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GENETIC DIVERSITY OF AN INVASIVE INVERTEBRATE IN AN URBAN ENVIRONMENT, AS EXEMPLIFIED BY THE HARLEQUIN LADYBIRD *HARMONIA AXYRIDIS* (PALLAS, 1773)

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Abstract.— Urbanised areas are capable of exerting a strong impact on the distribution of genetic diversity within populations of animals. Urban invertebrate species are currently either relicts from pre-urban ecosystems, or have immigrated during or following urbanisation. We analysed 10 microsatellite loci in 196 specimens of the harlequin ladybird (*Harmonia axyridis*), an invasive species in Poland sampled in three of the country's cities. Of Asiatic origin, this ladybird has been spreading through other continents, including Europe. Results showed that the Polish cities are being invaded by harlequin ladybirds that are uniform in terms of their genetic pool, with no significant genetic differentiation present between the urban populations investigated. Rapid spread and what are probably the large numbers of individuals colonising new areas allow this species to maintain high genetic diversity and avoid bottleneck effects. However, we suggest that urban populations differ in terms of genetic diversity. The highest genetic diversity characterises the most recently invaded area of Olsztyn in the north of Poland. Genetic data further suggest that this population could be still in an expansion phase. The earliest colonising population in Wrocław exhibits signs of a Wahlund effect, suggesting that gene flow among local groups within this urban area could be disturbed or impaired. We conclude that, in the case of an urban population of the harlequin ladybird, successful colonisation is followed by the onset of a decline in genetic diversity, with isolation between local sampling sites appearing. Further studies are required, however, if this process is to be elucidated.

Key words.— genetic diversity, population genetics, harlequin ladybird, *Harmonia axyridis*, invasive species, urban environment, microsatellites

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Introduction

Habitat fragmentation is known to have a strong impact on the distribution of genetic diversity within populations of animals. In theory, urban populations of wild species appeared to be fragmented, given that groups of individuals inhabiting patches of suitable habitat are more or less isolated, depending on their mobility, and ability to cross environmental barriers. Decreased genetic diversity has been found in urban populations of amphibians (Hitchings and Beebee 1998; Noël *et al.* 2007, Noël and Lapointe 2010, Munshi-South *et al.* 2013) but also in the case of more mobile species, such as foxes and birds (Wandeler *et al.* 2003, Rasner *et al.* 2004, Evans *et al.* 2009). Simultaneously, there are many other examples suggesting that reduced genetic diversity within an urban environment is by no means a certainty (Desender *et al.* 2005, Gardner-Santana *et al.* 2009, Rutkowski *et al.* 2009, Björklund *et al.* 2010, Munshi-South and Kharchenko 2010, Gortat *et al.* 2013). Such observations are usually explained as an after-effect of ongoing immigration into urban areas, multiple sources of colonisation and/or effective population sizes high enough to maintain a high level of genetic variation within suitable habitat patches. It has also proved possible to find certain locations in which far-reaching isolation due to roads, as well as a high-density built-up environment lead to the loss of quite a high proportion of a population's genetic diversity. On the other hand, urban sites situated close to city limits and bordering on to green areas may actually exhibit very high level of genetic diversity, even compared with those in large natural populations (Gortat *et al.* 2015, 2017).

Most relevant studies have underlined that genetic differentiation is more marked among local populations within urban areas, as opposed to those present in the natural environment. The genetic structure is therefore more pronounced (Rubin *et al.* 2001, Wood and Pullin 2002, Wandeler *et al.* 2003, Gardner-Santana *et al.* 2009, Björklund *et al.* 2010, Delaney *et al.* 2010, Vangestel *et al.* 2011, Munshi-South *et al.* 2013, Gortat *et al.* 2013, 2017), while particular species' genetic differentiation among locations within urban areas loses the isolation-by-distance pattern usually observed in a natural population (Wood and Pullin 2002, Noël *et al.* 2007, Munshi-South and Kharchenko 2010, Gortat *et al.* 2013 2015). These observations indicate that, compared with gene flow, genetic drift is a much stronger factor shaping population genetic structure in a fragmented urban population. Because at least some of the studied species exhibit little genetic structure in natural areas (e.g. Munshi-South and Kharchenko 2010; Gortat *et al.* 2015), it seems that urbanisation is a particularly potent factor affecting genetic differentiation as compared with natural fragmentation of the environment.

Present-day urban invertebrate species are either relicts of pre-urban ecosystems trapped in an urban environment, or have immigrated during or after urbanisation or have been introduced, often unintentionally (Sattler *et al.* 2011, Jones and Leather 2012). There are a few studies raising the issue of the genetic diversity and differentiation characterising the invertebrates (mainly the insects) inhabiting city areas. For beetles and butterflies, comparative studies indicate that the dispersal of habitat specialists and sedentary species is more affected by urbanisation than is that in mobile species that are habitat generalists (Takami *et al.* 2004, Desender *et al.* 2005). A suggestion has also arisen that, in some butterflies, availability of suitable habitat rather than the ability of species to disperse is what influences gene flow within an urbanised environment (Wood and Pullin 2002). On the other hand Leidner and Haddad (2010) indicated that in some cases, natural barriers can represent a much stronger factor limiting gene flow than level of urbanization. This suggests that dispersal rates of insects may actually be little affected by urbanisation, leaving the genetic structuring of an urban population as a subtle matter. As a result, isolation and genetic drift should not decrease genetic diversity of population inhabiting habitat patches within urban areas. However, the genetic structures characterising invertebrates in cities are still poorly understood, primarily given the still-small number of species analysed (Wood and Pullin 2002, Takami *et al.* 2004, Desender *et al.* 2005, Field *et al.* 2007, Leidner and Haddad 2010).

