# MICROSATELLITE POLYMORPHISM SUGGESTS HIGH GENETIC DIVERSITY BUT DISRUPTED GENE FLOW IN THE TWO-SPOT LADYBIRD *ADALIA BIPUNCTATA* (LINNAEUS, 1758) (COLEOPTERA: COCCINELLIDAE) POPULATIONS FROM DIVERSE ENVIRONMENTS

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**Abstract.—** The two-spot ladybird *Adalia bipunctata* (Linnaeus, 1758) (Coleoptera: Coccinellidae) is a widely distributed generalist, feeding preferably on aphids. The species inhabits various environments, including anthropogenic landscape, where pollution and microclimate enables to attain very high numbers of favorite prey. Recently, the abundance of the two-spot ladybird has declined, mainly as an after-effect of invasion of the competing species — the harlequin ladybird *Harmonia axyridis* (Pallas, 1773). High phenotypic polymorphisms made the two-spot ladybird classic model for population genetic studies of Coccinellidea. However, studies investigating diversity of the species at DNA level have Coccinellidae been scarce. In our investigation we used microsatellite markers to characterize genetic diversity in the two-spot ladybird populations from different environments. We made an attempt to amplify 13 loci, identified previously in the genome of the species, however due to lack of amplification, monomorphisms and presence of the null alleles, the final set of six markers useful for analysis on a population level was obtained. Analyzing genotypes of 124 individuals, we confirmed high genetic diversity of the species, suggested previously by studies, applying allozymes and mitochondrial DNA. Also, microsatellites revealed weakly pronounced genetic structure in a large scale (localities separated by a distance of 500 kilometers). However, we found some indications (signs of the Wahlund effect) that gene flow could be disturbed among sites in diverse environments (rural, suburban and urban). Our data suggested that invasion of the harlequin ladybird has not yet affected populations of the two-spot in such extent to alter genetic diversity or genetic structure.

**Key words.—** *Adalia bipunctata*, population genetics, microsatellites, urban environment

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## Introduction

The two-spot ladybird *Adalia bipunctata* (Linnaeus, 1758) (Coleoptera: Coccinellidae) (hereafter the two-spot) is a widely distributed invertebrate, inhabiting large areas in the Palaearctic (Kovář 2007) and Nearctic (Gordon 1985). The species is a habitat and food generalist with a preference to feed on aphids developing on deciduous trees and shrubs (Omkar and Pervez 2005). The two-spot thrives well in the anthropogenic environments, especially in urban areas, where pollution enables aphid populations to attain very high numbers (Sloggett 2017). Historically, the two-spot was very common in Europe and North America, however recently has been considered as a species under the highest threat from the harlequin ladybird (*Harmonia axyridis* Pallas, 1773) invasion, both in North America (Harmon *et al.* 2007) and Europe (Roy *et al.* 2012). Indeed, both species to a great extent share the same ecological niche, and an increasing body of evidence shows that populations of the two-spot do rapidly decrease their numbers after expansion of the harlequin (Roy *et al.* 2016). On the other hand, according to Sloggett (2017), the declining trends of the two-spot populations do not necessarily mean a real threat to this species, but reflect the process of replacement in the urban habitats of one synanthropic spe-cies by another. The artificially inflated numbers of *A. bipunctata* in towns and cities have presumably dropped to the levels characteristic of pre-industrial times.

Until recently, the two-spot was common and abundant throughout Poland, both in natural and disturbed environments (Burakowski *et al.* 1986). A survey carried out in the 1970s in and around Warsaw (Czechowska and Bielawski 1981) showed that the two-spot was a very clear dominant among Coccinellidae inhabiting tree crowns in the town (43% of all ladybirds recorded in city parks, 63% in a housing estate and 35% in the city centre). Research recently conducted in several city parks in Warsaw (Ceryngier *et al.* 2016, Romanowski *et al.* 2017) revealed that the contribution of the two-spot to the ladybird communities ranged between 0.3% and 2%, while that of the harlequin ladybird, between 25% and 64%.

