

Intense Browsing by Sika Deer (*Cervus Nippon*) Drives the Genetic Differentiation of Hairy Nettle (*Urtica Thunbergiana*) Populations

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Abstract

Many studies have inferred the way in which natural selection, genetic drift and gene flow shape the population genetic structures, but very few have quantified the population differentiation under spatially and temporally varying levels of selection pressure, population fluctuation and gene flow. In Nara Park (6.6 km²; NP), central Japan, where several hundred sika deer (*Cervus nippon*) have been protected for more than 1,200 years, heavily- or moderately-haired nettle (*Urtica thunbergiana*) populations have evolved probably in response to intense deer browsing. Here, we analysed the genetic structure of two populations from NP and five from surrounding areas using amplified fragment length polymorphism markers. A total of 546 marker loci were genotyped from 210 individuals. A Bayesian method estimated 5.5% of these loci to be outliers, which are putatively under natural selection. Neighbour-joining, Bayesian clustering and principal coordinates analyses using all-loci, non-outlier loci and outlier loci datasets showed that the two populations from NP formed a cluster distinct from the surroundings. These results indicate the genome-wide differentiation of the populations from NP and the surroundings. Moreover, these imply that: (1) gene flow is limited between these populations and thus genetic drift is a major factor causing the differentiation; and (2) natural selection imposed by intense deer browsing has contributed to some extent to the differentiation. In conclusion, sika deer seems to have counteracted genetic drift to drive the genetic differentiation of hairy nettles in NP. This study suggests that a single herbivore species could lead genetic differentiation among plant populations.

Introduction

Natural selection, genetic drift and gene flow interplay to influence genetic differentiation of populations (Templeton 2006; Futuyma and Kirkpatrick 2018; Freeland 2020). Natural selection can lead to local adaptation and then cause differentiation of traits or genes involved in the adaptation, while genetic drift can promote differentiation of neutral genes. On the other hand, gene flow generally impedes population differentiation. In recent decades, numerous empirical studies have analysed how natural selection, genetic drift and gene flow have shaped the genetic structure of plant populations occurring under different abiotic conditions such as altitude, temperature, precipitation and soil type using newly developed or improved molecular genetic techniques assisted with advanced mathematical and statistical methods (Müller et al. 2008; Gonzalo-Turpin and Hazard 2009; Westberg et al. 2013; Frei et al. 2014; Hamann et al. 2016; Di Pierro et al. 2016; Grdiša et al. 2019; Marshall et al. 2019; Hirao et al. 2020).

Many studies have also examined how biotic factors such as herbivory affect local adaptation (Pollard and Briggs 1982, 1984a, 1984b; Voure'h et al. 2001; Arany et al. 2009; Garrido et al. 2012; Didano et al. 2014; Lehndal and Ågren 2015; Martin et al. 2015; Skaïen and Arcese 2020), yet very few have evaluated the relative importance of natural selection, genetic drift and gene flow in shaping population genetic structure at different levels of their effects. A rare example is a series of studies of northern European populations of the perennial herb *Arabidopsis lyrata* which consists of glabrous and trichome-bearing forms (Kärkkäinen and Ågren 2002; Kärkkäinen et al. 2004; Kivimäki et al. 2007; Løe et al. 2007; Gaudeul et al. 2007; Leinonen et al. 2009). In this herb, populations under heavy herbivory by the diamond-back

moth *Plutella xylostella* are monomorphic with trichome-producing form, while those under its low herbivory are polymorphic in this trait. However, there is little genetic differentiation at the genome-wide level between monomorphic and polymorphic populations. These results suggest that gene flow may prevent the differentiation of most genes despite the fact that differential herbivory by the moth species leads to the differentiation of genes responsible for the production of trichomes.