The majority of ladybird beetles are predatory on various arthropods, such as aphids, coccids or tetranychid mites, which are often important agricultural pests (Giorgi *et al.* 2009). This accounts for the use of ladybirds as biological control agents for over a century now (Obrycki and Kring 1998). One species commonly deployed in biocontrol in the 20th century is the harlequin ladybird, *Harmonia axyridis* (Pallas). This aphidophagous species of Asiatic origin was released repeatedly post-1916, in various parts of the world. Nevertheless, the first feral population found to have been established outside the native range was that in Louisiana, USA, documented in 1988 (Chapin and Brou 1991). Following this initial establishment of an outpost, the population of the harlequin ladybird commenced with further spread in North America, before also extending its range to Europe, South America and Africa (Brown *et al.* 2011). Genetic analyses (Lombaert *et al.* 2010) indicate that the first invasive population noted in Louisiana was the only source of the invasive populations in the Americas and Africa, as well as the main source of the population now present in Europe, which also features a genetic admixture owing to the

biocontrol strains used in Europe in the 1990s and early 2000s.

Harlequin ladybird has been recorded as spreading in Europe since the late 1990s. First newly-established populations were those detected in Germany in 1999 (Brown *et al.* 2008). In neighboring Poland (western part), the harlequin ladybird was only recorded for the first time in autumn 2006 (Przewoźny *et al.* 2007), though it presumably arrived there earlier, as it was to be found in many others regions of the country by 2007 (Ceryngier 2008). Nonetheless, NE Poland seemed to remain free of the invader for some time: first records for the Białowieża Forest and Suwałki region came as late as in 2012 and 2013 respectively (Jędryczkowski and Gutowski 2014; Ceryngier *et al.* 2015). Despite such minor differences in apparent times of colonisation, the overall conclusion would seem to be that the harlequin ladybird spread very rapidly across Poland. While relatively effective as a biocontrol agent, the harlequin ladybird exerts many adverse effects in the areas it invades. As an efficient competitor and intraguild predator, it poses a serious threat to native arthropods, most especially to other predatory ladybirds (Yasuda *et al.* 2004, Pell *et al.* 2008, Roy *et al.* 2012). Furthermore, it may be a pest in its own right in orchards and vineyards, due to its later-summer and autumn habit of eating ripe fruit. (Kovach 2004, Galvan *et al.* 2008). This species may often pose a nuisance because of its tendency to overwinter in large aggregations in buildings. It may also occasionally bite people, and/or cause allergic reactions (Kovach 2004, Huelsman and Kovach 2004).

The invasive character of the harlequin ladybird has stimulated molecular studies whose focus is the identification of sources and routes of invasion in Europe, the Americas and Africa; as well as characterisation of genetic structure in the native area (Lombert *et al.* 2010, 2011). Genetic analysis in fact indicated the presence of two genetic clades within the native range, with this separating the individuals from Russia and Kazakhstan from those sampled in China, Japan and North Korea (Lombaert *et al.* 2011, Zakharov *et al.* 2011). It is thus reasonable to suggest that the species in question has well-recognised phylogeography, as well as routes of colonisation. Equally, the genetics of small-scale populations of the harlequin ladybird has been regarded as relatively unknown.

The work detailed here thus sought to investigate genetic diversity in urban populations of the harlequin ladybird. Specifically, research questions addressed (i) possible differences in levels of genetic diversity in populations of different Polish cities; and (ii) potential genetic differentiation between individual urban populations. The three Polish cities of Wrocław, Warsaw and Olsztyn were selected for study (Fig. 1). Wrocław officially recorded its first harlequin ladybird in 2007 (Ceryngier 2008), though the city was very likely settled earlier. In Warsaw, in turn, there have been frequent records of the species since 2008 (P. Ceryngier, unpubl.) with numerous adults, larvae and pupae also found close to the capital (in Dziekanów Leśny) as early as in autumn 2007 (Ceryngier 2008). Olsztyn was only colonised by the harlequin ladybird at a later date, though first individuals (larvae, pupae and adults) were noted in autumn 2008 (Jadwiszczak 2008). While the species under study has clearly spread rapidly in Poland, it was nevertheless assumed that particular urban areas did differ in time of colonisation, and of the establishment of a stable population. Most specifically, the south-western cities (e.g. Wrocław) were deemed to have been colonized earlier than urban areas in the north and north-east (e.g. Olsztyn).