The two-spot is highly polymorphic for its elytral and pronotal pattern. Due to this, since the early 20th century it has been the subject of numerous studies on the inheritance and dominance of colour forms (Sloggett and Honěk 2012). Various mechanisms affecting the spatial and temporal variability in the frequency of melanic and non-melanic forms has been proposed, including thermal (Brakefield and Willmer 1985) and industrial melanism (Mikkola and Albrecht 1988), non-random mating (O'Donald and Muggleton 1979) and Müllerian mimicry (Brakefield 1984). Also, this phenotypic polymorphisms created basis for numerous studies in the field of population and ecological genetics of the species (Zakharov 1995). The high level of within-population genetic diversity was suggested, and clinal variation was found, indicating that natural selection affects geographical distribution of color morphs (Majerus 1994). Recently, genetic techniques, allowing to investigate polymorphisms on molecular level were also applied. Krafsur *et al.* (1996) used allozymes to investigate gene flow and genetic diversity in Eurasian and North American populations. It was shown that the two-spot is highly polymorphic at the molecular level and that there is a substantial level of gene flow among populations. However, despite high genetic diversity, Krafsur *et al.* (1996) found also heterozygosity deficiency at population level in some loci, and attributed this mainly to the Wahlund effect.

The first studies, analyzing genetic diversity of the two-spot at DNA level, focused on nucleotide polymorphisms in mitochondrial genes (Schulenburg *et al.* 2002, Palenko *et al.* 2018). It was confirmed, that genetic diversity is high within the species. Moreover, there was no clear geographical division among identified haplotypes, however in central Europe frequency and evolutionary relationships among haplotypes were typical for species with sudden expansion of geographical range (star-like type of mitochondrial phylogeny). Throughout the continent, the south-north cline of haplotype frequency was identified, however genetic differentiation among geographically distinct populations was very low (Palenko *et al.* 2018). These results support previous observation, indicating high within population diversity and high gene flow among populations of the two-spot (Krafsur *et al.* 1996).

In 2002 large set of highly polymorphic microsatellite markers were developed for the two-spot (Haddrill *et al.* 2002). These molecular markers, due to high polymorphism at species level and high resolution power, provide convenient molecular tool to estimate genetic diversity, analyse genetic structure and identify individuals (Selkoe and Toonen 2006, Reiner *et al.* 2019). The microsatellite analysis of the two-spot focused on the extent and consequences of female multiple mating (Haddrill *et al.* 2008). However, according to our knowledge, microsatellites have not been used for investigation of population genetics of the species.

Hence, the main purpose of the study was to apply microsatellites – molecular markers with high resolution power – to study declining populations of the two-spot. Specifically, we aim to (i) characterize microsatellite polymorphisms of the two-spot at the population level; (ii) investigate genetic diversity in the populations of the species from two regions differing in time of the harlequin invasion: (a) Wrocław in the south-west, which was probably colonized earlier than (b)

Olsztyn area, in the north-eastern Poland; (iii) analyse genetic structure of population, inhabiting diverse environment: highly transformed urban habitat and rural areas.

## Material and methods

The individuals of the two-spot were collected in two regions of Poland: in suburbs of Wrocław city (south-western Poland; 51°06'00"N; 17°01'59"E); including two sampling sites: Kazanów near Pilczycki Forest [51°09'11"N; 16°57'36"E] and Psie Pole [51°08'45"N; 17°02'08"E]); and from several locations around and within borders of Olsztyn city (north-eastern Poland; 53°46'47"N; 20°29'38"E). In the case of Wrocław 25 individuals were collected in Kazanów near Pilczycki Forest [WR-Site1] in May 2014, and 4 during summer 2015, and 7 at Psie Pole in June 2014. Altogether 36 individuals were collected from Wrocław suburbs. In the case of Olsztyn area 88 specimens were collected throughout rural, suburban and urban environment, including several sampling locations [each approx. 1 $km^2$], where from 3 to 12 ladybirds were collected. All individuals were collected in 2014. In total, we collected 124 specimens of the two-spot belonging to three colour forms: f. *typica*, f. *quadrimaculata* and f. *sexpustulata* (Fig. 1). Forma *typica* was the most common (84% of all individuals) followed by f. *quadrimaculata* (9%) and f. *sexpustulata* (7%). Beetles were collected from trees between May and August. After being collected in the field, specimens were stored in plastic vials, while after delivery to the laboratory they were kept in a freezer at -4°C.