The Japanese stinging nettle *Urtica thunbergiana* (Rosales, Urticaceae) is a wind-pollinated, perennial herb with stinging hairs on leaves and stems. Nettle populations in Nara Park (hereafter NP; 6.6 km²), which is adjacent to the urban area of Nara City, central Japan, are heavily haired (Fig. 1A, B), whereas those in the surrounding areas are lightly haired (Fig. 1C, D; Kato et al. 2008; Shikata et al. 2013). In NP, several hundred sika deer (*Cervus nippon* (Artiodactyla, Cervidae)) have been protected for more than 1,200 years as sacred animal. Our previous work has revealed that heavily-haired nettles in NP are resistant to sika deer but not herbivorous insects such as the red admiral *Vanessa indica* (Kato et al. 2008; Iwamoto et al. 2014) and that variation in hairiness among nettle populations are genetically based (Kato et al. 2008; Hirata et al. 2019). These findings suggest that heavy hairiness has evolved through natural selection under intense browsing by sika deer. In addition, nettles from NP exhibit lower plant height, higher degree of branching, earlier and longer flowering, higher resource allocation to leaves and inflorescences, and higher proportion of monoecious individuals than those from a surrounding area where deer are rarely found (Hirata et al. 2019). Most of these traits are assumed to have evolved to cope with intense browsing by deer as well as hairiness, although some traits may also have been influenced by other factors (Hirata et al. 2019). Nettles are patchily distributed in NP and surrounding areas, and thus their evolution and differentiation should be influenced by genetic drift and gene flow. Therefore, nettle populations from NP and surrounding areas provide an excellent system for studying how natural selection, genetic drift and gene flow interplay to shape the evolution of phenotypic traits.

This study aims to assess the relative importance of natural selection, genetic drift and gene flow to the genetic differentiation of heavily-, moderately- and lightly-haired populations from NP and surrounding areas. For this purpose, we performed a comprehensive genetic analysis using amplified fragment length polymorphism (AFLP) markers. More specifically, we (1) compared genetic diversity among nettle populations, (2) examined the degree of genetic differentiation among populations of NP and the surrounding areas, (3) identified F_{ST} outlier loci, or signals of natural selection, on AFLP markers, (4) examined phylogenetic relationships among populations, and (5) quantified the population genetic structure.

Materials And Methods

Sampling Sites

Nara Park is adjacent to the town of Nara City, central Japan, having an area of 6.6 km² (Fig. 2). World Heritage Sites such as Todaiji Temple, Kofukuji Temple and Kasugataisha Shrine are located on the west side of NP, and grassy and woody areas spread in the remaining areas. In NP, hundreds of sika deer

(*Cervus nippon*) have been protected for 1200 years for religious reasons (Torii and Tatsuzawa 2009), and its number reached 1095 in 2011 (Foundation for the Protection of Deer in Nara Park 2020). In the grassy and woody areas, patchy colonies of *U. thunbergiana* are found at a distance of one to a few hundred metres from one another (Kato 2001; Shikata et al. 2013). Here we refer to each colony as a population for expediency.

We sampled leaves of *U. thunbergiana* for DNA extraction from seven sites (Fig. 2): Todaiji (110 m a.s.l.) located in the central area of NP; Notogawa (150 m) in a marginal area of NP; Kohbohji (100 m) in a vacant lot of a residential area close to NP; Nara Women's University campus (80 m) in the urban area near NP; Sakurai (140 m), Takatori (550 m) and Kouchidani (280 m) at the edges of cedar or cypress forests located 17 to 28 km from NP (see Shikata et al. 2013 for topographical details). Of them, the Todaiji population (hereafter TO; patch size $4 \times 10^3 \text{ m}^2$) is subjected to the most intensive browsing by sika deer (Shikata et al. 2013). The Notogawa population (NO; $1.4 \times 10^3 \text{ m}^2$), which grows on steep banks of Notogawa Stream, avoids intense deer browsing. The Kohbohji population (KO; $0.38 \times 10^3 \text{ m}^2$) is almost free from deer browsing because a wire fence prevents the access by deer. The population in Nara Women's University campus (NWU; $2.4 \times 10^3 \text{ m}^2$) also scarcely suffers deer browsing because the campus is enclosed by woody fences. On the other hand, no or few deer were found around the Sakurai (SA; $0.2 \times 10^3 \text{ m}^2$), Takatori (TA; $22.5 \times 10^3 \text{ m}^2$) and Kouchidani (KC; $2.5 \times 10^3 \text{ m}^2$) populations before 2011 (Shikata et al. 2013), but they have been frequently observed around TA and KC since 2016 (HS, personal observation).