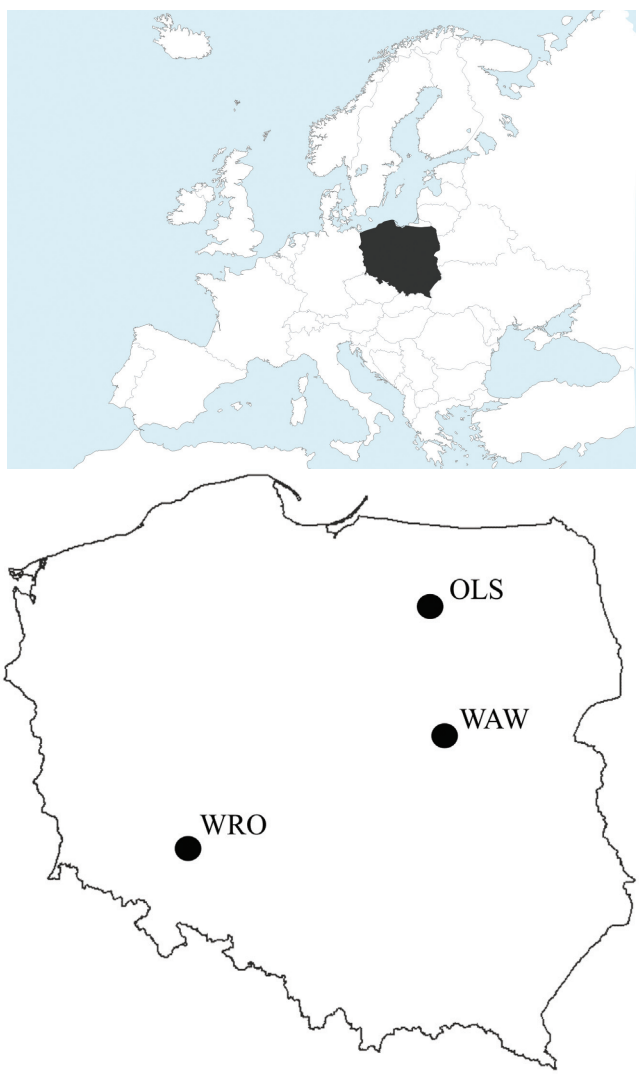

Figure 1. Distribution of investigated urban populations of the harlequin ladybird *Harmonia axyridis*. OLS – Olsztyn, WAW – Warsaw, WRO – Wrocław.

Material and methods

The individuals of harlequin ladybird were collected in Wrocław (denoted subsequently as WRO; south-western Poland); Warsaw (WAW; central Poland) and Olsztyn (OLS, north-eastern Poland). In each city, we selected three distinct sites to collect samples (Fig. 1). In Wrocław: (i) surroundings of Wrocław University of Environmental and Life Sciences (denoted subsequently as WRO-1; 51°06'41"N; 17°03'50"E), (ii) Kozanów near Pilczycki Forest (WRO-2, 51°09'11"N; 16°57'36"E); (iii) Psie Pole (WRO-3; 51°09'03"N; 17°09'55"E), in Warsaw: (i) Cardinal Stefan Wyszyński University in Warsaw (WAW-1; 52°18'42"N; 20°55'04"E), (ii) the Saxon Garden (WAW-2; 52°14'25"N; 21°00'30"E); and (iii) vicinity of Kamionkowskie Lake (WAW-3; 52°14'43"N; 21°03'17"E), in Olsztyn: (i) Jaroty (OLS-1, 53°43'39"N; 20°28'30"E); (ii) Central Park at Emilia Plater Street (OLS-2; 53°46'30"N; 20°28'56"E), and (iii) green area near the Upper Gate (OLS-3, 53°46'39"N; 20°28'39"E). We collected about 20 specimens from each sampling site (Table 1). Beetles were collected from various trees between May and August. In total 196 harlequin ladybirds were collected. 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 10 microsatellite loci, described by Loiseau *et al.* (2009), i.e. Ha-244; Ha-201; Ha-281; Ha-605; Ha-267; Ha-194b; Ha-215; Ha-223; Ha-253; and Ha-105. The markers were amplified in two multiplex reactions, using primers and mix compositions designed by Loiseau *et al.* (2009). Each forward primer was labelled with one or other of the fluorescent dyes Dye2, Dye3 and Dye4 (from WellRead Dyes, distribution in Poland by Sigma-Aldrich Poland). The reaction mixture contained 1.5 μl of the mixture of primers ('forward' and 'reverse' for each locus, 2 pmol/μl) 7.5 μl PCR MasterMix (QIAGEN, distribution in Poland by Syngen Biotech), and 1 μl 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 57°C, 90 sec 72°C 1 cycle: 30 s at 94°C, 90 s 57°C, 10 min at 72°C. As in the case of the extraction process, we sought to control contamination in the course of PCR. Thus, alongside the DNA extracts, each PCR series received a 'blind' sample (with all reagents but no DNA). The genotyping analyses were performed using a CEQ 8000 sequencer (BECKMAN COULTER, distribution in Poland by Comesa-Polska).

To identify and estimate possible genotyping errors 25% of the samples were genotyping twice.

Table 1. Comparison of parameters of genetic diversity in harlequin ladybirds (N = 196), sampled in nine localities in three cities in Poland. n — sample size; A — number of alleles; R_A — allelic richness; R_P — private allelic richness, H_O — heterozygosity observed; H_E — heterozygosity expected; *HWE* — P-values for *HWE* exact test for heterozygote deficiency/excess; F_{IS} — fixation index (* — F_{IS} value significant after Bonferroni correction. Sampling site and city abbreviations as in Material and methods.

Locality	n	A	R_A	R_P	H_O	H_E	*HWE*	F_{IS}
OLS-1	18	6.40	6.20	0.04	0.560	0.623	0.006	0.130
OLS-2	25	6.70	6.10	0.4	0.592	0.660	0.033	0.123
OLS-3	21	6.30	5.91	0.28	0.615	0.663	0.808	0.097
WAW-1	29	6.40	5.65	0.16	0.624	0.652	0.482	0.060
WAW-2	23	6.10	5.63	0.11	0.622	0.649	0.513	0.065
WAW-3	20	5.80	5.54	0.28	0.646	0.661	0.070	0.049
WRO-1	20	5.80	5.57	0.02	0.586	0.651	0.196	0.125
WRO-2	17	5.60	5.57	0.01	0.610	0.677	0.075	0.130
WRO-3	23	6.50	5.96	0.31	0.613	0.655	0.073	0.086
OLS-ALL	64	7.80	6.07	0.75	0.591	0.670	<0.001	0.126*
WAW-ALL	72	7.70	5.61	0.54	0.629	0.665	0.474	0.061
WRO-ALL	60	7.30	5.70	0.42	0.603	0.678	<0.001	0.119*
TOTAL	196	9.20			0.609	0.675	<0.001	0.101*

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 200 randomisation, and Bonferroni correction for multiple comparison. These analyses were performed using GenAlEx version 6.501 (Peakall 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 urban population separately (OLS-ALL, WAW-ALL, WRO-ALL): we assessed mean allelic diversity (A) and allelic richness (R; Petit *et al.* 1998), mean number of private alleles (R_A), private allelic richness (R_P), H_O and H_E. The F_{IS} for each urban population was also calculated. These analyses were performed using GenAlEx, FSTAT, Genepop and HP-RARE (Kalinowski 2005). Finally, we repeated these analysis for each sampling site (OLS1-3; WAW1–3; WRO1-3). The significance of differences between mean values of A, R (calculated across separate sampling sites within each urban population), F_{IS}, and F_{ST}, and H_O were tested using the permutation procedure as implemented in FSTAT.