### Laboratory procedures

DNA extractions from a single leg were performed using NucleoSpin Tissue Kits (MACHEREY-NAGEL, distribution in Poland by AQUA LAB) in line with a standard protocol. Prior to extraction, the top of the workstation was cleaned with alcohol. Each time DNA was extracted from 15 samples and one 'blind' sample (all reagents without biological material) to control for the possibility of contaminated reagents. Following the extraction, all pipettes and additional equipment were cleaned with alcohol and autoclaved.

All the extracts, including the 'blind' samples, were made subject to PCR. We aimed to amplify 12 microsatellite loci, using primers and conditions described by Haddrill *et al.* (2002), i.e. Ab-3; Ab-4; Ab-7; Ab-9; Ab-11; Ab-14; Ab-15; Ab-19; Ab-27; Ab-31; Ab-32; and Ab-35. Initially, these markers were amplified in separate reactions in randomly selected 25 individuals of the two-spot. Amplification was performed in 13 $\mu$l of reaction mix, containing: 1.5 $\mu$l of DNA extract, 6.25 $\mu$l REDTaq PCR ReadyMix (Sigma-Aldrich), 3.75 $\mu$l of water and 10 pmol of each primer. PCR reaction was performed under the following conditions — initial denaturation: 94°C in 3 min.; 30 cycles: 94°C in 45 s; 52–60°C in 45 s; 72° C in 45 s; final elongation: 72°C in 5 min. Each forward primer was fluorescently labelled with one or other of the dyes: Dye2, Dye3 and Dye4 (from WellRead Dyes, Sigma-Aldrich). The results of the amplification were assessed using a CEQ 8000 sequencer (BECKMAN COULTER). When the length of the PCR product, detected by sequencer, was similar to the size of alleles described in the literature (Haddrill *et al.* 2002), and the electropherograms were typical for microsatellites, the loci were classified as

Figure 1. Colour forms of the two-spot ladybird recorded in this study. From left to right: *Adalia bipunctata* f. *typica*, *A. bipunctata* f. *quadrimaculata* and *A. bipunctata* f. *sexpustulata*.

a successfully amplified. Then, the selected markers were combined into two multiplexes, according to the length of the PCR product and the fluorescent dye. The first multiplex contained primers for loci Ab14, Ab9, Ab31, Ab32 and Ab19; the second: Ab35, Ab3, Ab4, Ab7 and Ab11. Each reaction mixture contained 1.5 $\mu$l of the mixture of primers ('forward' and 'reverse' for each locus, 2 pmol/$\mu$l) 7.5 $\mu$l PCR MasterMix (QIAGEN, distribution in Poland by Syngen Biotech), and 1.5 $\mu$l of DNA extract. The reactions were performed in the following conditions: 15 min at 95°C, 40 cycles of 30 s at 94°C, 90 s 60°C, 90 sec 72°C 1 cycle: 30 s at 94°C, 90 s 60°C, 10 min at 72°C. Again, the genotyping was performed using a CEQ 8000 sequencer. To identify and estimate possible genotyping errors 25% of the samples were genotyping twice.

### Statistical analysis

Analysis of molecular data were performed on three levels. First, we described microsatellite polymorphism for the total sample. In this analysis, we estimated number of alleles, observed heterozygosity ($H_O$) and unbiased expected heterozygosity ($H_E$) (Nei and Roychoudhury 1974) for each locus. Fixation index ($F_{IS}$) for each locus was calculated and its significance was tested under 160 randomisation, and Bonferroni correction for multiple comparison. These analyses were performed using GenAlEx version 6.501 (Paekall and Smouse 2001) and FSTAT version 2.9.3 (Goudet 2001). For each locus, the deviation from the Hardy-Weinberg equilibrium (hereafter *HWE*) and linkage disequilibrium (*LD*) were assessed using Fisher's exact test in Genepop v.4.0.10 (Raymond and Rousset 1995, Rousset 2008), with the following settings: 10,000 dememorisation, 1000 batches and 10,000 iterations.