Leaf traits

We determined the among-population difference in leaf area and the number of stinging hairs on a leaf by common garden experiments and re-analysis of Shikata et al.'s (2013) data on wild populations. Shikata et al. (2013) randomly chose 32 *U. thunbergiana* individuals for each of the above-mentioned seven populations and collected two intact, fully grown leaves on the second or third node from the tip of main shoots of each individual in late May 2008. We carried out common garden experiments according to Hirata et al. (2019). Briefly, we collected 20 to 40 seedlings from all locations except Kouchidani in mid-March 2015 and were grown in a greenhouse at Nara Women's University (nettles from Kouchidani were not raised because of the limitation of greenhouse space). The structure and light conditions of the greenhouse were described in Hirata et al. (2009). We planted seedlings individually in flowerpots (12.5 cm in diameter and 13.3 cm in depth) with potting soil (Takii-no-baiyodo, Takii & Co., Ltd., Kyoto, Japan). We paired two seedlings from different sites and placed in a plastic tray (30 cm in width, 42 cm in length and 5 cm in depth) filled with water. We then arrayed trays on tables in the greenhouse and rotated once every week to avoid position effects. On the same day, we supplied water to trays. On 18 June 2015, we collected two leaves on the second or third node from the tip of the main shoot from each surviving individual.

We scanned the upper surface of collected leaves at 600 dpi using an image scanner (EPSON GT-X770, Seiko Co., Suwa, Japan). We visually counted the number of stinging hairs on the leaf surface from the digital images, and measured leaf area using free software LIA for Windows95 ver. 0.373 (Yamamoto 1997). We calculated the number of stinging hairs per unit area (i.e., stinging hair density) by dividing the number of hairs by leaf area. We tested among-population differences in leaf area, stinging hair number and stinging hair density using sequential Šidák multiple comparison post hoc test ($\alpha = 0.05$) implemented in generalized linear mixed models (GLMMs). All GLMMs included population as fixed factor and individual as random factor. In the analysis for stinging hair density, we used its number as response variable and the logarithm of leaf area as offset term. Error distributions and link functions were Gaussian and identity-link for leaf area, and Poisson and log-link for stinging hair number and density. We performed GLMMs using IBM SPSS ver. 25 (IBM SPSS 2017).

DNA extraction and AFLP analysis

We randomly chosen 30 *U. thurgiana* plants in each population on 1 or 2 June 2010, and sampled one young leaf from each plant for DNA analyses. We extracted total genomic DNA using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with the manufacturer's protocol.

We performed AFLP analysis following the methods described in Kohyama et al. (2014). In brief, we conducted the restriction, adapter ligation, pre-selective and selective amplification using the AFLP Plant Mapping Kit (Applied Biosystems, Foster City, CA, USA). For selective amplification, we used combinations of 12 primer pairs (EcoRI+/MseI+: AC/CTA, TC/CTT, TG/CTG, TA/CTC, AA/CAA, AG/CAT, AT/CAG, TT/CAC, AT/CTC, AC/CAG, TA/CTG, and TT/CAT). We analysed the amplified fragments by an Applied Biosystems 3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) and GeneScan 500 ROX dye Size Standard (Thermo Fisher Scientific). We made the scoring of AFLPs using the PeakScanner ver. 1.0 (Thermo Fisher Scientific) and the RawGeno ver. 2.0-1 (Arrigo et al. 2012). We randomly selected thirty individuals (about 14% of the total 210 individuals), and loci with a reproducibility rate of 85% or higher were used for subsequent analyses.