To deduce the demographic history of the populations we attempted to identify possible genetic effects of fluctuations in the effective population size, following colonization. We tested for heterozygosity excess using the BOTTLENECK, ver. 1.2.02 program (Cornuet and Luikart 1996). The two-phase mutation (TPM) model with 10% multistep mutations was applied (Di Rienzo *et al.* 1994). We used a Wilcoxon signed rank test to determine which population had significant heterozygote excess across loci. Subsequently we evaluated whether or not the allele frequency was normal (L-shaped), suggesting stability of effective population size or shifted, i.e., indicating bottleneck.

To describe the genetic structure of the studied populations, we applied hierarchical and non-hierarchical AMOVA procedures using Arlequin software (version 3.5.1.2) (Excoffier and Lischer 2010). The number of different alleles (F_{ST}, Weir and Cockerham 1984) was used to estimate genetic distance. The significance of fixation indices (F_{ST}, F_{SC}, F_{CT}) was estimated using 1000 permutations. In addition, we obtained overall and pairwise F_{ST} estimates of genetic differentiation using FSTAT. The significance of pairwise F_{ST} was tested by 720 permutations with Bonferroni correction for multiple comparisons (Bonferroni corrected P-value at $\alpha = 0.05$ was 0.0014). The 95% confidence intervals for overall 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 (1–9), 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. We then examined ΔK statistics that identify the largest change in the estimates of K produced by STRUCTURE, as ΔK may provide a more realistic estimation of K than methods based on likelihoods (Evanno *et al.* 2005).

To visualize STRUCTURE results we used STRUCTURE HARVESTER (Earl and von Holdt 2012) and applied CLUMPP (Jakobsson and Rosenberg 2007) to average the multiple runs given by STRUCTURE and to correct for label switching. The output from CLUMPP was visualized with DISTRUCT version 1.1 (Rosenberg 2004) to display the results.

Results

The ten analysed microsatellites proved to be polymorphic in all urban populations and at all sampling sites (Appendix 1). In total, 92 alleles were found in 196 individuals. We failed to amplify locus Ha-267 in four samples, Ha-223 in one sample and Ha-253 in two samples. Repeat analyses of microsatellite genotypes indicated very limited genotyping error: among 10 loci in 24 specimens we found only four genotyping errors (1.6%), all being of the 'large allele drop-out' type. We therefore feel justified in assuming that genotyping errors had only a minor influence on our results.

Analysis for the whole population revealed seven out of ten loci deviating from *HWE* (Appendix 1). Similarly, the overall analysis indicated significant heterozygosity deficiency (Table 1). In turn, analysis at the level of the urban population (OLS-ALL, WAW-ALL and WRO-ALL) revealed that only WAW was in *HWE*. Analysis of particular sampling sites in turn revealed significant deviation from *HWE* (heterozygote deficiency) at OLS-1 and OLS-2 only, though none of the F_{IS} values achieved significance (Table 1).

There was no significant *LD* among the investigated loci after Bonferroni correction (the adjusted P-value for the 5% nominal level was 0.000123, with 8100 permutations).

Allelic diversity (A) was similar in different urban populations (7.30–7.80) and at different sampling sites

(5.60–6.70). Nevertheless, allelic richness (R_A), calculated across separate sampling sites within each urban population proved to be significantly greater in OLS than in WAW (one-sided test, 1000 permutations, P = 0.016), as well as in OLS as compared with WRO (one-sided test, 1000 permutations, P = 0.029). We found no significant differences in heterozygosity between either urban populations or sampling sites, though F_{IS} was significantly higher in OLS than in WAW (one-sided test, 1000 permutations, P = 0.017), as well as in WRO as opposed to in WAW (two-sided test, 1000 permutations, P = 0.047). Private allelic richness (R_P) was highest in OLS, and lowest in WRO (Table 1).

We did not confirm a bottleneck effect, either by analysing the total dataset (Wilcoxon signed rank test for heterozygosity excess, P = 0.442), or after dividing samples among the three urban populations ($P > 0.10$). However, analysing separate sampling sites we found significant heterozygosity excess in OLS-3, as well as at two sampling sites in WRO: WRO-1 and WRO-2 (Wilcoxon signed rank test for heterozygosity excess, $P < 0.05$). All sampling sites, urban populations and the overall dataset were all found to be characterised by a normal L-shaped distribution for the frequency of occurrence of microsatellite alleles.

Non-hierarchical grouping indicated small but significant genetic differentiation (F_{ST}) among sampling sites in OLS and WRO (F_{ST} = 0.019 and 0.016, respectively; $P < 0.05$) (Table 2). While there was no significant differentiation between sampling sites assigned to groups by urban area (G1: OLS1-3; G2: WAW1-3; G3: WRO1–3), small though significant differentiation was noted among sampling sites within groups, and within sampling sites (Table 2).

Pairwise comparison pointed to very limited genetic differentiation between sampling sites (Table 3). The overall F_{ST} was 0.01 (95%CI = 0.002–0.02), with only three values for pairwise F_{ST} indicative of significant differentiation: OLS1 – WRO3; OLS3 – WRO2; and WAW1 – WRO3 (Table 3).