Secondly, we calculated genetic diversity for each region separately (Olsztyn and Wrocław). We assessed mean allelic diversity (*A*) and allelic richness (*R*; Petit *et al.* 1998), and private allelic richness (*RP*), $H_O$ and $H_E$. The $F_{IS}$ for each region was also calculated. These analyses were performed using GenAlEx, FSTAT, Genepop and HP-RARE (Kalinowski 2005). Finally, we repeated these analysis for one location, where relatively large number of samples was collected during single season (WR-Site1 2014).

Because 'null alleles' could significantly affect microsatellite data, we analysed identified genotypes in the PopGenReport V. 3.0.0 package in the R environment (R Core Team 2017) to identify possible problems, interlinked with 'nulls'. We used a method after Brookfield (1996), as this performs better when all genotyped individuals have at least one allele detected (i.e. there are no missing data). 95% confidence intervals were estimated using the bootstrap method. The analyses were performed for the overall dataset, for both regions and only for WR-Site1 2014. Additionally, possible problems connected with 'large allele drop-out' and typographic errors were identified using MicroChecker v. 2.2.3 (Van Oosterhout *et al.* 2004).

To describe the genetic structure of the investigated populations, we obtained pairwise $F_{ST}$ estimates of genetic differentiation between Wrocław and Olsztyn, using FSTAT. The significance of $F_{ST}$ was tested by permutations procedure. The 95% confidence intervals for $F_{ST}$ were also estimated in FSTAT.

Bayesian-clustering method (STRUCTURE ver. 2.3.4; [Pritchard *et al.* 2000]) was used to examine how well the investigated locations corresponded to genetic groups (*K*). STRUCTURE was run 15 times for each user-defined *K* (1–4), with an initial burn-in of 100,000, and 1,000,000 iterations of the total data set. The admixture model of ancestry and the correlated model of allele frequencies were used. Sampling location was not used as prior information.

## Results

Separate PCR of 12 microsatellite loci indicated successful amplification of 10 among them in 25 individuals. We failed to amplify Ab-15 and Ab-27, despite testing wide range of annealing temperatures. Successfully amplified loci were combined in two multiplex reactions, and tested in all 124 specimens of the two-spot. Analysis indicated that locus Ab-4 was monomorphic, whereas locus Ab-7 did not yield the product in 64% specimens. Hence, these two loci were also excluded from further analysis. In this way, we obtained a set 8 polymorphic microsatellites for analysis of the two-spot at population level (Table 1). Repeated genotyping of 31 individuals indicated very low error rate of c.a. 1% (3 cases among 248 locus x individual combination).

For the total sample (all the individuals pooled together), we found very high number of alleles in particular loci (from 6 to 22), and high overall and within-locus heterozygosity (in majority of loci higher than 60%), however 5 out of 8 loci deviated significantly for *HWE*. For these loci $F_{IS}$ was high, positive and significant, indicating heterozygote deficiency. Accordingly, overall *HWE* test and $F_{IS}$ showed significant heterozygote deficiency (Table 1). Although we amplified all loci in all individuals, the method of Brookfield (1996) indicated the possibility of there being null alleles for loci Ab-9 (frequency of 15%), and Ab-3 (18%) both in the case of the pooled dataset and region-based analysis. Hence, we excluded also these two loci from further calculations. MicroChecker did not suggest problems with either stuttering or large-allele drop-out. There was no significant linkage disequilibrium (*LD*) noted

