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ORIGINAL PAPER

# Fine scale population structure of hoverfly pollinator, *Eristalis arbustorum*: an integrative study

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## Abstract

Determination of the factors influencing the population structure and adaptive tolerance to environmental pressures of the synanthropic hoverfly *Eristalis arbustorum* is of essential importance in understanding how pollinator populations could respond to climate change or ecosystem management. We addressed the issue of connectivity among conspecific populations sampled in Bosnia and Herzegovina. Twenty environmental factors, mitochondrial DNA sequences of the cytochrome *c* oxidase subunit I gene (*COI* mtDNA), allele frequencies at allozyme loci and wing traits (size and shape) were compared for characterization of population structure and environmental niches. Additionally, patterns of within-individual asymmetry (fluctuating asymmetry; FA) in wing size and shape within and among conspecific populations were studied. In line with the overall similarity of the environmental factors extracted for our study sites, the results of *COI* mtDNA diversity and STRUCTURE allozyme data provide evidence for shallow differentiation among conspecific populations. In contrast, georeferenced Bayesian clustering methods (BAPS and GENELAND) and population-based approaches (pairwise $F_{ST}$ values and AMOVA) indicate that the dispersal potential of *E. arbustorum* may be limited across the study area. Along with a significant FA in wing size and shape, a consistent level of FA regardless of urban/rural sampling origin is an indication of the great potential of *E. arbustorum* for local adaptation, because increased FA levels can be considered to be a way of expression of phenotypic variation and, hence, may contribute to adaptive responses in populations facing changing environments. Thus, by using a combined genetic-morphological approach, we significantly contributed to the understanding of the fine-scale genetic structure of the synanthropic generalist pollinator *E. arbustorum*.

**Keywords** Environmental niche characterization · Fluctuating asymmetry · Landscape genetics · Spatial structure · Wing geometric morphometrics

## Introduction

There has been growing concern over many pollinator groups, which are thought to be declining in intensively managed and transformed landscapes (e.g. Powney et al. 2019; Sánchez-Bayo and Wyckhuys 2019). Indeed, multiple studies have pointed out that the number of pollinating insects is continuously declining due to exposure to pesticides, land use changes, decreasing genetic diversity and climate change (e.g. Potts et al. 2010; Ratnieks and Carreck 2010). For instance, in Western Europe, there has been a noticeable decrease in the abundance of *Eristalis* (Diptera, Syrphidae) pollinators during the 1990s, which has been caused by habitat loss and fragmentation, and widespread use of compounds such as systemic helminthicides (González-Varo et al. 2013; Speight et al. 2017). This has great implications for conservation efforts because, along

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with bees, species of the family Syrphidae are considered to be important pollinators in modern landscape systems (e.g. Hennig and Ghazoul 2012; Baldock et al. 2015 Klecka et al. 2018). However, the characteristics of urban areas may be detrimental to most hoverfly species. For instance, a negative response of hoverflies has been reported due to urban land use (Bates et al. 2011; Deguines et al. 2012), indicating that for hoverflies, resources for feed and development may be lacking in urban areas. In contrast, it has been shown that hoverfly species richness in urban areas did not differ significantly to farmland and nature reserves (Baldock et al. 2015). In addition, pollinator richness and specialization were found to increase in urban areas compared to agricultural areas, further implying the importance of conservation of plant and insect communities, as well as flower visitor networks in urban environments (Theodorou et al. 2017). Finally, there is also evidence that habitat edges might act as a barrier to the movement of syrphids between fields. Namely, even for apparently mobile and highly dispersive insects such as hoverflies, field boundaries may restrict their movement on a finer landscape scale (Wratten et al. 2003). However, a molecular study provided evidence of local adaptation to urbanization and an overall lack of genetic differentiation among urban populations of the highly mobile pollinator *Bombus lapidarius*, suggesting persistent gene flow (Theodorou et al. 2018).

Hence, to contribute to our understanding of a species important for maintaining pollination services, we performed population genetic and spatial analyses of *Eristalis arbustorum* (Linnaeus, 1758) (Supp. Fig. S1), a widespread and relatively efficient pollen disperser of 65 plant taxa (Lucas et al. 2018), including: *Senecio inaequidens* (invasive) and *S. jacobaea* (native); Asteraceae (Vanparys et al. 2008); the crops *Brassica napus* (Stanley et al. 2003) and *Paeonia lactiflora* (Yu and Qiang 2004); and low-growing plants and shrubs (De Buck 1990). Because of its easy availability, abundance, high dispersal abilities (Gatter and Schmid 1990), and potential for long-distance movement (Speight et al. 2017), it represents a highly suitable synanthropic species for research on foraging activities in farmland, urban parks and gardens, wetlands and alluvial softwood forests (Speight et al. 2017; Lucas et al. 2018). The flight period of *E. arbustorum* extends from March into autumn, and rat-tailed larvae (larvae have a long, telescopic, three-segmented respiratory tube at their posterior end by which they breathe at the water surface) have been found in a variety of areas in organically-rich, shallow, standing water, as well as in cow-dung and silage pits etc. (Speight et al. 2017). Thus, here we address the issue of connectivity among *E. arbustorum* populations by integrating genetic and phenotypic data.

First, based on 20 environmental factors, we aimed to characterize the environmental niches of the studied samples from three sites in Bosnia and Herzegovina, which have been gradually urbanized: from the most natural (Pljeva) to Banja Luka and finally Prijedor as the most urbanized city. We choose to study urban areas because they are considered to be the most pervasively changed landscapes. In addition, land use change from natural habitats to human-managed landscapes is generally perceived as having a negative impact on wildlife (Jones and Leather 2012). Specifically, the massive disturbances created by city growth not only destroy the habitat of native species but also create habitat for the relatively few species that are adapting to urban and suburban conditions. This process of replacing localized native species with increasingly widespread non-native species promotes biotic homogenization on several spatial scales (McIntyre 2000; McKinney 2004). Within an urban environment, the maintenance of viable populations of flowering plants may therefore depend on insect pollinators that the urban environment supports. However, it should be taken into account that urban habitats are becoming a refuge for insect pollinators, since they offer better, more hospitable conditions (e.g. lower pesticide use, a variety of forage and nesting sites in the cities) than rural ones (Hall et al. 2017).

Our second aim was based on the fact that a reliable estimate of gene flow in a given landscape is the ultimate measurement of landscape connectivity, and genetic tools allow such estimates through space and time (e.g. Segelbacher et al. 2010). Hence, we address the question of landscape connectivity using molecular (mitochondrial cytochrome *c* oxidase I gene—*COI* mtDNA and allozyme loci) and phenotypic data (wing size and shape) on the spatial and dispersal patterns of hoverflies. We are interested in whether samples of *E. arbustorum* from localities with different levels of urbanization in Bosnia and Herzegovina can be genetically and phenotypically differentiated using nuclear and mitochondrial DNA markers and wing geometric morphometrics. Regarding the complex landscape of the studied samples, we hypothesized that there is a population structure of this important beneficial species.