Analyses of genetic Data

We quantified genetic diversity within populations by the proportion of polymorphic loci and expected average heterozygosity. In addition, we examined genetic differentiation among populations by pairwise F_{ST} value and Nei's genetic distance (Nei 1972). We computed these genetic diversity and differentiation indices using the AFLP-SURV ver. 1.0 (Vekemans 2002).

To test the significance of the isolation-by-distance (Wright 1943) among nettle populations, we examined the correlation between pairwise F_{ST} and pairwise geographic distance using a Mantel test with 10,000 permutations as implemented in the vegan package ver. 2.5-6 (Oksanen et al. 2019) in R ver. 3.6.2 (R Core Team 2019).

To detect F_{ST} outlier loci, which are putatively under natural selection, we employed a Bayesian approach implemented in BayeScan ver. 2.1 (Foll and Gaggiotti, 2008) using the all-loci dataset. We run this analysis with a burn-in of 100,000 iterations, a thinning interval of 20, and a sample size of 300,000. We considered loci to be outliers when the logarithm of the posterior odds [$\log_{10}(PO)$] (i.e., the ratio of the posterior probability of the selection model to the neutral model) was greater than 0.5.

We inferred phylogenetic relationships among populations by the neighbour-joining method based on Nei's genetic distance for the all-loci, non-outlier loci and outlier loci datasets using PHYLIP ver. 3.6 (Felsenstein 2005).

To detect population genetic structure, we applied the Bayesian model-based cluster analysis implemented in STRUCTURE ver. 2.3.4 (Pritchard et al. 2000) for the three datasets. We did not determine the optimal number of actual genetic clusters using $\ln P(D)$ (Pritchard et al. 2000) or ΔK (Evanno et al. 2005) and, instead, examined grouping patterns across k values (cluster numbers). This is because there is a large degree of uncertainty in the estimation of k and there are few biological reasons to assume an only single value for k (Meirmans 2015).

Furthermore, to investigate genetic relationships among populations, we performed coordinates analysis (PCoA) based on pairwise Euclidian distances between individuals for all-loci, non-outlier loci and outlier loci datasets. We conducted PCoA using scikit-bio ver. 0.5.6 (The Scikit-Bio development team 2020) in Python ver. 3.8.6.

Results

The pattern of among-population variation in leaf area differed between wild and common-garden nettles (Fig. 3A, B). Wild nettles from TO and SA had significantly smaller leaf area than those of the other populations NO, KO, NWU, TA and KC, but common-garden nettles of the five populations TO, SA, NO, KO and TA did not significantly differ from each other (Fig. 3A). In contrast, wild and common-garden nettles showed similar variation patterns in stinging hair number and density (Fig. 3C-F); whether wild or raised in the common garden, TO was most heavily haired followed by NO and KO, while the other were only lightly haired.

We genotyped a total of 546 AFLP loci in 210 individuals from the seven populations. The proportion of polymorphic loci was highest in TO (75.5%), followed by NO (67%), and lowest in NWU (31.7%) (Table 1). Likewise, expected heterozygosity was highest in TO (0.200), followed by NO (0.195), and lowest in NWU (0.128).