Table 1. Microsatellite polymorphism in the two-spot ladybird ($N$ = 124). Size – a range of a length (in base pairs) of PCR products; $A$ – number of alleles (in the last row total number of identified alleles is given); $H_O$ – observed heterozygosity and $H_E$ – expected heterozyogsity (in the last row mean values are given); *HWE* – the results of *HWE* exact test for heterozygote deficiency/excess (in the last row the results of the test over all loci and individuals was given); $F_{IS}$ – fixation index (in the last row overall value was given); * – significant deviation from *HWE* ($P < 0.05$) or significant $F_{IS}$ value after Bonferroni correction (Bonferroni corrected $P$-value at $\alpha = 0.05$ was 0.00417).

| | Locus | Size | $A$ | $H_O$ | $H_E$ | *HWE* | $F_{IS}$ |
|---|---|---|---|---|---|---|---|
| MIX I | Ab-14 | 135–159 | 11 | 0.640 | 0.788 | * | 0.19* |
| | Ab-9 | 211–275 | 22 | 0.523 | 0.842 | * | 0.38* |
| | Ab-31 | 298–316 | 6 | 0.209 | 0.224 | | 0.07 |
| | Ab-32 | 164–200 | 13 | 0.778 | 0.829 | * | 0.07 |
| | Ab-19 | 206–228 | 9 | 0.699 | 0.692 | | -0.01 |
| MIX II | Ab-35 | 215–271 | 21 | 0.667 | 0.868 | * | 0.24* |
| | Ab-3 | 137–177 | 22 | 0.667 | 0.869 | * | 0.24* |
| | Ab-11 | 292–354 | 19 | 0.781 | 0.866 | | 0.10 |
| | | | 123 | 0.620 | 0.747 | * | 0.174* |

for the investigated loci following Bonferroni correction (adjusted $P$-value for the 5% nominal level = 0.003 300 permutations).

All the analyses were then performed based on data from six microsatellite loci. Again, we found high polymorphisms and heterozygote deficiency for the pooled dataset. However, after reducing samples from Wrocław to single location and season (WR-Site1 2014), there was no disequilibrium, and overall $F_{IS}$ was low and not significantly different from zero (Table 2). Only one locus was not in *HWE*, but this time due to heterozygote excess. The opposite situation was found in the second region. In Olsztyn we found five loci with significant heterozygote deficiency. The overall metrics also indicated that this population was not in *HWE* (Table 2).

Further comparing Wrocław and Olsztyn, we found higher number of microsatellite alleles and higher observed heterozygosity in Wrocław (Tukey HSD test: $P = 0.035$ and $P = 0.012$ for $A$ and *Ho* respectively). Similarly, $F_{IS}$ was significantly higher in Olsztyn than in WR-Site1 2014.

We found significant ($P < 0.05$) although small genetic differentiation between two investigated regions ($F_{ST} = 0.016$, 95%CI -0.003–0.032).

Our STRUCTURE analysis of all samples from two regions indicated a gradual decrease of mean likelihoods from $K$=1 to $K$=4, with an accompanying increase of variance among iterations. The analysis of likelihood of ancestry did not result in any assignment of individuals to groups, which is to say that each could be assigned to 2, 3 or 4 clusters with equal probability (data not shown). This indicated a lack of justification for any division of samples into separate genetic groups.

## Discussion

We tested a set of polymorphic microsatellites, identified by Haddrill *et al.* (2002) to characterize markers potentially useful for analysis of the two-spot ladybird at the population level. Although preliminary amplification showed polymorphism and reliable amplification of 8 markers, further analysis of identified genotypes suggested presence of null alleles in two of these loci. As the calculated frequency of the 'nulls' in our data clearly exceeded the threshold below which the impact of null alleles on estimation of genetic diversity and differentiation is considered insignificant (c.a. 8%; Chapuis and Estoup 2007), we had to exclude locus Ab-9 and Ab-3 from further analysis. Indeed, both of these loci generated significant heterozygote deficiency at the population level. However, it should be noted that many factors could affect the level of heterozygosity in populations, often erroneously suggesting that this deviation was associated with presence of null alleles in particular loci (Dąbrowski *et al.* 2015). In the original paper Haddrill *et al.* (2002) attributed heterozygote deficiency in some microsatellite loci of the two-spot to inbreeding rather than to presence of the 'nulls'. In our study we found high frequency of null alleles in two loci characterized by the highest polymorphism (22 alleles each). Usually, in such microsatellite loci high heterozygosity is expected. Hence, even slight deviation from values expected by *HWE* generates significant signal of deficiency, and accordingly, in subsequent analysis — suggestion of the presence of the null alleles. Investigating range of allele sizes, identified in locus Ab-9, we found several gaps in some size classes. Indeed, this could be attributed to the presence of 'nulls'. However, in locus Ab-3 we