The third aim was to study asymmetry in wing traits. Namely, genetic (a high level of homozygosity and disruption of co-adapted gene complexes) and/or extreme environmental conditions and/or environmental deterioration (Palmer and Strobeck 1992; Willmore et al. 2007) experienced by individuals during larval development can influence developmental precision, resulting in suboptimal phenotypes, which, consequently, will result in asymmetry in bilaterally symmetrical organs (such as wings) (Van Dongen 2006). A frequently used metric of developmental instability is fluctuating asymmetry (FA; non-directional subtle differences between the left and right sides of bilaterally symmetrical traits), which is considered to be the only form of asymmetry that can serve as a useful and reliable indicator of individuals exposed to

environmental/genetic stress (Palmer and Strobeck 1986; Leary and Allendorf 1989). In fact, studies suggest FA level disparity exists across species, traits and stressors (Beasley et al. 2013). Given that trait asymmetry has been shown to be a measure of stress and fitness (Lens et al. 2002a; Van Dongen et al. 2009), and related to the potential for adaptive evolution in hoverflies (Ludoški et al. 2012), differences in the levels and patterns of FA might be expected in *E. arbustorum* as well. Because of this, we hypothesized that if FA is a stress "indicator", levels of FA would be higher in the most urbanized city (Prijedor) compared to the most natural locality (Pljeva). To test this hypothesis, we assessed, for the first time, patterns of within-individual asymmetry (FA), within and among conspecific populations.

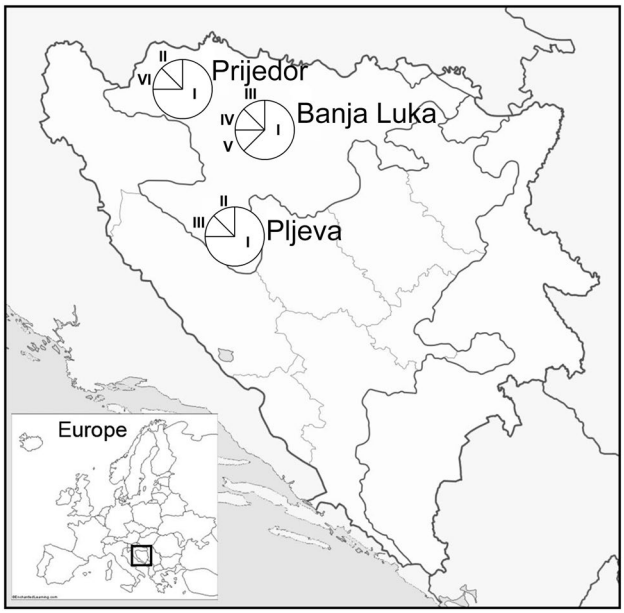


**Fig. 1** Map of *Eristalis arbustorum* sampling locations in Bosnia and Herzegovina and *COI* mtDNA haplotype distribution. The size of each pie slice represents the number of individuals with that haplotype

## Materials and methods

### Sample collection and study sites

Samples of 131 flies were collected during the entire active season, from May to October in 2012, from three localities in Bosnia and Herzegovina (Collectors Milankov V. and Lukač M.) (Fig. 1; Table 1). Specimens were identified based on the morphological characters of adults as defined for *E. arbustorum* (Hippa et al. 2001). The study area is crisscrossed by a network of roads, various watercourses and settlements, and agriculture and forestry are the main human, non-industrial activities in the region. We selected two urban sampling sites (Prijedor and Banja Luka) and one rural site (Pljeva), separated by a distance of 45 (Prijedor-Banja Luka), 59 (Banja Luka-Pljeva) and 85 km (Prijedor-Pljeva) (Supporting Information).

Adult flies were collected by hand netting while they were feeding on flowers or resting on the bare ground or vegetation, and frozen live for subsequent morphological and genetic analyses. Allozyme variation, mtDNA *COI* sequences and differences in wing traits were analyzed using the same individuals. To date, both individual- and population-based genetic analyses have been used to understand the spatial and dispersal patterns of hoverflies (Milankov et al. 2013; Francuski and Milankov 2015). These studies have shown that the mitochondrial cytochrome *c* oxidase I gene (*COI* mtDNA) and allozyme loci are highly informative and useful tools for studying intraspecific spatial heterogeneity on Syrphidae (e.g. Milankov et al. 2009; Ståhls et al. 2016). We also used wing geometric morphometrics as a complementary tool to genetic data to assess the population structure. It is considered to be an effective approach that can infer recent population events (e.g. migration; Dujardin 2011) and has been shown to be capable of detecting population heterogeneity and structuring for very close populations in urban areas (e.g. Carvajal et al. 2016). Over the past decade, wing geometric morphometrics have also been utilized as an alternative technique to determine population structures in hoverflies (e.g. Francuski et al. 2009; Milankov et al. 2013), yielding results that are consistent with hypotheses based on molecular data (Ludoški et al. 2008; Francuski

**Table 1** The number of *Eristalis arbustorum* individuals sampled from three localities in Bosnia and Herzegovina, and assayed by wing geometric morphometrics, mtDNA and allozyme analysis

| Population | Longitude and latitude | mtDNA sequencing | Allozyme analysis | Wing traits | | Allozyme + wing analysis | |
|---|---|---|---|---|---|---|---|
| | | | | ♀ | ♂ | ♀ | ♂ |
| Prijedor | 44° 58′ N 16° 42′ E | 8 | 34 | 16 | 18 | 16 | 18 |
| Banja Luka | 44° 46′ N 17° 11′ E | 8 | 35 | 36 | 27 | 20 | 13 |
| Pljeva | 44° 14′ N 17° 02′ E | 8 | 35 | 17 | 17 | 17 | 17 |
| Total | | 24 | 104 | 69 | 62 | 53 | 48 |

et al. 2014). Specifically, because it has been shown that wing shape exhibits a high level of heritability (Bitner-Mathé and Klaczko 1999), the distribution of phenotypic variations across and within populations likely correlates with patterns of genetic variation.

## DNA extraction and amplification

Legs of 24 specimens that were previously used for allozymic and wing morphometric studies were also used for DNA sequencing (Table 1). DNA was extracted from the legs of fly specimens using a Nucleospin Tissue DNA extraction kit (Machery-Nagel, Düren, Germany) following the manufacturer's protocols and then re-suspended in 50 µl of ultra-pure water. Remains of specimens were deposited at the Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad (Novi Sad, Serbia).