Our STRUCTURE analysis of nine sampling sites from three urban populations indicated a gradual decrease of mean likelihoods from K=1 to K=9, with an accompanying increase of variance among iterations (Fig. 2A). ΔK suggested that the observed genetic variability was best explained at K=5, though the value remained low, and was close to those for K=3 and K=7 (Fig. 2B). 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 3, 5 or 7 clusters with equal probability (data not shown). This indicated a lack of justification for any division of samples into separate genetic groups.

Discussion

We analysed microsatellite polymorphism in populations of the harlequin ladybird considered at the levels of the city (the three urban populations) and the sampling site. At the urban-population level, the genetic data imply a sudden expansion of the harlequin ladybird across Poland, with significant genetic structure not noted within the investigated population. Notwithstanding the sampling from three distinct urban areas, the groups investigated are found to constitute a uniform genetic population. A pattern similar to the above was in fact obtained for another invertebrate expanding its range rapidly in Europe, including into Poland (Wawer *et al*. 2017). In some species it has proved possible to confirm that particular urban areas were colonised independently of neighbouring ex-urban areas (e.g. birds, Evans *et al*. 2009). Such a pattern results in significant genetic differentiation between separate urban populations, and stands in contrast to the 'leapfrog' model, whereby descendants of a group of individuals colonising one urban area disperse into another, while avoiding any non-urban environment (Evans *et al*. 2010). The lack of genetic structure in the harlequin ladybirds under study may also suggest

Table 2. Results of AMOVA. In each non-hierarchical grouping F_{ST} describes variation among locations within each city. In each hierarchical grouping F_{CT} describes variation among groups of locations, F_{SC} variation among locations within groups and F_{ST} variations within locations. P-values were estimated using 100 permutations. Sampling site abbreviations as in Material and methods.

Fixation indices	Non-hierarchical grouping		
	OLS1–OLS3	WAW1–WAW3	WRO1–WRO3
F_{ST}	0.019 (P = 0.003)	0.005 (P = 0.144)	0.016 (P = 0.014)
	Hierarchical grouping		
	G1: OLS1–3; G2: WAW1-3; G3: WRO1–3		
F_{CT}	-0.001 (P = 0.504)		
F_{SC}	0.013 (P < 0.001)		
F_{ST}	0.012 (P < 0.001)		

Table 3. Genetic differentiation (pairwise F_{ST}) among all studied locations. In bold – values significant after Bonferroni correction. Sampling site abbreviations as in Material and methods.

	OLS-2	OLS-3	WAW-1	WAW-2	WAW-3	WRO-1	WRO-2	WRO-3
OLS-1	0.0169	0.0247	0.0049	0.0062	0.0176	0.0071	0.0125	**0.0323**
OLS-2	0.0000	0.0097	-0.0024	-0.0038	0.0100	-0.0010	0.0107	0.0141
OLS-3		0.0000	0.0171	0.0092	0.0118	-0.0005	**0.0235**	-0.0011
WAW-1			0.0000	-0.0067	0.0094	-0.0001	0.0098	**0.0178**
WAW-2				0.0000	0.0115	-0.0010	0.0070	0.0156
WAW-3					0.0000	0.0077	0.0209	0.0272
WRO-1						0.0000	0.0091	0.0076
WRO-2							0.0000	0.0170

invasion of urban areas in line with the 'leapfrog' model. This pattern could be explained by adaptation to or imprinting upon an urban habitat (Mabry and Stamps 2008). In the case of insects, imprinting is in fact rather unlikely, and the harlequin ladybird indeed inhabits many different habitats, and is observed commonly in an extra-urban environment (Brown *et al.* 2011, Roy *et al.* 2016). Hence, the only possibility for 'leapfrog' colonisation of urban areas to occur would seem to involve human agency (Cristescu 2015). Indeed, this pattern could be a major factor inducing invasion by the species. (e.g. Majerus *et al.* 2006). On the other hand, non-significant genetic differentiation between separate urban populations may also suggest that the colonisation has proceeded in a single wave involving a uniform genetic pool, continuing independently of the type of environment. Indeed, there are some insects for which urbanisation does not represent a significant barrier to dispersal (Leidner and Haddad 2010). The comparison of urban populations with ex-urban sampling sites should offer further elucidation of this process.

Hierarchical grouping of samples (i.e. from the total dataset through to the individual sampling site) indicates a gradual decrease in the number of loci deviating from *HWE*, suggesting a Wahlund effect. As AMOVA did not confirm significant genetic differentiation among urban populations, this should probably be attributed to geographical distance among sampling sites. Indeed, significant pairwise genetic differentiation was found almost exclusively between sampling sites in the most widely-separated cities (Wrocław and Olsztyn, 380 km apart). This supports the finding from a previous investigation into the harlequin ladybird (Lombaert *et al.* 2011), that genetic differentiation was very limited among sampling sites within the native range of the species, despite the large geographical distances separating them (2700 km on average). However, genetic differentiation was present on a moderate level within the invasive range (Lombaert *et al.* 2011). Inclusion of more sampling sites should allow for elucidation of the isolation-by-distance pattern in the investigated population from Poland.