Table 2. Summary of genetic diversity indices at 6 microsatellite loci present among the two-spot populations. *N* – sample size; *A* – number of alleles per locus; *R* – allelic richness, estimated for the sample size of 25 individuals; *RP* – mean private allelic richness, estimated for the sample size of 25 individuals; $H_O$ – heterozygosity observed; $H_E$ – heterozygosity expected; *HWE* – exact test for heterozygote deficiency/excess (* – significant deviation, $P < 0.05$); $F_{IS}$ – inbreeding coefficient (* – values significant after Bonferroni correction, 240 randomisations, adjusted *P*-value=0.004, aside from overall value).

| Locus | *A* | *R* | *RP* | $H_O$ | $H_E$ | *HWE* | $F_{IS}$ |
|---|---|---|---|---|---|---|---|
| Olsztyn *N* = 88 | | | | | | | |
| Ab-14 | 10 | 6.99 | 2.05 | 0.621 | 0.779 | * | 0.209* |
| Ab-31 | 6 | 3.74 | 0.38 | 0.182 | 0.210 | * | 0.139 |
| Ab-32 | 12 | 8.72 | 1.64 | 0.776 | 0.808 | * | 0.045 |
| Ab-19 | 8 | 6.46 | 0.26 | 0.667 | 0.684 | | 0.032 |
| Ab-35 | 15 | 10.69 | 3.07 | 0.655 | 0.863 | * | 0.246* |
| Ab-11 | 18 | 11.23 | 2.06 | 0.736 | 0.834 | * | 0.124* |
| Mean | 11.5 | 7.97 | 1.58 | 0.606 | 0.696 | * | 0.135* |
| Wrocław *N* = 36 | | | | | | | |
| Ab-14 | 7 | 6.55 | 0.125 | 0.704 | 0.772 | | 0.107 |
| Ab-31 | 5 | 4.51 | 0.41 | 0.296 | 0.267 | | -0.092 |
| Ab-32 | 9 | 8.83 | 0.00 | 0.783 | 0.853 | * | 0.104 |
| Ab-19 | 8 | 7.57 | 0.13 | 0.808 | 0.712 | | -0.116 |
| Ab-35 | 16 | 13.77 | 1.04 | 0.704 | 0.860 | * | 0.200* |
| Ab-11 | 14 | 13.26 | 0.19 | 0.926 | 0.908 | | -0.001 |
| Mean | 9.8 | 9.08 | 0.32 | 0.703 | 0.729 | * | 0.054 |
| WR-Site1 2014 *N* = 25 | | | | | | | |
| Ab-14 | 6 | 6 | 0.19 | 0.680 | 0.762 | | 0.127 |
| Ab-31 | 4 | 4 | 0.11 | 0.280 | 0.252 | * | -0.091 |
| Ab-32 | 9 | 9 | 0.09 | 0.762 | 0.862 | | 0.140 |
| Ab-19 | 8 | 8 | 0.21 | 0.875 | 0.724 | | -0.188 |
| Ab-35 | 16 | 16 | 1.49 | 0.760 | 0.861 | | 0.137 |
| Ab-11 | 14 | 14 | 0.31 | 0.920 | 0.909 | | 0.008 |
| Mean | 9.50 | 9.50 | 0.40 | 0.713 | 0.728 | | 0.042 |

identified all possible alleles within a range of 137–177 base pairs. Hence, the presence of the null alleles in this locus seems rather doubtful. Further analyses are needed, preferably investigating pattern of inheritance, to verify problems associated with null alleles in the two-spot. At this stage, we suggest careful examination of Ab-9 locus during population genetic studies of the two-spot.