PCR and sequencing followed protocols described in Milankov et al. (2009). Universally conserved primers for amplifying and sequencing the cytochrome oxidase *c* subunit I mitochondrial DNA (*COI*) fragment were C1-J-2183 (5′-CAA CAT TTA TTT TGA TTT TTT GG-3′) (alias JERRY), TL2-N-3014 (5′-TCC AAT GCA CTA ATC TGC CAT ATT A-3′) (alias PAT) (Simon et al. 1994). Amplification products were checked for the expected product size by standard 1.5% agarose gel electrophoresis. The remaining product was purified using Exonuclease I and Shrimp Alkaline Phosphatase enzymes according to the manufacturer's instructions (Fermentas). All sequencing reactions were performed using the Big Dye Terminator Kit v. 3.1. (Applied Biosystems) according to the manufacturer's protocol, and sequences were generated on an ABI 3730xl DNA Analyzer (Applied Biosystems). All analyzed hoverflies were bi-directionally sequenced using the same primers used for amplification. Sequences were deposited in GenBank, under accession numbers MF510195-MF510218. Chromatograms obtained by mtDNA sequencing were edited in Chromas 2.6 (Tehnelysium Pty Ltd) for erroneously called bases, while sequence alignment and identification of variable positions and haplotypes was performed in BioEdit 7.2.5 (Hall 1999).

## Allozyme analysis

A total of 104 specimens (Table 1) were included for allozyme analysis by vertical polyacrylamide gel electrophoresis. Allozyme polymorphism was studied at five different loci: aldehyde oxidase (1.2.3.1. AO; *Ao*), aspartate amino transferase (2.6.1.1. AAT; *Aat*), β-hydroxy acid dehydrogenase (1.1.1.30. HAD; *Had*), malate dehydrogenase (1.1.1.37. MDH; *Mdh*-2) and malic enzyme (1.1.1.40. ME; *Me*). A trisborate-EDTA acid buffer system (pH 8.9) was used to assay AO and ME, whereas a tris-citrate buffer system (pH 7.1) was used for analysis of AAT, HAD and MDH. Details of buffer systems and staining procedures are given in Munstermann (1979) (HAD, MDH and ME) and Pasteur et al. (1988) (AO and AAT) and running conditions are described in detail in Francuski et al. (2011). Specimens from all samples were analyzed concurrently on all gels to facilitate comparison of electrophoretic mobility. Allele frequencies were calculated directly from observed banding patterns based on the genetic interpretation of zymograms (Supporting Information Table S1).

## Bayesian genotypic clustering techniques

For tests considering the population structure of *E. arbustorum* in the study area we applied three different programs using Bayesian approaches. Each of these Bayesian methods estimates genetic structure based on different assumptions of theoretical inheritance and from multi-locus genotypes without assuming pre-defined populations.

Firstly, the clustering program STRUCTURE 2.3.4 software (Pritchard et al. 2000) was implemented in individual-based allozyme analysis. The correlated allele frequencies model and the admixture model was selected with a burn in period of 100,000 and sampling from 100,000 Markov chain Monte Carlo steps. To check the consistency of results between runs with the same $K$, ten runs were performed for each value of $K$. We estimated the number of clusters and the assignment of individuals into clusters using two methods: (1) the most likely number of clusters was estimated by determining the change in the marginal likelihood of the data $Pr(X|K)$ when the numbers of clusters ($K$) was fixed to different values; (2) the $\Delta K$ method sensu Evanno et al. (2005) was implemented to detect the amount of structuring. $\Delta K$ is the second-order rate of change of the marginal likelihood function, and takes into account both the gain in posterior probabilities over a range of $K$-values and the variance between independent runs at given values of $K$. Therefore, $\Delta K$ cannot be calculated for $K = 1$ (in our data $K$ is likely to equal one, see "Results"). Results of all runs were summarized using STRUCTURE HARVESTER, version 0.6.92 (Earl and vonHoldt 2012). For the optimal value of $K$ over the ten independent runs, we calculated the mean coefficient membership to each cluster per locality and the proportion of individuals per locality assigned to each cluster (i.e.: individuals whose coefficient membership to a particular cluster is at least 80%).

Secondly, we used the Bayesian clustering method implemented in the program BAPS 6.0 (Corander et al. 2004, 2008). This method enables a more hierarchical analysis by treating the partition among groups of individuals as the main parameter of interest. It treats both allele frequencies of allozyme loci and the number of genetically diverged groups in a population as random variables, and uses stochastic optimization to infer the posterior mode of the genetic structure.

As results suggested small, but significant differences among *E. arbustorum* samples, we performed the genetic mixture analysis at the level of populations instead of individuals ("spatial clustering of groups"). Namely, the statistical power to correctly detect the underlying population structure is increased by conditioning on the sample groups when it is biologically feasible (Corander and Marttinen 2006). When the molecular information is weak, e.g. the number of available marker loci is small or the markers have low levels of polymorphism, inferences can be strengthened by utilizing the sample design information in the prior specification as in Corander et al. (2003). BAPS was run with the maximal number of groups (*K*) set to 2 and 3. Each run was replicated ten times, and results were averaged according to resultant likelihood scores.

To estimate potential locations of discrete population structure in the allozyme data, we also applied the Bayesian clustering algorithm employed in GENELAND 4.0.3 (Guillot et al. 2005), implemented in R software 3.4.3 (R Development Core Team 2017), which, like BAPS, takes into account the spatial location of sampling sites and estimates the optimal number of population clusters. We ran ten replicates for two million iterations (thinning = 200) with the number of possible clusters set to 2 and 3 and checked for consistency. We processed a final run on a landscape of 100 × 100 cells and with a burn-in of 2000 iterations, fixing the maximum *K* to two, the value with the highest posterior density from all preliminary runs (e.g. Leaché 2011). GENELAND incorporates spatial data directly under the assumption that populations are spatially organized. However, this model does not assume admixture and any genetic boundaries found are assumed to separate *K* random mating populations (Guillot et al. 2005; Manel et al. 2007). Improvements to the model suggest the ability to increase the detection of clusters having low genetic differentiation (Guillot et al. 2008). The dataset, consisting of 104 specimens genotyped at allozyme loci and their morphometric data (individual CVA scores), was analyzed using data combinations of phenotypic and genetic data under the spatial model. Ten independent Markov chain Monte Carlo runs of 100,000 iterations were performed, discarding the first 10,000 iterations as burn-in (Guillot et al. 2005, 2008).

The software Alleles in Space (AIS) was used to detect barriers to gene flow between sampled locations (Miller 2005) using both allozyme data and *COI* mtDNA haplotypes. This procedure locates barriers to gene flow by iteratively identifying sets of contiguous, large genetic distances along a connectivity network. The steps of this analysis consist of the following: (1) connecting adjacent geographical positions of individuals using Delaunay triangulation (Brassel and Reif 1979; Watson 1992), resulting in a connectivity network; (2) calculating genetic distances between neighbouring samples and associating these distances to each edge of the network; and (3) using Monmonier's maximum difference algorithm to identify boundaries, as described in detail elsewhere (Monmonier 1973; Manel et al. 2003).

