that coincide with a clear change in mating behavior in *Sepsis duplicata* and *Perochaeta dikowi* (Puniamoorthy et al., 2008). Similarly, sexual dimorphism in fore femur width, and its condition dependence, co-varies with the mating system in sepsids, suggesting sexual selection to act on fore femur morphology (Rohner & Blanckenhorn, 2018). Nevertheless, the effect of sexual selection on fore femur size or shape (as opposed to body size per se: Blanckenhorn, 1999; Blanckenhorn et al., 2004; Blanckenhorn et al., 2000) remains to be directly demonstrated in *S. cynipsea* and *S. neocynipsea* (compare similar studies in *S. punctum*: Dmitriew & Blanckenhorn, 2012). That femur shape can respond to selection is evident from significant standing genetic variation encoding for the trait (Dmitriew & Blanckenhorn, 2014).

Wing shape often varies among populations and species and can even be used for taxonomic inference (Sontigun et al., 2017). However, compared to size, shape differentiation among populations and closely related species is usually weak and often attributed to (stabilizing) natural selection (Gilchrist et al., 2000; Gilchrist & Partridge, 2001). Despite the close relatedness of all lineages, we here found wing shape to show considerable differentiation similar in magnitude to that of fore femur shape. Sexual selection on wing shape has been reported for *D. melanogaster*, favoring males with elongated wings (Menezes, Vigoder, Peixoto, Varaldi, & Bitner-Mathé, 2013). However, whereas *D. melanogaster* use their wings to produce courtship songs (Greenspan & Ferveur, 2000; Markow & Grady, 2005), the functionality of the wings during courtship in the sepsids studied here is minor. Wings seem to play a role in mating and aggressive behavior in some related sepsids (Busso & Blanckenhorn, 2018; Eberhard, 2002; Puniamoorthy et al., 2009), and may thus also affect copulation success in *S. cynipsea* and *S. neocynipsea*. Irrespective of the concrete evolutionary forces acting on either trait, our study clearly demonstrates that wing shape is as informative as femur shape in delimiting these closely related species/lineages. One reason why male femur but not wing shape is used for taxonomic inference within the sepsids (Pont & Meier, 2002) might relate to human visual recognition capabilities; that is, it might be easier to recognize small differences in the relative positioning of spines and extrusions of the femur than to recognize a shift in a whole set of wing vein positions.

To explain the similarity of population and lineage differentiation of wings and femora, it would be necessary to directly assess sexual selection on both traits (Blanckenhorn et al., 2004). At this point, we are not able to distinguish between stabilizing (sexual) selection on either trait versus high levels of gene flow preventing within-lineage population differentiation. Since indirect evidence suggests sexual selection is acting on fore femora (op. cit.), and it is not unlikely that sexual selection on the femur may be stabilizing (if the fore femur is indeed stimulating the female at the wing base: Eberhard, 2002, 2013), stabilizing natural or sexual selection on the wing may occur as well. A study investigating strength and direction of sexual selection on femur and wing shape in these species would therefore be highly informative. Until such direct evidence for selection is presented, the null hypothesis of genetic drift substantially driving differentiation of the three lineages studied here remains intact (Figures 5 and 6).

Our common garden rearing additionally revealed that flies raised at lower temperatures developed larger fore femora, a plastic response in accordance with the temperature–size–rule, which applies to most ectotherms (Atkinson, 1994). In contrast, temperature effects on wing size or shape were largely non-significant, suggesting that femur morphology is more plastic (i.e., less canalized) than wing morphology (Kjærsgaard et al., 2007). Nevertheless, (temperature) plasticity seems slight in comparison with geographic morphological differentiation in the sepsids studied here, thus limiting the scope of alternative mechanisms of (genetic) differentiation or adaptation to explain our results (cf. above).