Table 1 Genetic diversity based on 546 AFLP loci

Population	<i>N</i>	PLP	H_j	S.E. (H_j)
NA	30	75.5	0.200	0.0063
NO	30	67	0.195	0.0066
KO	30	48	0.146	0.0076
NWU	30	31.7	0.128	0.0067
SA	29	53.8	0.175	0.0076
TA	30	36.6	0.155	0.0075
KC	30	49.5	0.167	0.0079

N, sample size; PLP, percentage of polymorphic loci; H_j , expected average heterozygosity or Nei's gene diversity. See Fig. 2 for abbreviations of populations

All pairwise F_{ST} values among the seven populations were significantly different from zero ($P < 0.001$; Table 2), indicating that genetic differentiation occurred among populations overall. However, the value between TO and NO in NP was very low (0.031; Table 2). Despite that, there was no significant correlation between pairwise F_{ST} values and pairwise geographic distances (Mantel's $r = 0.066$, $P = 0.44$; Suppl 2); that is, the isolation-by-distance model (Wright 1943) was not supported in this study.

Table 2 Geographic distances (km; upper triangle) and pairwise F_{ST} values (lower triangle) between seven nettle populations in Nara Park (TO and NO) and its surrounding populations (KO, NWU, SA, TA and KC)

Population	TO	NO	KO	NWU	SA	TA	KC
TO	-	1.94	0.41	1.10	16.65	28.99	28.33
NO	0.031	-	2.33	2.46	14.93	27.43	26.66
KO	0.132	0.109	-	1.20	17.06	29.39	28.61
NWU	0.158	0.168	0.147	-	16.46	28.63	29.10
SA	0.116	0.112	0.126	0.181	-	12.79	23.48
TA	0.107	0.096	0.084	0.173	0.109	-	30.66
KC	0.154	0.117	0.107	0.241	0.140	0.115	-

F_{ST} values are based on 546 AFLP loci, and all of them were significantly different from zero with $P < 0.001$. See Fig. 2 for abbreviations of populations

The BayeScan programme identified 30 AFLP loci (5.5% of total loci) as F_{ST} outliers with explicit differentiation among populations ($\log_{10}(PO) > 0.5$; Suppl 1). These outlier loci may be linked to genes that are under natural selection. On the other hand, the non-outlier loci could be putatively regarded as neutral with respect to selection.

We constructed neighbour-joining trees with three datasets (i.e., those which are composed of all loci, non-outlier loci or outlier loci). TO (heavily haired population) and NO (one of the two moderately haired populations) located in NP were grouped together into a strongly supported cluster in the trees constructed with the all-loci and non-outlier loci datasets (bootstrap values were 100%; Fig. 4A, B), whereas their cluster was moderately supported in the tree constructed with the outlier-loci dataset (77%; Fig. 4C). The other five populations did not form distinct clusters (bootstrap values $< 73\%$). Note that KO (another moderately-haired population) and NWU (a lightly haired population) close to or near NP were consistently distinct from TO and NO in all trees.

We analysed the genetic structure of the seven populations using STRUCTURE for the three datasets. In the results, TO and NO in NP consistently remained in the same group irrespective of datasets and k values, and they formed a group together at $k \geq 3$ in the all-loci and non-outlier loci datasets (Figs. 4A, B and Suppl 3a, b) and at $k \geq 4$ in the outlier loci dataset (Figs. 4C and Supple 3c).

We also examined the genetic similarity among the populations using PCoA for the three datasets. In biplots for the all-loci and non-outlier loci, TO and NO in NP were clearly separated from the other populations along the first principal component, forming a group, while NWU near NP was distinguishable from the others along the second component (Figs. 6A, B and Suppl 4a, b). For the outlier

loci dataset, TO and NO were identifiable along not the first but second component, while NWU was separated along the first component (Figs. 6C and Suppl 4c).

Discussion

Our previous study have reported that nettles in NP are heavily haired, while those in the surrounding areas are lightly haired, and also suggested that this variation is genetically based (Kato et al. 2008; Shikata et al. 2013; Hirata et al. 2019). This notion is supported by the present study; common-garden individuals showed the pattern of among-population differences similar to wild individuals (Fig. 3C-F). On the other hand, leaf area varied a little among common-garden individuals from different populations compared with wild individuals (Fig. 3A, B). This suggests that among-population variation in leaf area is influenced largely by environmental factors. Despite that, the among-population variation in the number of stinging hairs on a leaf was robust to environmental differences. The present study populations, therefore, can be assigned TO as heavily haired, NO and KO moderately haired, and NWU, SA, TA and KC lightly haired.