## Population-based approaches

For population comparisons, we employed Wright's *F*-statistics (Wright 1951; Conner and Hartl 2004) to measure genetic differentiation and index the extent of gene flow among populations. $F_{ST}$ is a widely used, low variance measure that performs better than other estimates of population structure when the number of loci and the number of samples are low (Gaggiotti et al. 1999), as is the case in this study. The pairwise $F_{ST}$ values between populations (for both allozyme and *COI* mtDNA data) were calculated by Arlequin 3.5 (Excoffier and Lischer 2010) and their P-values calculated with 10,000 permutations.

For both molecular markers, allozyme and *COI* mtDNA, analysis of molecular variance (AMOVA, Excoffier et al. 1992) was also performed using the Arlequin 3.5 software. Ten thousand permutations were used to determine significance of variance components.

## Wing geometric morphometrics

To study variations in wing size and shape by applying geometric morphometrics, both the left and right wings of 131 specimens (Table 1) were removed and mounted in Hoyer's medium between microscope slides, and digital images were taken with a Leica DFC320 camera connected to a Leica MZ12.5 stereomicroscope. A set of 16 landmarks positioned at vein intersections or terminations were collected using TpsDig 2.26 (Rohlf 2016) and expressed as *x,y* coordinates in Cartesian space (Fig. 2). Each wing was digitized two times by the same person. Because this species exhibits sexual dimorphism (see "Results"), wing trait analyses were performed separately for males and females.

Raw landmark coordinates were superimposed using a full Procrustes fit procedure (Dryden and Mardia 1998;

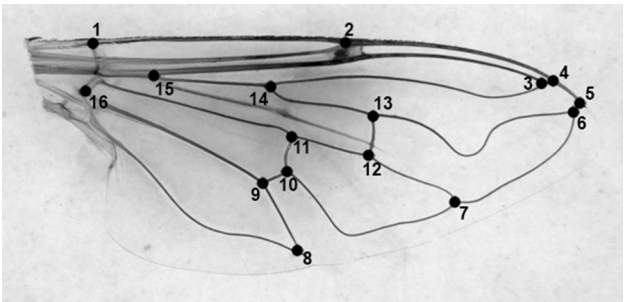


**Fig. 2** The locations of 16 landmarks selected for geometric morphometric analysis of *Eristalis arbustorum*

Klingenberg and McIntyre 1998), and centroid size (CS) and shape information (Procrustes coordinates) were extracted. Procrustes fits were performed on the whole data set (females and males pooled in the same file). Both centroid size and Procrustes coordinate data were subjected to a Grubbs test for outlier detection (http://www.graphpad.com/quickcalcs/Grubbs1.cfm), which revealed the absence of outliers in our data set. Because they are bilaterally symmetrical organs, wings have matched symmetry, and therefore the total shape variation of landmark configurations comprises two components of variation: a symmetric component and asymmetry, which can be partitioned and analyzed separately (Klingenberg et al. 2002). Given that each individual is represented with landmark configurations of the left and right wing, the symmetric component includes among-individual variation in the average of right and left side configurations, while the asymmetry component represents within-individual variation expressed as differences between the right and left wing (Klingenberg et al. 2002).

To examine individual variation (regardless of asymmetry) in wing size and shape, centroid size and Procrustes coordinates obtained by averaging left and right side values within each individual were used (symmetric component of variation; Klingenberg et al. 2002). Non-parametric ANOVA on centroid size (One-way PERMANOVA in Past) was used to test for differences in wing size between populations. Pairwise comparisons between all samples were performed with a permutational test (9999 replicates) followed by Bonferroni correction. Multivariate regression of Procrustes coordinates against centroid size on pooled within-group (pooled by population) variation was used to assess allometric effects of size on shape variation. Interpopulation wing shape variation was analyzed using canonical variate analysis (CVA). Pairwise differences were quantified using Procrustes distances and compared with a permutation test with 10,000 iterations.

Wing size and shape asymmetry was investigated following procedures described in Klingenberg and McIntyre (1998) and statistical analysis detailed in Ludoški et al. (2012, 2014). To evaluate the contribution of individual variation, fluctuating asymmetry (FA), and measurement error on the overall variation, Procrustes ANOVA on centroid size and Procrustes coordinates for the complete data set (with population and sex as the additional fixed effects) and separate ANOVAs for each population of both sexes were performed. Data were also checked for antisymmetry (deviation from symmetry toward either the right or left side) using the Kolmogorov–Smirnov test and an absence of antisymmetry was found. Levene's test of homogeneity of variances was applied to explore differences between populations in the amounts of FA in size and shape. To evaluate whether size has an effect on shape asymmetry (an allometric effect) multivariate regressions of shape FA on size FA estimates were performed. To compare patterns of the shape FA variation matrix correlation between covariance matrices (calculated both with and without diagonal blocks of the covariance matrices) was used. The null hypothesis of complete dissimilarity of matrices was tested with 10,000 random permutations of landmarks. Additionally, principal component analysis (PCA) on the asymmetric component of shape variation was performed to visualize the pattern of variation of asymmetry in the positions of landmarks throughout the wing.

All statistical analyses were done using MorphoJ version 1.06d (Klingenberg 2011) and PAST (Paleontological Statistics) version 3.20 (Hammer et al. 2001) software.

### Characterization of environmental niches

The geographical coordinates of the populations were imported into DIVA-GIS 7.5 software to extract the environmental factors for each site (Hijmans et al. 2001). Environmental data sources were retrieved from the WorldClim Global Climate database at a 30-s resolution (Hijmans et al. 2005). This data source, with 20 environmental factors, contains annual trends, annual seasonal trends, seasonality and extreme or limiting environmental factors, and includes elevation (m), annual mean temperature (all temperatures in °C), mean monthly temperature range, isothermality, temperature seasonality, maximum temperature of the warmest month, minimum temperature of the coldest month, annual temperature range, mean temperature of the wettest quarter, mean temperature of the driest quarter, mean temperature of the warmest quarter, mean temperature of the coldest quarter, annual precipitation (all precipitation levels in mm), precipitation of the wettest month, precipitation of the driest month, seasonal precipitation, precipitation of the wettest quarter, precipitation of the driest quarter, precipitation of the warmest quarter and precipitation of the coldest quarter. All environmental data were log10-transformed to eliminate dimensional differences among the data (Luxbacher and Knouft 2009). Then, we performed principal components analysis (PCA) in PAST version 3.20 (Hammer et al. 2001) on the environmental data to characterize the environmental niche (i.e., the environmental space) occupied by each population (Knouft et al. 2006; Bai et al. 2016).