In conclusion, our study demonstrates significant divergence in wing and foreleg morphology among the two closely related, widespread sepsid fly species *S. cynipsea* and *S. neocynipsea*. Patterns of shape differentiation largely followed the biological species concept, with greater divergence between sister species than between continental *S. neocynipsea* populations, suggesting a primary role of neutral evolution in shaping male femur and wing shape. Nevertheless, based on our results, at least subspecies status of European versus North American *S. neocynipsea* could be warranted. Most crucially, however, our study does not support the often-raised prediction that sexually selected traits (here the male foreleg) evolve faster than naturally selected traits (here the wing; Arnqvist, 1998; Arnqvist & Rowe, 2005). Given that all investigated European *S. neocynipsea* populations are sympatric with *S. cynipsea*, we additionally detected slightly stronger fore femur differentiation in sympatry versus cross-continental allopatry (presumably due to character displacement: Giesen et al., 2017; Giesen et al., 2019). Whether sexual selection acts not only on the male forelegs but perhaps also the male wings remains to be directly assessed.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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APPENDIX 1

Sampling locations, geographic coordinates, and sample sizes of *Sepsis cynipsea* and *Sepsis neocynipsea* used for the microsatellite analysis

Continent	Location	Latitude	Longitude	$N_{\text{individuals}}$
S. neocynipsea				
North America	Tucson, AZ	32.2	−111.1	7
	La Veta, CO	33.7	−117.7	15
	Raleigh, NC	35.9	−78.8	4
	Lexington, KY	38.1	−84.6	20
	Sierraville, CA	39.6	−120.4	8
	Meeker, CO	40.1	−107.9	18
	Fort Hall, ID	43	−112.5	15
	Syracuse, NY	43	−76.1	4
	Lamar Valley, WY	44.9	−110.2	11
	Belgrade, MT	45.8	−111.2	7
Europe	Maggia, CH	46.3	8.7	8
	Geschinen, CH	46.5	8.3	14
	Hospental, CH	46.6	8.6	25
	Oberwald, CH	46.6	8.4	24
	Sörenberg, CH	46.8	8	20
	Zürich, CH	47.4	8.6	6
S. cynipsea				
Europe	Petroia, I	43.2	12.6	18
	Asturias, ESP	43.5	−5.9	16
	Monte Ceneri, CH	46.1	8.9	14
	Maggia, CH	46.3	8.7	16
	Geschinen, CH	46.5	8.3	25
	Lenzerheide, CH	46.7	9.6	25
	Sörenberg, CH	46.8	8	25
	Zürich, CH	47.4	8.6	25
	Nordrach, GER	48.4	8.1	25
	Dillenburg, GER	50.7	8.3	25
	Reading, UK	51.5	−1	17
	Berlin, GER	52.5	13.2	14
	Sheffield, UK	53.4	−1.5	18
	Stirling, UK	56.1	−3.9	23
	Killin, UK	56.5	−4.3	12
	Huddinge, SWE	59.2	17.9	25
	Pehka, EST	59.5	26.3	15

Note: In the analysis presented in the main document, we pooled the *S. cynipsea* populations from Reading and Sheffield (SUK), Stirling and Killin (NUK), and Mt. Ceneri and Maggia (Maggia).

APPENDIX 2

Sampling locations and sample sizes of *Sepsis cynipsea* and *Sepsis neocynipsea* populations used to quantify geographic patterns of morphological differentiation. Microscopy slides for all individuals are stored at the University of Zurich