The TO in NP is conspicuously, heavily haired compared to the other populations (Figs. 1 and 3C-F). Evidently, this characteristic has evolved as a result of adaptation to intense deer browsing from our present and previous findings that (1) stinging hairs serve as defence against sika deer but not herbivorous insects such as the red admiral butterfly (Kato et al. 2008; Iwamoto et al. 2014), (2) among-population variation in stinging hair number and density is genetically based (Fig. 3C-F; Kato et al. 2008; Hirata et al. 2019), and (3) populations subjected to intense deer browsing are heavily haired, whereas populations almost free from browsing are lightly haired (Fig. 3C-F; Kato et al. 2008; Shikata et al. 2013). Another population from NP, or NO, is moderately haired. This is probably ascribed to the fact that NO is less accessible to deer because it grows on steep banks (Shikata et al. 2013).

All pairwise F_{ST} values among the seven nettle populations based on the all-loci dataset were significant (Table 2), indicating that these populations are well differentiated from one another. TO and NO in NP are, however, relatively close according to neighbour-joining analysis, STRUCTURE and PCoA (Figs. 4A, 5A and 6A). The differentiation among populations cannot be explained by the isolation-by-distance theory (Wright 1943), because the genetic and geographical distances were not significantly correlated (Suppl 2). Instead, the differentiation appears to be related to the difference in stinging hair number and density (Fig. 3C-F). Genetically similar TO and NO are heavily or moderately haired, while the other populations but KO are lightly haired. This would lead to the inference that the differentiation of TO and KO in NP from the others is influenced by natural selection.

In populations that are adapted to different habitats but experience gene flow, genes involved in the expression of adaptive traits may show larger differentiation than those which are neutral to natural selection. Such genes under strong selection could be detected as outlier loci (Holderegger et al. 2010; Nosil 2012). Neutral genes and DNA regions, meanwhile, would be assigned as non-outlier loci, and they are expected to show non-adaptive differentiation because of the interplay of gene flow and genetic drift

(Nosil 2012; Freeland 2020). In the following sections, we discuss how natural selection, gene flow and genetic drift interplay to influence the genome-wide differentiation of NP populations on the basis of results obtained by neighbour-joining analysis, STRUCTURE and PCoA for the non-outlier and outlier loci datasets.

Natural selection

The neighbour-joining analysis, STRUCTURE and PCoA for the non-outlier loci dataset indicate that TO and NO in NP are genetically separated from the other populations including KO and NWU located close to NP (Figs. 4B, 5B and 6B), implying that gene flow occurs more frequently between the two populations from NP than between populations in NP and the surrounding areas. Similarly, those analyses for the outlier dataset show similar patterns, but somewhat unclearly; in the neighbour-joining tree, the two populations from NP form a moderately-defined clade with a bootstrap value of 77% (but 100% for the non-outlier loci datasets; Fig. 4); in STRUCTURE, they belong to the same genetic cluster at $k \geq 4$ (but $k \geq 3$; Figs. 5 and One Resource 3B); in PCoA, they are separated from the other populations along the second principal component (but the first one; Fig. 6 and Suppl 4b). Thus, the genetic differentiation of the two populations from NP in outlier loci would not be so large as that in non-outlier loci. This could be explained by a substantial difference in the selection pressure imposed by deer browsing between the two populations from NP. As mentioned above, TO is subjected to intense deer browsing. This should exert a strong selective pressure on this population to maintain not only heavy hairiness but also small stature, high degree of branching, early and long flowering period, high resource allocation to leaves and flowers, and dominance of monoecy (Hirata et al. 2019). These characteristics would help to enhance resistance and tolerance to sika deer and to reduce damages on growth and reproduction (Hirata et al. 2019). In contrast, NO suffers only moderate browsing. Therefore, if alleles causing less effective defence are brought into the populations by gene flow, they would be rapidly eliminated from TO but may remain longer in NO, resulting in the differentiation of genes involved in the defensive traits (i.e., candidates of outlier loci) between the two populations.