## Results

### Genetic variation among populations

A total of 24 sequences, each containing 779 base pairs of the *COI* mtDNA fragment, were obtained for individual specimens of *E. arbustorum*. Five nucleotide sites were found to be variable in the region sequenced, resulting in

**Table 2** Pairwise $F_{ST}$ estimates among populations of *Eristalis arbustorum* included in the study performed on allozyme (upper matrix) and *COI* mtDNA data (lower matrix). Shaded boxes indicate significant (P < 0.05) pairwise comparisons

| Population | Prijedor | Banja Luka | Pljeva |
|---|---|---|---|
| Prijedor | – | 0.025 | 0.157 |
| Banja Luka | 0.048 | – | 0.109 |
| Pljeva | –0.067 | 0.006 | – |

**Table 3** AMOVA results for *Eristalis arbustorum* samples performed on allozyme and mtDNA data

| Source of variation | Sum of squares | Variance components | Percentage of variation |
|---|---|---|---|
| mtDNA | | | |
| Among populations | 0.667 | 0.001 | 0.46 |
| Within populations | 6.750 | 0.321 | 99.54 |
| Total | 7.417 | 0.322 | |
| Fixation Index $F_{ST}$ = 0.005; P = 0.221 | | | |
| Allozymes | | | |
| Among populations | 12.436 | 0.082 | 13.25 |
| Within populations | 110.007 | 0.537 | 86.75 |
| Total | 122.442 | 0.619 | |
| Fixation Index $F_{ST}$ = 0.132; P < 0.001 | | | |

six haplotypes (HI-HVI). All populations sampled share a dominant haplotype HI. Haplotype II was shared by specimens from Prijedor and Pljeva, while HIII was found in both Banja Luka and Pljeva samples. HVI was found only in the Prijedor population, while HIV and HV were specific to the Banja Luka population (Fig. 1). In spite of private haplotype records, all pairwise $F_{ST}$ values were non-significant and below 0.05 (Table 2) indicating that the populations represent a homogeneous group across the study area. Moreover, AMOVA of *COI* mtDNA corroborated $F_{ST}$ estimates, and showed no significant difference among samples (Table 3).

Individual-based allozyme analysis performed on the software STRUCTURE supports the existence of a unique genetic cluster in the *E. arbustorum* data set. Namely, most of the individuals per locality could not be assigned to a particular cluster (i.e. their highest membership coefficient to a particular cluster was lower than 80%). Thus, STRUCTURE analysis resulted in no underlying genetic groups in the data set corresponding to a urban/rural sample division (Supp. Fig. S2). The $K$ value that best fit our genetic data detected using the highest likelihood method was $K = 1$ (Supp. Fig. S3; empirical and simulation evidence suggests that a biologically meaningful number of $K$ may be indicated by a declining rate of increase in $\Pr(X|K)$ as $K$ increases, rather than by the absolute maximum likelihood; Pritchard et al. 2000; Evanno et al. 2005). Resolving Evanno's $\Delta K$ through our STRUCTURE data analyses, we found that use of the $\Delta K$ method (Evanno et al. 2005) was not applicable because in our data $K$ was likely to equal one. Namely, $\Delta K$ value represents the second-order rate of change of the likelihood functions with respect to $K$ and therefore cannot be calculated for $K = 1$ (Evanno et al. 2005).

In addition, a population-based approach was used to gain insight into spatial patterns of population connectivity based on allozyme data. Estimated pairwise $F_{ST}$ values ranged between 0.025 and 0.157 and were significantly greater than zero for Pljeva/Banja Luka ($F_{ST} = 0.109$; P = 0.011) and Pljeva/Prijedor ($F_{ST} = 0.157$; P = 0.008) sample pairs (Table 2). In addition, the AMOVA results based on allozyme data revealed that most of the total variation occurred within populations (86.75%). The remainder of the variation (13.25%) was significantly different from zero ($F_{ST} = 0.132$; P < 0.001) and resulted from differences among populations (Table 3).

## Phenotypic variation within and among populations

In total 131 specimens (69 females and 62 males) from three localities were used to assess wing size and shape variation. Procrustes ANOVA performed on the whole sample of individuals showed statistically significant among-individual and within-individual (FA) variation for both wing size and shape (Table 4). Also, additionally tested effects revealed significant intersexual and interpopulational differences for both wing traits (Table 4). Therefore, further analyses on wing size and shape variation were performed for each sex separately. Comparing mean square values it was revealed that measurement errors for wing size and shape were negligible (Table 4).

To explore whether among-individual variation differs between populations, symmetric components of variation (symmetric centroid size and symmetric component of shape) were used. Non-parametric ANOVA on centroid size revealed significant interpopulation difference for females (F = 4.91, P = 0.01), but not for males (F = 3.03, P = 0.06). In both sexes, pairwise comparisons assessed by the permutational test followed by Bonferroni correction showed significant differences for the Banja Luka/Pljeva pair (female: P < 0.01, male: P < 0.05) (Supp. Fig. S4). The mean centroid size was 4.72% (females) and 3.88% (males) larger in Banja Luka than in Pljeva. Multivariate regression of symmetric shape variables on symmetric centroid size was significant (P < 0.001) and accounted for 7.16% and 4.99% of the overall shape variation in females and males, respectively. To remove allometric effects, size-corrected shape variables (the residuals from regressions) were used in subsequent shape analyses. Canonical variate analysis revealed differences in wing shape among populations and

**Table 4** Procrustes ANOVA of centroid size (CS) and wing shape (SH) for *Eristalis arbustorum*

| | Effect | SS | MS | df | F | P |
|---|---|---|---|---|---|---|
| CS | Population | 783,345.78 | 391,672.89 | 2 | 8.59 | 0.0003 |
| | Sex | 196,507.49 | 196,507.49 | 1 | 4.31 | 0.0399 |
| | I | 5,790,860.94 | 45,597.33 | 127 | 593.33 | <0.0001 |
| | S | 1463.19 | 1463.19 | 1 | 19.04 | <0.0001 |
| | I×S | 9990.42 | 76.85 | 130 | 52.48 | <0.0001 |
| | E | 383.67 | 1.46 | 262 | | |
| SH | Population | 0.01119701 | 0.00019995 | 56 | 3.61 | <0.0001 |
| | Sex | 0.06525149 | 0.00233041 | 28 | 42.07 | <0.0001 |
| | I | 0.19700168 | 0.00005540 | 3556 | 15.12 | <0.0001 |
| | S | 0.00071005 | 0.00002536 | 28 | 6.92 | <0.0001 |
| | I×S | 0.01333287 | 0.00000366 | 3640 | 17.43 | <0.0001 |
| | E | 0.00154146 | 0.00000021 | 7336 | | |

We presented sum of squares (SS), mean squares (MS), degree of freedom (df), F and P-values for the random effect "Individuals" (I), fixed effect "Side" (S) and "Individuals × Sides" interaction (I × S) which assesses fluctuating asymmetry

pairwise comparisons assessed by the permutational test on Procrustes distances showed that Banja Luka mean shape configurations were significantly different from Pljeva in both sexes (Fig. 3, Supp. Table S2; female: Procrustes distance = 0.0084, $P < 0.05$; male: Procrustes distance = 0.0082, $P < 0.05$). Namely, in females, along the first canonical variable (CV1) which accounted for 67.3% of total shape variation, Pljeva was separated from the other two populations, while Banja Luka and Prijedor were partially separated along CV2 (32.3%). In males, canonical axes accounted for 68.9% (CV1) and 31.1% (CV2) of total shape variation, and contributed to partial separation of the analyzed populations. In females, all but landmark 13 contributed to shape differences between the populations, while in males displacement of landmarks 1, 12, 13 and 15 had the largest contribution (Fig. 3).