Biological invasions are usually connected with the colonisation of new areas by a limited number of individuals, with the result being a bottleneck effect and decreased genetic diversity (Bock *et al.* 2015). Such a pattern has in fact been confirmed in several species (Dlugosh and Parker 2008). However, in some cases, newly-established populations proved capable of restoring lost diversity, for example through multiple colonisations (Bock *et al.* 2015). The previous study found that invasive populations of the harlequin ladybird in fact exhibit high levels of genetic diversity, even if these remain slightly below those characterising native populations (Lombaert *et al.* 2011). As our estimates of allelic richness (R = 5.5–6.0) are similar to values reported by Lombaert et al. (2011), we can assume that the studied urban populations of the harlequin ladybird did not experience a reduction in genetic diversity. We also confirmed previous observations (Lombaert *et al.* 2010), indicating that the invasion of the harlequin ladybird has not been linked with bottleneck effects in the newly-established populations. In the case of urban areas investigated in our study, high genetic diversity probably arose due to a substantial number of founding individuals, or else rapid demographic recovery of the species, rather than through the admixture of genetically distinct sources in the course of multiple introductions. However, we did note the genetic after-effects of bottlenecks at some of the sampling sites in Olsztyn and Wrocław. This may suggest that certain populations experience demographic processes affecting genetic diversity, on the local scale at least. In an urban environment in particular, this could be linked with colonisation of a suitable habitat patch by a limited number of individuals, as followed by a period of isolation (Gortat *et al.* 2017). On the other hand, we failed to confirm bottleneck effects at sampling sites in Warsaw, a heavily built-up city in which effects of fragmentation ought to be pronounced. Moreover, our data suggested that genetic structuring,

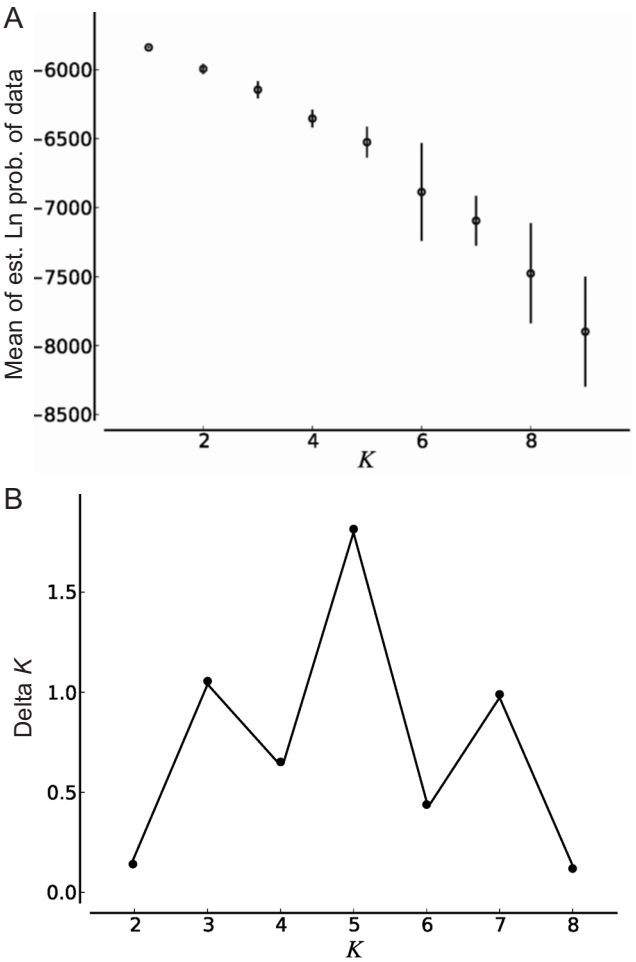

Figure 2 (A–B). Results of analysis in STRUCTURE. (A) estimated mean (and SD) likelihoods; (B) ΔK curves as a function of K. ΔK suggested the presence of five genetic clusters, however the mean likelihood was the highest and had the smallest variance for $K = 1$.

although slight, was more pronounced in Olsztyn and Wrocław than in Warsaw, with urban sampling sites in Olsztyn in fact characterised by significantly greater allelic richness. While these differences could reflect chance selection of sampling sites that differ in terms of the level of genetic diversity in populations of the species, the three urban areas investigated in our study were probably colonised by the harlequin ladybird during different periods. Although this has not been determined precisely, we can predict that Wrocław in the south-west was colonised first, followed by Warsaw in the centre of the country, and then Olsztyn in the north (Ceryngier 2008, Jadwiszczak 2008). If such a pattern is true, then we observe the highest genetic diversity in the urban population established most recently. Moreover, at two sampling sites in Olsztyn we found populations with a significant deviation from *HWE*, along with a third one displaying significant bottleneck effects. This could suggest that the urban population under study is still in a phase of expansion (Cornuet and Luikart 1996). Significant deviation from *HWE* was also noted for an urban population in Wrocław. However, unlike in Olsztyn, an excess of heterozygosity at the level of the urban population rather reflected a Wahlund effect, as all sampling sites in Wrocław supported populations in equilibrium. As Wrocław was colonised sooner than Olsztyn, the hypothesis would hold that, in the case of an urban population of the harlequin ladybird, successful colonisation is followed by the onset of a decrease in the genetic diversity of the established populations, with this reduction perhaps accompanying increasing isolation between local sampling sites within the urban environment. To elucidate whether observed differences result from the specific locations of sampling sites, or rather from differences in colonisation time and/or pattern between Wrocław, Warsaw and Olsztyn, further study involving more sampling sites within particular urban areas will be required.

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Appendix 1. Characterization of polymorphisms in 10 microsatellite loci in the harlequin ladybirds (N = 196), collected in nine sampling sites in three cities in Poland. n — sample size; A — number of alleles; R_A — allelic richness; R_P — private allelic richness, H_O — heterozygosity observed; H_E — heterozygosity expected; *HWE* — *P*-values for *HWE* exact test for heterozygote deficiency/excess; in bold — deviation significant at α = 0.05; F_{IS} — fixation index. * — F_{IS} values significant after Bonferroni correction. Sampling site and city abbreviations as in Material and methods.