Continent	Population	Abbreviation	Latitude	Longitude	18°C	24°C	Sampling date	Rearing date
					N_{Lines} (N_{Ind})	N_{Lines} (N_{Ind})		
S. neocynipsea								
North America	Tucson, AZ	AZ	32.1	−110.6	5 (9)	9 (18)	Jun-15	Apr-16
	Lexington, KY	KY	38	−84.5	10 (20)	11 (20)	Jun-12	Jul-12
	Zephyr Cove, NV	NV	39	−119.6	6 (20)	8 (20)	Jun-12	Jul-12
	Syracuse, NY	NY	42.9	−76.9	7 (20)	5 (20)	Jun-12	Jul-12
	Sheridan, WY	WYa	44.5	−106.6	3 (9)	NA	Aug-15	Apr-16
	Lamar Valley, WY	WYb	44.6	−110.5	8 (20)	8 (20)	Jun-13	Jul-13
	Belgrade, MT	MT	45.5	−111.1	4 (10)	4 (10)	Aug-15	Apr-16
	Charlottetown, PEI	PEI	46.2	−63.1	4 (20)	4 (20)	Sep-14	Apr-16
Europe	Maggia, CH	TI	46.3	8.7	6 (12)	6 (12)	May-13	Jul-13
	Hospental, CH	VS	46.5	8.4	5 (18)	7 (20)	Jun-14	Aug-14
	Sörenberg, CH	LU	46.8	8	10 (20)	10 (20)	May-13	Jul-13
S. cynipsea								
Europe	Petroia, I	IT	43.2	12.3	6 (14)	7 (14)	Sep-14	Apr-16
	Maggia, CH	TI	46.3	8.7	5 (11)	9 (16)	May-13	Jul-13
	Sörenberg, CH	LU	46.9	8.3	10 (20)	10 (20)	May-12	Jul-12
	Zürich, CH	ZH	47.2	8.32	3 (20)	3 (12)	May-12	Jul-12
	Reading, UK	SUK	51.3	−0.6	5 (20)	4 (20)	May-13	Jul-13
	Stirling, UK	NUK	56.1	−3.9	7 (20)	6 (20)	May-13	Jul-13
	Pehka, EST	EE	59.5	26.4	6 (14)	7 (12)	May-13	Jul-13

APPENDIX 3

Sample sizes, number of alleles per locus (N_a), mean observed (H_O) and expected (H_E) heterozygosity and global F_{ST}-values at nine microsatellite loci across European populations of *Sepsis cynipsea* and North American as well as European populations of *Sepsis neocynipsea*

	Europe					Europe					North America				
	S. cynipsea (N_{pop} = 17)					*S. neocynipsea* (N_{pop} = 6)					*S. neocynipsea* (N_{pop} = 11)				
Locus	N_{Ind}	N_a	H_O	H_e	F_{ST}	N_{Ind}	N_a	H_O	H_e	F_{ST}	N_{Ind}	N_a	H_O	He	F_{ST}
K55	337	7	.651	.657	.008	97	4	.052	.081	.020	115	3	.035	.043	−.032
K11	331	16	.088	.862	.014***	96	12	.073	.838	−.011	114	6	.079	.239	−.031
J60	328	18	.454	.891	.020***	92	14	.663	.749	.015	115	11	.765	.782	.070***
H94	335	19	.397	.781	.012	96	22	.688	.891	.049***	115	6	.435	.547	.006
H26	337	13	.605	.634	.009*	97	11	.680	.790	.026*	115	10	.548	.678	.067***
G67	331	24	.801	.920	.013***	96	13	.833	.895	.003	114	11	.566	.829	.018
G53	327	74	.801	.975	.004*	81	37	.630	.954	−.005	112	33	.580	.950	.040***
E81N	311	11	.241	.810	.025**	96	12	.490	.872	.027*	113	11	.455	.667	.006
E67	332	12	.214	.303	−.010	96	6	.375	.465	.002	113	19	.717	.900	.032**
Overall	338					97					116				

Note: In total, we sampled 551 individuals of both species and both continents. Nuclear DNA was isolated from entire flies using DNeasy Blood and Tissue Kit (Qiagen AG) following manufacturer's protocol. PCR amplification for nine microsatellite markers was done using the M13-tail PCR method (Schuelke, 2000) described in detail in Greminger et al. (2009). Primers for markers J60, E67, and G53 were developed de novo and are listed above. Amplifications were conducted with 15-min initial denaturation at 95°C, 35 cycles of 30 s denaturation at 94°C, 45 s annealing at 60°C (except for H94 with 56°C and H26 with 54°C), and 45 s at 72°C, followed by eight cycles of 30 s at 94°C, 30 s at 53°C, 45 s at 72°C, and finally ended with a final extension of 30 min at 60°C. Fluorescent-labeled PCR fragments were separated on an ABI Prism 3730 capillary sequencer, and allele lengths were scored using GeneMapper V 4.0 (both Applied Biosystems).* $p < .05$. ** $p < .01$ *** $p < .001$.