Another possible explanation may be that either or both of TO and NO have been affected by genetic drift. However, it is unlikely that genetic drift drives the differentiation of all outlier loci underlying defensive traits. Effects of genetic drift will be considered again later.

The presence of outlier loci may be a result of natural selection (Nosil 2012). The proportion of loci identified as outliers in this study was 5.5%. This figure is roughly in concordance with a survey of Strasburg et al. (2012), who found that the proportion of outliers ranged from 0.4 to 35.5% with a median of 4.2% through review of 22 plant genome scan studies. It is not known what functions our outlier loci have, but some may be related to the production of stinging hairs, while others to the expression of the plant defensive traits mentioned above. Loci underlying these traits remain to be identified. Nevertheless, our sampling design was insufficient to filter out outlier loci undergoing selection, because nettles with

defensive traits against deer browsing are limited to occur in the narrow area of NP. Hence, some of these outlier loci probably also contain region-specific loci that are unrelated to adaptation to deer browsing.

Gene flow

The neighbour-joining analysis, STRUCTURE and PCoA for the non-outlier dataset (Figs. 4B, 5B and 6B) show that the genetic cluster of TO and NO in NP are separated distinctly from the surrounding populations. This implies that gene flow between populations in NP and the surrounding areas is limited.

The KO is interesting, because despite being moderately haired and occurring close to NP, it does not belong to the genetic cluster of NP populations regardless of datasets (Figs. 4, 5 and 6). This population is protected from deer browsing by a wire fence. Hence its moderate hairiness cannot be regarded as local adaptation to deer browsing. Hairiness of KO could be accounted for by founder effect. KO colonized at a vacant lot in 1980's when the house was removed and the ground was cleaned up. Its founders may have originated from a hairy population of NP which has a different set of neutral genes from those which TO and NO have. Although their descendants were released from selection imposed by deer browsing, the time since colonization may be too short for the reduction of hairs to proceed. This hypothesis can be confirmed by searching populations in NP with the same set of neutral genes as KO.

It is also worth noting that although TO and NWU are only 1.1 km apart from each other, they are morphologically and genetically distinct (Figs. 4-6). This implies that gene flow between the two populations is hindered despite the spatial closeness. Given this, it is natural that gene flow is almost absent between populations from NP and more distantly surroundings such as SA, TA or KC. Such a situation would act to maintain the genetic distinctness of populations in NP.

Gene flow in nettles can occur through pollen and seed dispersal; pollen is dispersed by wind, while seeds are probably transported by mammals (Pollard and Briggs 1984b). Pollen of European stinging nettle, *U. dioica*, may be blown several hundred metres in open lands (Pollard and Briggs 1984b). Because nettle fruits are very small (ca. 1 mm in length) and their surface (perianth) is hairy, they would adhere to the fur of mammals. In addition, mature seeds falling on the ground would adhere to mammal's toes with soil. In the present study system, however, pollen dispersal should be hindered considerably in urban areas and forests (Holderegger et al. 2010; Miles et al. 2019), and seed vectors may hardly migrate across these areas.

The level of gene flow among populations in NP should be high, because TO located in the centre of NP and NO located in a marginal area of NP (1.9 km apart; Table 1, Fig. 2) have similar compositions of neutral loci (Figs. 4B, 5B and 6B). Probably nettle populations in NP form a metapopulation in which constituent populations are connected with one another by gene flow via intervening populations. Nevertheless, gene flow level may not be so high as to hinder their genetic differentiation from one another; otherwise, the pairwise F_{ST} between TO and NO should have been statistically insignificant (Table 2).