Locus	n	A	R_A	R_P	H_O	H_E	*HWE*	F_{IS}
				OLS-1				
Ha-244	18	11	10.66	0.32	0.889	0.873	0.685	0.011
Ha-201	18	8	7.66	0.04	0.833	0.779	0.242	-0.041
Ha-281	18	8	7.77	0.00	0.611	0.810	**0.019**	0.272
Ha-605	18	12	11.30	0.01	0.778	0.762	0.296	0.008
Ha-267	17	6	6.00	0.00	0.765	0.715	0.811	-0.040
Ha-194b	18	2	2.00	0.00	0.444	0.475	1.000	0.093
Ha-215	18	6	5.99	0.00	0.667	0.688	0.242	0.060
Ha-223	18	4	3.87	0.00	0.167	0.252	0.065	0.363
Ha-253	18	3	2.99	0.00	0.222	0.329	0.110	0.349
Ha-105	18	4	3.78	0.05	0.222	0.551	**0.002**	0.615
				OLS-2				
Ha-244	25	13	11.35	1.99	0.880	0.882	0.448	0.023
Ha-201	25	8	7.49	0.11	0.760	0.742	0.534	-0.003
Ha-281	25	7	6.75	0.00	0.680	0.803	0.307	0.173
Ha-605	25	14	11.94	0.64	0.720	0.840	**0.006**	0.163
Ha-267	25	6	5.64	0.00	0.800	0.790	0.329	0.007
Ha-194b	25	3	2.64	0.64	0.240	0.510	**0.004**	0.544
Ha-215	25	6	5.83	0.00	0.680	0.710	0.931	0.063
Ha-223	25	3	2.83	0.00	0.200	0.185	1.000	-0.062
Ha-253	25	5	4.52	0.64	0.480	0.644	0.122	0.274
Ha-105	25	2	2.00	0.00	0.480	0.493	1.000	0.046
				OLS-3				
Ha-244	21	9	8.50	0.00	0.762	0.850	0.941	0.128
Ha-201	21	8	7.75	0.76	0.810	0.805	0.989	0.019
Ha-281	21	10	9.22	1.00	0.905	0.842	0.972	-0.050
Ha-605	21	11	9.98	0.00	0.714	0.837	0.142	0.170
Ha-267	19	7	6.68	0.84	0.579	0.745	0.068	0.249
Ha-194b	21	2	2.00	0.00	0.524	0.459	0.666	-0.117
Ha-215	21	5	4.76	0.00	0.667	0.740	0.399	0.124
Ha-223	21	3	2.99	0.00	0.333	0.291	1.000	-0.120
Ha-253	21	5	4.29	0.23	0.429	0.533	0.553	0.219
Ha-105	21	3	2.95	0.00	0.429	0.526	0.573	0.209
				OLS-ALL				
Ha-244	64	14	13.62	2.11	0.844	0.876	0.421	0.045
Ha-201	64	9	8.91	1.41	0.797	0.780	0.861	-0.013
Ha-281	64	10	9.91	0.92	0.734	0.839	0.417	0.132
Ha-605	64	15	14.88	0.93	0.734	0.870	**<0.001**	0.163*
Ha-267	61	7	6.95	0.52	0.721	0.769	0.163	0.071
Ha-194b	64	3	2.91	0.45	0.391	0.485	0.112	0.203
Ha-215	64	6	6.00	0.01	0.672	0.755	0.134	0.118
Ha-223	64	4	4.00	0.06	0.234	0.241	0.232	0.036
Ha-253	64	6	5.81	0.73	0.391	0.552	0.231	0.299*
Ha-105	64	4	3.91	0.31	0.391	0.530	0.073	0.270
				WAW-1				
Ha-244	29	10	8.91	0.00	0.724	0.837	**0.018**	0.152
Ha-201	29	7	6.54	0.00	0.793	0.743	0.417	-0.051

Appendix 1. Continued.

Locus	*n*	*A*	R_A	R_P	H_O	H_E	*HWE*	F_{IS}
Ha-281	29	7	6.73	0.00	0.724	0.805	0.513	0.118
Ha-605	29	14	10.95	0.80	0.828	0.796	0.860	-0.022
Ha-267	29	6	5.53	0.00	0.724	0.765	0.374	0.070
Ha-194b	29	3	2.55	0.55	0.483	0.513	0.585	0.077
Ha-215	29	6	5.35	0.17	0.655	0.732	0.843	0.122
Ha-223	29	3	2.55	0.00	0.207	0.216	0.114	0.059
Ha-253	29	5	4.57	0.00	0.552	0.583	0.801	0.072
Ha-105	29	3	2.80	0.03	0.552	0.532	1.000	-0.019
				WAW-2				
Ha-244	23	11	10.22	0.70	0.870	0.871	0.657	0.023
Ha-201	23	7	6.52	0.00	0.826	0.783	0.848	-0.033
Ha-281	23	7	6.98	0.00	0.826	0.824	0.916	0.020
Ha-605	23	11	9.58	0.00	0.739	0.796	0.484	0.093
Ha-267	23	7	6.30	0.21	0.826	0.733	0.652	-0.104
Ha-194b	23	2	2.00	0.00	0.522	0.491	1.000	-0.039
Ha-215	23	5	4.69	0.00	0.739	0.704	1.000	-0.027
Ha-223	23	3	2.89	0.00	0.130	0.199	0.089	0.365
Ha-253	23	4	3.69	0.17	0.348	0.555	**0.028**	0.392
Ha-105	23	4	3.39	0.06	0.391	0.538	0.172	0.293
				WAW-3				
Ha-244	20	8	7.75	0.00	0.800	0.828	0.501	0.059
Ha-201	20	7	6.40	0.80	0.700	0.626	0.881	-0.092
Ha-281	20	9	8.69	0.01	0.650	0.839	**0.002**	0.249
Ha-605	20	9	8.55	1.00	0.850	0.768	0.202	-0.082
Ha-267	19	5	4.84	0.00	0.632	0.723	0.059	0.153
Ha-194b	20	2	2.00	0.00	0.400	0.480	0.636	0.191
Ha-215	20	7	6.76	1.00	0.900	0.808	0.714	-0.089
Ha-223	19	4	3.84	0.00	0.474	0.515	0.280	0.107
Ha-253	20	4	3.80	0.00	0.500	0.569	0.424	0.146
Ha-105	20	3	2.80	0.00	0.550	0.454	0.726	-0.188
				WAW-ALL				
Ha-244	72	11	10.80	0.63	0.792	0.855	0.168	0.081
Ha-201	72	8	7.81	0.45	0.778	0.752	0.601	-0.027
Ha-281	72	9	8.77	0.06	0.736	0.834	0.409	0.125
Ha-605	72	16	15.58	1.60	0.806	0.796	0.396	-0.005
Ha-267	71	7	6.78	0.21	0.732	0.754	0.746	0.036
Ha-194b	72	3	2.81	0.40	0.472	0.498	0.461	0.059
Ha-215	72	8	7.61	1.09	0.750	0.753	0.957	0.011
Ha-223	71	4	4.00	0.04	0.254	0.306	0.110	0.179
Ha-253	72	6	5.80	0.62	0.472	0.573	0.127	0.182
Ha-105	72	5	4.73	0.30	0.500	0.527	0.684	0.058
				WRO-1				
Ha-244	20	10	9.72	0.00	0.800	0.868	0.228	0.103
Ha-201	20	6	6.00	0.00	0.750	0.778	0.194	0.061
Ha-281	20	8	7.76	0.01	0.750	0.839	0.213	0.131
Ha-605	20	11	10.29	0.00	0.850	0.850	0.941	0.026
Ha-267	20	5	4.99	0.00	0.750	0.753	0.564	0.029
Ha-194b	20	2	2.00	0.00	0.450	0.469	1.000	0.066
Ha-215	20	5	4.80	0.00	0.750	0.758	0.330	0.036
Ha-223	20	3	2.60	0.00	0.100	0.096	1.000	-0.013