For within-individual variation, Procrustes ANOVAs on wing size and shape (asymmetric component of variation) performed for each population separately revealed statistically significant variation due to FA in both wing traits (Supp. Tables S3 and S4). Levene's test of variances indicated that differences in the amount of size and shape FA among populations were not significant for both females (size FA: $F_{(2,66)} = 1.78$, $P = 0.18$; shape FA: $F_{(2,66)} = 1.31$, $P = 0.28$) and males (size FA: $F_{(2,59)} = 0.98$, $P = 0.38$; shape FA: $F_{(2,59)} = 1.19$, $P = 0.31$). Multivariate regression of shape asymmetry on size FA was performed for each analyzed population and non-significant allometry was found. With regard to patterns of shape FA variation among populations, comparison of covariance matrices revealed a significant matrix correlation for all population pairs in both analyses. The matrix correlation values in females ranged from 0.80 to 0.82 and from 0.54 to 0.65 with and without the diagonal block, respectively, and in males from 0.61 to 0.75 and from 0.36 to 0.48 with and without the diagonal block, respectively (for all comparisons $P < 0.0001$) (Supp. Tables S5 and S6). PCA showed that most variation was concentrated in a few dimensions with about a third of the total variances (31–44%) accounted for by the first PC. PC plots display quite similar patterns of asymmetry variation in the positions of landmarks throughout the wing; namely, displacement of landmarks at the wing base (landmarks 1, 15, 16) and landmarks 2 and 8 mostly pertaining to PC1 (Fig. 4).

## Spatial structure of genetic and phenotypic variation

Spatial Bayesian clustering analysis with BAPS gave a probability of 98.1% that there are two genetic clusters in the study area (Fig. 5). One cluster consisted of Prijedor and Banja Luka, while the rural sample (Pljeva) represented its own genetic unit, Cluster 2. Similarly, Bayesian analysis performed on the software GENELAND supports the existence of two unique genetic clusters (not shown). The final Bayesian implementation was performed using GENELAND on geo-referenced phenotypic and genetic data. The estimated number of clusters was two across the ten independent runs. From this, integrated genetic and morphometric data suggested groupings of samples from Prijedor and Banja Luka (Cluster 1) and Pljeva (Cluster 2) clusters (Fig. 5).

To identify potential barriers to dispersal within the study area, we used the implementation of Monmonier's algorithm in AIS. Visualization of the spatial arrangement of *COI* mtDNA haplotypes revealed a geographical barrier to gene flow, which separates the Banja Luka population from the other two samples. On the other hand, Monmonier's algorithm using allozyme genotypes suggest the separation of Pljeva (Fig. 6).

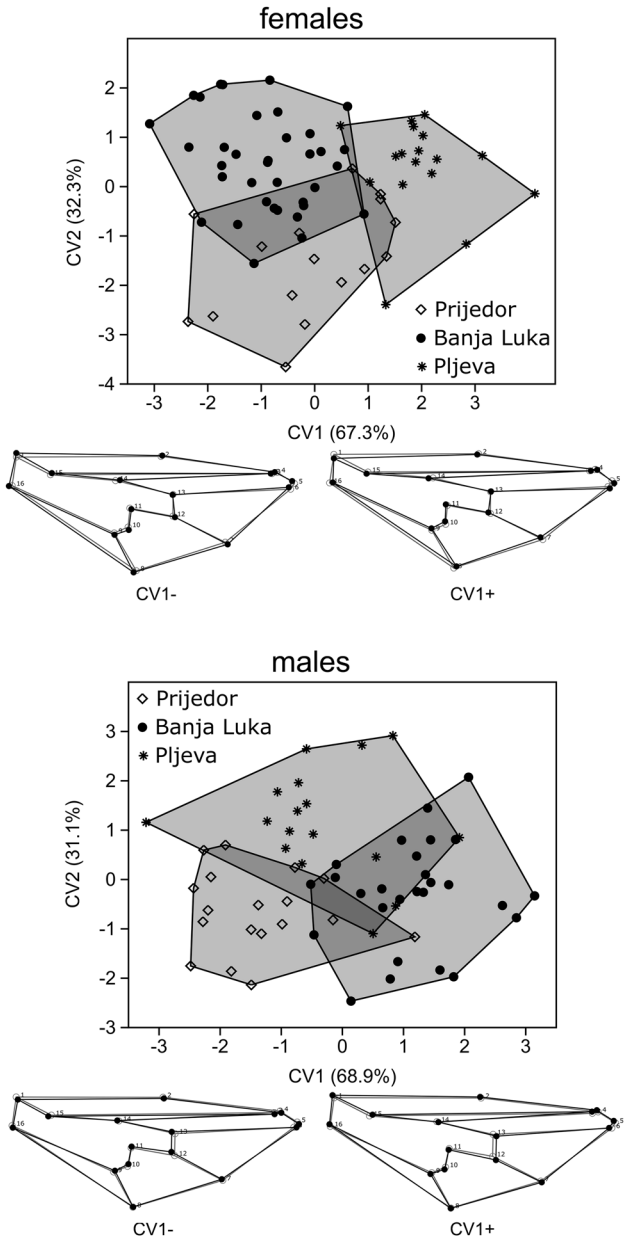


**Fig. 3** Scatterplots of individual scores for the first two canonical variates (CV1 and CV2) with the percentage of explained variance in parentheses. Wireframe visualisation of the average wing shape for populations of *Eristalis arbustorum* showing shape changes (black lines) from the consensus configuration of landmarks (grey lines) to each negative (CV1 −) and positive (CV1 +) extreme along the first canonical axis

### The influence of ecological parameters at population differentiation

In order to provide ecological insights into population differentiation, we characterized environmental niches in the studied populations originating from urban and rural environments. The PCA method was used to analyze 20 environmental factors associated with *E. arbustorum* populations. The two PCs, explaining 85.8% and 14.2% of the total variation, illustrate the low overall impact of environmental factors (Table 5). For PC1, all factors had loadings below 0.7 thresholds, while for PC2, the only factor with a relevant coefficient greater than 0.7 was the mean temperature of the wettest quarter (Bio 8) (Table 5). Therefore, the observed ecological uniformity of the collecting sites had no significant influence on the observed genetic and phenotypic diversity among urban and rural populations.

## Discussion

With respect to our first goal, in the present study we observed a lack of significant difference in ecological parameters among collecting sites using 20 environmental factors. However, it is important to highlight two points: first, we analyzed three populations from geographically close localities (45–85 km); and second, environmental factor uniformity presents averaged values over a longer period and may not reflect ecological factor variations for the stage of the season and the particular year of study. Bearing in mind that urbanization is coupled with a temperature increase in cities (Oke 1982; Arnfield 2003), such as is the case in this study (Banja Luka and Prijedor cities), additional studies should consider longer-term experimental designs incorporating multiple seasons, denser sampling and precise measurements of ecological factors. Indeed, it has been previously suggested that the daytime temperature (indicated by the maximum temperature) is an important factor influencing *E. arbustorum* phenotype, activity and probably fitness (Ottenheim et al. 1999). In addition, plasticity in thorax length, abdominal color patterns and pupal development time were a means of adjusting the *E. arbustorum* phenotype to prevailing day-time conditions (Heal 1981; Ottenheim et al. 1996).