Considering population genetics of the two-spot three main conclusions emerged from our study: (i) the species exhibits high genetic diversity; (ii) there is a gradual decrease in deviation from *HWE* as the grouping of individuals is reduced; (iii) geographically distant populations show little genetic differentiation.

We found high number of alleles in the investigated microsatellite loci and high level of heterozygosity. The level of genetic diversity of the two-spot is comparable with that found in populations of the invasive harlequin ladybird (*Harmonia axyridis*) (Lombaert *et al.* 2011, Rutkowski *et al.* 2017). This aphidophagous species of Asiatic origin was commonly used for biocontrol purposes in the 20th century and repeatedly released in various parts of the world since 1916 (Brown *et al.* 2011). Following the initial establishment in the late 1980s in Louisiana (USA) (Chapin and Brou 1991), the feral populations of the harlequin started spreading in North America and then also in Europe, South America and Africa (Brown *et al.* 2011). It was suggested that the invasion of the species was probably connected with high number of colonizers and rapid demographic recovery after invasion (Goryacheva and Blekhman 2017). These lead to high effective population size, which could alleviate the effects of the demographic and genetic bottlenecks, usually expected in the invasive species (Lee 2002). Also other scenarios

assume, that invasive populations maintain high genetic diversity (Puillandre *et al.* 2008).

Our data indicated that the recent significant decrease in the abundance of the two-spot (Sloggett *et al.* 2017) has not been interlinked with reduction of genetic diversity. During sample collection for our study, it was obvious that the two-spot significantly decrease its abundance, being replaced by the invasive harlequin. There are several reasons for this, for example cannibalism associated with crowding and deficiency of food (Dimetry 1976), intraguild predation (Thomas *et al.* 2013) and development strategy (Hodek and Ceryngier 2000) which is also related with shifts during the development of both species. On the same trees the two-spot is in the pupal stage while the harlequin is in the larval stage thus pupae might be attacked by the larvae (Sakuratani *et al.* 2000). Also, research on bacteria killing the two-spot males (Balayeva *et al.* 1995) and the immunity of the harlequin to parasite infestation or infection (Dudek *et al.* 2017) should not be overlooked. Although the observed decline of the two-spot is usually attributed to the interactions with the harlequin, some authors suggest that, at least in North America, this declining trend might have already begun before the establishment of the harlequin in the range of the two-spot (Harmon *et al.* 2007).

It is possible that the population size reduction has been too recent to decrease previously very reach genetic pool. Indeed, it was shown that the two-spot presents high genetic diversity in general, both in the cities (Palenko *et al.* 2018) as well rural environments (Krafsur *et al.* 1996). Our sampling sites were located around or within borders of urbanized landscape. Majority of samples from Olsztyn were collected in sampling sites, located in green spaces in the centre of the city. Urban and industrial areas are favourable environment for ladybirds, especially for the generalist species, such as the two-spot (Sloggett 2017). In such environment the two-spot usually reaches high densities. Such numerous population could bear diverse genetic pool. Moreover, high genetic diversity could also be maintained by breeding strategy of the two-spot. In the wild the females are highly polyandrous and store sperm from multiple males to fertilize their eggs (Haddrill *et al.* 2008). Such a breeding pattern could increase heterozygosity level in the prodigy. On the other hand, the ladybirds winter in large aggregations and frequently breed even before dispersal phase in the early spring, during overwintering period (Susset *et al.* 2018). Breeding before dispersal could lead to copulation with related (genetically similar) males, for example with the same colony or population. Such a process should lead to increase of homozygosity due to inbreeding. Nonetheless, the mechanisms of aggregation formation in invertebrates are still poorly understood, so otherwise it is possible that genetic diversity is already created during aggregation formation in autumn, for example if aggregation consist of genetically diverse individuals from different colonies or subpopulations. Moreover, it was shown that avoiding both relatedness and familiarity is important mating strategy in ladybirds (Saxena *et al.* 2017). It was also suggested, that presence of recessive lethal alleles should ensure high genetic diversity in populations (Hodek and Honěk 1996). In general, high genetic diversity characterizes many populations of ladybirds, both native and introduced species (Krafsur *et al.* 2005). Hence our results for the two-spot support previous observations, confirming rich genetic pool in the members of this family of beetles.