Appendix 1. Continued.

Locus	*n*	*A*	R_A	R_P	H_O	H_E	*HWE*	F_{IS}
Ha-253	19	4	3.82	0.00	0.263	0.544	**0.008**	0.536
Ha-105	20	4	3.76	0.19	0.400	0.561	0.255	0.311
				WRO-2				
Ha-244	17	8	8.00	0.00	0.706	0.849	0.149	0.198
Ha-201	17	6	6.00	0.00	0.647	0.785	0.447	0.205
Ha-281	17	8	8.00	0.00	0.765	0.830	0.496	0.109
Ha-605	17	9	8.88	0.00	0.824	0.794	0.371	-0.007
Ha-267	17	5	5.00	0.00	0.706	0.775	0.344	0.119
Ha-194b	17	2	2.00	0.00	0.353	0.360	1.000	0.050
Ha-215	17	6	6.00	0.00	0.765	0.779	0.858	0.048
Ha-223	17	3	3.00	0.00	0.294	0.424	0.132	0.333
Ha-253	16	5	5.00	0.04	0.688	0.623	0.598	-0.071
Ha-105	17	4	3.88	0.10	0.353	0.554	0.013	0.389
				WRO-3				
Ha-244	23	9	8.85	0.00	0.696	0.860	**0.033**	0.213
Ha-201	23	6	5.97	0.00	0.609	0.774	0.293	0.235
Ha-281	23	9	8.27	0.01	0.696	0.825	0.063	0.179
Ha-605	23	13	10.67	0.70	0.913	0.818	0.216	-0.095
Ha-267	23	7	6.67	0.21	0.913	0.820	0.533	-0.091
Ha-194b	23	2	2.00	0.00	0.348	0.386	0.613	0.120
Ha-215	23	8	7.22	1.22	0.739	0.784	**0.042**	0.079
Ha-223	23	4	3.39	0.70	0.261	0.235	1.000	-0.086
Ha-253	23	4	3.67	0.00	0.391	0.518	0.272	0.265
Ha-105	23	3	2.91	0.28	0.565	0.532	0.685	-0.040
				WRO-ALL				
Ha-244	60	10	9.97	0.06	0.733	0.879	**0.004**	0.174*
Ha-201	60	6	6.00	0.00	0.667	0.798	0.204	0.173
Ha-281	60	9	9.00	0.30	0.733	0.852	**0.016**	0.148
Ha-605	60	15	14.90	0.87	0.867	0.876	0.464	0.019
Ha-267	60	7	6.97	0.29	0.800	0.802	0.215	0.010
Ha-194b	60	2	2.00	0.00	0.383	0.413	0.546	0.081
Ha-215	60	8	7.97	1.05	0.750	0.788	**0.005**	0.057
Ha-223	60	5	4.93	0.49	0.217	0.254	0.240	0.157
Ha-253	58	5	5.00	0.12	0.431	0.562	0.074	0.241
Ha-105	60	6	5.96	1.01	0.450	0.553	**0.031**	0.194
				ALL				
Ha-244	196	15			0.791	0.876	**<0.001**	0.100*
Ha-201	196	10			0.750	0.781	0.471	0.042
Ha-281	196	10			0.735	0.846	**0.003**	0.134*
Ha-605	196	18			0.801	0.856	**0.025**	0.067
Ha-267	192	8			0.750	0.781	**0.028**	0.042
Ha-194b	196	4			0.418	0.475	0.087	0.122
Ha-215	196	9			0.724	0.769	**0.009**	0.061
Ha-223	195	5			0.236	0.270	0.058	0.128
Ha-253	194	7			0.433	0.564	**0.002**	0.235*
Ha-105	196	6			0.449	0.537	**0.036**	0.166