In line with our second goal, *COI* mtDNA intraspecific analysis revealed non-significant pairwise $F_{ST}$ values, where less than 1% of the variation was partitioned among populations (AMOVA analysis). These findings were further supported by the high degree of admixture revealed by allozyme-based clustering analysis in STRUCTURE. However, Bayesian implementation on geo-referenced data (BAPS and GENELAND) and population-based analyses of allozyme data ($F_{ST}$ parameter and AMOVA) showed sample division. In this sampling scheme, separation of Banja Luka (based on the spatial arrangement of *COI* mtDNA haplotypes) and Pljeva (based on allozyme genotype data) were revealed based on Monmonier's maximum difference algorithm and confirmed the presence of genetic discontinuities among the samples. However, a previous study with the same sampling design and allozyme methodology revealed stronger homogeneity of another highly dispersive *Eristalis*


**Fig. 4** PCA of variation in landmark positions for fluctuating asymmetry in females and males of the *Eristalis arbustorum* populations. The diagram visualizes PC coefficients of each landmark in the *x* and *y* directions as lines originating at the mean location of the landmark (dots). The lengths of the lines correspond to a shape change of 0.1 Procrustes units

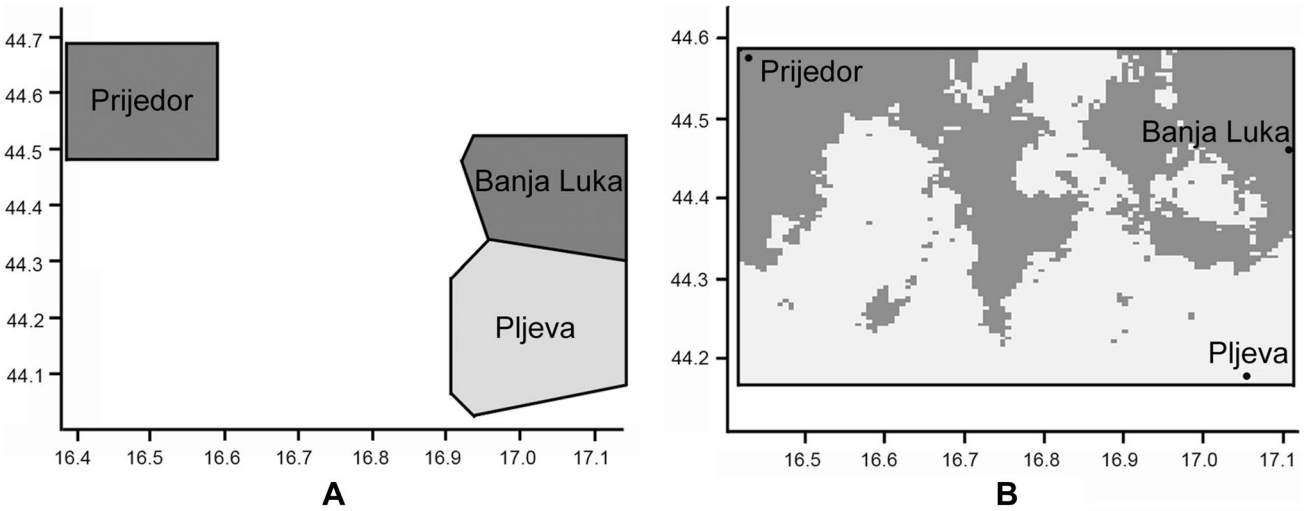


**Fig. 5** Spatially derived genetic clusters based on allozyme data of *Eristalis arbustorum* inferred using the program GENELAND (**a**) and BAPS (**b**). The different colors of sample plots indicate heterogeneous genetic composition: dark gray—Prijedor and Banja Luka, and light gray—Pljeva

species, *E. tenax* (Francuski and Milankov 2015). However, it must be kept in mind that results from the present study represent data from only three populations.

Moreover, in both genders, Banja Luka individuals had considerably larger wings than specimens from the rural Pljeva sample, which resulted in significant differentiation between these two samples. Hence, results obtained by using wing size geometric morphometrics are in agreement with genetic data and the spatial patterns of molecular and phenotypic variation (BAPS) that differentiated *E. arbustorum* from Banja Luka city and Pljeva (rural site). Phenotypic and genetic similarity of populations from Banja Luka and Prijedor can be a result of the geo-morphological attributes of the region. Namely, comparing to the Vrbas river that connects Banja Luka and Pljeva, Sana river valley could be a more efficient corridor for dispersal. Taking into account that the geographic distance between Banja Luka and Pljeva is smaller than that between Prijedor and the rural site, Pljeva, as well as the presence of overall similarity among environmental factors extracted for the three study sites implies

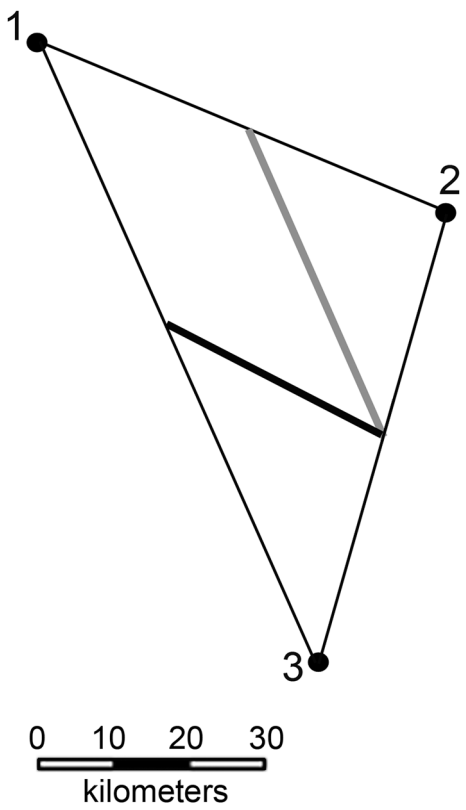


**Fig. 6** Geographical locations of *Eristalis arbustorum* samples. The lines between locations represent the connectivity network created by Delaunay triangulation. Monmonier's algorithm was used to detect genetic barriers: a thick black line separates the Pljeva population (based on allozyme data) and a thick gray line separates the Banja Luka population (based on COI mtDNA) from other samples

broader analyses. Indeed, understanding the spatial distribution of wing traits that are thought to have different genetic properties is important, because both wing size and shape are potentially linked to a number of fitness components (Reeve et al. 2000; Kölliker-Ott et al. 2003). For example, growing evidence shows that dispersal ability often positively correlates with indices of body size and wing size/length (e.g. Sekar 2011; Stevens et al. 2012). Indeed, wing length was positively associated with dispersal in some hoverflies (Rotheray et al. 2014), but whether this is a factor affecting *E. arbustorum* has yet to be investigated. Compared to wing shape, wing size has been shown to be under less strict genetic control and to be strongly affected by environmental variation (Bitner-Mathé and Klaczko 1999; Matta and Bitner-Mathé 2004). For example, the influence of temperature changes on wing size variation has been extensively reported in *E. arbustorum* (Ottenheim et al. 1998; Ottenheim and Volmer 1999). In breeding experiments, investigations on *E. arbustorum* showed that longer wings develop in warmer conditions than at lower temperatures (Ottenheim and Volmer 1999).