The second conclusion is gradual decrease in deviation from *HWE* as the grouping of individuals was reduced: analysing relatively large number of individuals from single location, we found no evidence for heterozygote deficiency; on the other hand, treating individuals from various sites dispersed throughout large area, as a single population, resulted in significant deviation from *HWE* due to heterozygote deficiency. There could be several reasons, causing heterozygote deficiency in natural populations, including the Wahlund effect, temporal heterogeneity and, especially in the case of microsatellites, errors interlinked with failure of amplification of some alleles (Waples 2015, 2018, De Meeûs 2018). We claim that a pattern detected in the two-spot is consistent with Wahlund effect (Gillespie 2004). The reduction of intensive gene flow between sites in Olsztyn could result from sampling of individuals in different environments. For example, there could be some level of isolation between urban and rural environment, or locations within highly transformed urban environment could be isolated. Within the cities, green patches, occupied by the two-spot during dispersal phase are separated by large areas of build-up environment. This could lead to fragmentation of population. Indeed, such a pattern resulting in Wahlund effect, was confirmed in several animal species, occupying urban environment (e.g. Rubin *et al.* 2001, Wood and Pullin 2002, Bjørklund *et al.* 2010, Gortat *et al.* 2013, 2015, 2017). For beetles and butterflies, comparative studies indicate that the dispersal of habitat specialists and sedentary species are more affected by urbanization than in mobile species that are habitat generalists (Takami *et al.* 2004, Desender *et al.* 2005). However, urban environment generates the Wahlund effect even in highly expansive and mobile harlequin ladybird (Rutkowski *et al.* 2017). The two-spot is probably a species with relatively low dispersal ability. Although, according to our knowledge, the flight performance has not been studied in this species, it certainly does not belong to long-distance migrants, such as the harlequin, *Hippodamia convergens* or

*Ceratomegilla undecimnotata* (Hodek *et al.* 1993). Hence it is possible that fragmented environment within urbanized landscape generates small-scale genetic structure.

Another factor, which possibly influence heterozygosity at the population level could be biased sex ratio. In the ladybirds male-killing microorganisms are common, particularly in the two-spot, which harbours at least four different intracellular, maternally inherited symbionts (Schulenburg *et al.* 2002). These symbionts kill male embryos or larvae, intensifying their spread throughout population through infected females. As a result, in some populations of the two-spot the sex-ratio is strongly female-biased. Although a phenomenon of male-killing is considered as a mechanism leading to decrease rate of inbreeding (Hurst and Jiggins 2000), selective elimination of male can halve the number of viable offspring and consequently reduce the effective population size and heterozygosity (Engelstädter and Hurst 2007). We therefore conclude that heterozygosity deficiency observed in the two-spot could reflect some level of impaired gene flow among different sites, however other factors, creating such a pattern are also possible. This issue should be further investigated.

Although some indications suggest the Wahlund effect in the investigated population of the two-spot, we did not find evidence for large-scale genetic structure in the species. We found very little differentiation between two geographically distinct regions: Wrocław and Olsztyn, separated by distance of 500 kilometres, and analysis in STRUCTURE supported existence of single, panmictic population. Although $F_{ST}$ was significant, the value indicate very small differences in the frequency of the alleles between two regions. Probably, estimated differentiation was associated with Isolation-By-Distance, commonly observed in natural populations. This supports previous data indicating genetic homogeneity of the two-spot from different geographical populations (Krafsur *et al.* 1996, Palenko *et al.* 2018). Indeed, intensive gene flow and accompanying lack of genetic structure seem to be characteristic for many ladybird species (Krafsur *et al.* 2005), including invasive harlequin ladybird.

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