Our final aim was to study the amount and pattern of asymmetry in wing traits, in order to test whether different populations experienced perturbations during larval development. Results presented herein show highly significant FA in wing size and shape in *E. arbustorum* sampled from all three sampling sites. In addition, consistent levels of FA across the analyzed populations were also found. A substantial contribution of particular landmarks located at the wing base (landmarks 1, 15, 16) and landmarks 2 and 8 in overall shape variability reflected the same pattern of shape FA variation in three samples used in our study. Since FA has often been correlated with intrinsic (genetic) and extrinsic (environmental) stress (e.g. Palmer and Strobeck 1986; Carter et al. 2009), our results suggest that the individuals we studied may have possibly been exposed to a variety of stressors during their development. Although some evidence suggests that individuals with more symmetric wings would have greater dispersal potential (e.g. Nouvellet et al. 2011), the influence of FA on hoverfly dispersal abilities and fitness has yet to be investigated. For example, in some studies individuals with phenotypes that deviated from perfect symmetry exhibited lower fitness (Lens et al. 2002b; Knierim et al. 2007), but a lack of consequences on individual fitness associated with FA has also been reported (e.g. Markow 1995; Clarke 1995). It has even been proposed that individuals with highly unstable traits and thus more variability could have a selective advantage in new environments (Simons and Johnston 1997; Juste et al. 2001). Because the effect of perturbations on phenotypic development is a complex issue, the reasons for the uniformity we observed for *E. arbustorum* with respect to amounts and patterns of shape FA at the population level are probably not straightforward. Indeed, *Eristalis* hoverflies are robust and resilient to a range of external and internal factors (Thyselius and Nordström 2016) that make them successful in providing current ecosystem services in urban environments, and also support their use in long-term urban ecosystem management.

Also, it should be taken into account that this lack of interpopulation differences in wing size and shape FA could be correlated with sample size. Namely, data simulation studies indicated that the accuracy and precision of FA estimation would increase with increasing sample size and/or number repeats, especially when measurement error was high (e.g. Van Dongen, 1999). Thus, sample size that was heterogeneous across the populations we analyzed (lower in Prijedor and Pljeva comparing to Banja Luka) could have potentially affected the degree of FA and produced misleading results. Still, considering the low measurement errors we obtained for both wing traits in all samples comparing to individual variation and FA, and our application of a non-parametric approach (permutational test) for statistical analyses of morphometric data, low sample size issues could be overcome.

**Table 5** Principal component loadings from analysis of environmental data extracted from the localities of the *Eristalis arbustorum* populations (elevation in m a.s.l., all temperatures in °C and all precipitation levels in mm)

| Variable code | Variable type | Prijedor | Banja Luka | Pljeva | PC1 | PC2 |
|---|---|---|---|---|---|---|
| Alt | Elevation of site | 133 | 317 | 791 | −0.498 | 0.332 |
| Bio 1 | Annual mean temperature | 11.1 | 10.3 | 8.5 | 0.093 | −0.032 |
| Bio 2 | Mean monthly temperature range | 10.3 | 10.5 | 8.9 | 0.060 | 0.036 |
| Bio 3 | Isothermality: (Bio2/Bio7) × 100 | 33.8 | 34.2 | 31.3 | 0.032 | 0.021 |
| Bio 4 | Temperature seasonality (STD × 100) | 741.9 | 748.3 | 719.8 | 0.013 | 0.012 |
| Bio 5 | Maximum temperature of warmest month | 27.3 | 26.8 | 23.9 | 0.049 | 0.001 |
| Bio 6 | Minimum temperature of coldest month | −3.1 | −3.8 | −4.5 | 0.289 | −0.193 |
| Bio 7 | Temperature annual range (Bio5–Bio6) | 30.4 | 30.6 | 28.4 | 0.027 | 0.015 |
| Bio 8 | Mean temperature of wettest quarter | 6.8 | 17.7 | 4.4 | 0.310 | 0.912 |
| Bio 9 | Mean temperature of driest quarter | 3.1 | 2.3 | 0.6 | 0.593 | −0.051 |
| Bio 10 | Mean temperature of warmest quarter | 20.1 | 19.4 | 17.3 | 0.053 | 0.012 |
| Bio 11 | Mean temperature of coldest quarter | 1.7 | 0.9 | −0.6 | 0.422 | −0.091 |
| Bio 12 | Annual precipitation | 992 | 989 | 1041 | −0.019 | −0.009 |
| Bio 13 | Precipitation of wettest month | 105 | 108 | 124 | −0.060 | 0.003 |
| Bio 14 | Precipitation of driest month | 64 | 64 | 70 | −0.035 | −0.012 |
| Bio 15 | Precipitation seasonality (coefficient of variation) | 16.2 | 16.7 | 20.8 | −0.093 | −0.006 |
| Bio 16 | Precipitation of wettest quarter | 285 | 286 | 333 | −0.060 | −0.017 |
| Bio 17 | Precipitation of driest quarter | 199 | 197 | 217 | −0.035 | −0.020 |
| Bio 18 | Precipitation of warmest quarter | 261 | 274 | 247 | 0.029 | 0.051 |
| Bio 19 | Precipitation of coldest quarter | 231 | 224 | 264 | −0.056 | −0.045 |

## Conclusions

Spatial analysis in the present study revealed a lack of association between the uniformity of ecological parameters among collecting sites and observed genetic and phenotypic interpopulation differences in *Eristalis arbustorum*. For instance, for genetic and phenotypic population differentiation at a small spatial scale (60 km between Banja Luka and Pljeva), our results underline the discrepancy between population spatial patterning and the dispersal power of the study species. Indeed, *E. arbustorum* possess many characteristics that are predicted to limit differentiation at both the fine and broad scale, such as current and historical abundance, a continuous distribution, strong dispersal abilities, and high population densities (Speight et al. 2017). Although preliminary, our presented data of the spatial patterns of molecular and phenotypic variation imply the possible presence of hidden population structuring of *E. arbustorum* at a larger scale.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Research involving human participants and/or animals** This work did not involve human subjects or experiments on animals.

**Informed consent** Informed consent statement does not apply to this work since it did not involve human subjects.

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