Genetic drift and genetic diversity

In general, the effect of genetic drift on the differentiation of neutral loci becomes apparent under reduced gene flow. As mentioned earlier, non-outlier loci identified in this study account for 94.5% and are substantially differentiated between the populations from NP and the surrounding areas. Considering that gene flow is almost absent or very low between the two groups of populations and that non-outlier loci are probably selectively neutral (Nosil 2012), the differentiation of these non-outlier loci may have been generated mostly by genetic drift.

The TO and NO in NP show higher proportions of polymorphic loci and higher degrees of expected heterozygosity than the surroundings, having higher genetic diversity (Table 1). This is apparently inconsistent with the hypothesis that natural selection operates strongly in nettle populations of NP, because selection for fittest genotypes should lead to the elimination of all the other genotypes (Freeland 2020). However, given that the proportion of outlier loci, which are putatively subject to natural selection, is 5.5%, this inconsistency may be negligible. There are also three other possible explanations for higher genetic diversity in the populations from NP. First, as mentioned before, patchily distributed populations in NP are connected to one another by weak gene flow, forming a large meta-population. This can reduce the effect of genetic drift and help to maintain relatively high genetic diversity. Second, populations in NP as a whole seem to have experienced neither bottlenecks nor resulting loss of genetic variation in the past, because the environment and landscape of NP are believed to have persisted without considerable changes for 1200 years. Third, although deer browsing pressure is intense in NP as a whole, the extent actually varies according to fine-scale topography. For instance, nettles growing on steep slopes, such as NO on the banks of a stream, can avoid heavy browsing, and accordingly they are moderately haired (Shikata et al. 2013). In addition, the surrounding populations (NO, NWU, SA, TA and KC) are isolated and their colony sizes are far small compared with that of the metapopulation of NP (see "Sampling Sites"). Thus, high genetic diversity of TO and NO could be explained by meta-population structure, population stability and environmental heterogeneity. In plant populations, polyploidy has also been known as a factor causing high genetic diversity (Sampson and Byrne, 2012). However, polyploidy seems to be unlikely in the two populations from NP, because nettles from TO and a surrounding population, TA, produce fertile seeds in reciprocal crosses (unpublished data).

Conclusion

This study confirmed the Hirata et al's (2019) notion that among-population variation in the number of stinging hairs on a leaf in nettles is genetically based. Furthermore, we demonstrated that *U. thunbergiana* populations from NP, which clearly exhibit local adaptation to deer browsing, have differentiated genome-widely from the surroundings which are almost free from browsing. In addition, our data show relatively fine-scale genetic differentiation; it occurs between populations located 1.1 km apart from each other. Based on the proportion of putatively neutral loci (94.5%), divergence of most loci may be due to genetic drift under limited gene flow. Differentiation of outlier loci (5.5%), which are putatively subjected to selection, should have been promoted partly by one herbivorous animal, sika deer. Several

studies demonstrated that a single herbivore species causes plant species to evolve resistant traits (e.g., the relationship between diamond-back moth *Plutella xylostella* and perennial herb *Arabidopsis lyrata*; see Introduction) but no studies revealed that the herbivore even leads genetic differentiation among plant populations. Nettles under intense browsing by sika deer exhibit not only heavy hairiness but also low plant height, high degree of branching, early and long flowering, high resource allocation to leaves and inflorescences, and dominance of monoecy compared with those almost free from deer browsing (Hirata et al. 2019). These characteristics would help to enhance resistance and tolerance to sika deer and to reduce damages on growth and reproduction (Hirata et al. 2019). The expression of these traits should be under the control of many genes, most of which may be located on the outlier loci assigned in this study. Identification of these genes remains to be performed but is very challenging.

Declarations

Authors' declaration

The authors declare that they have no conflict of interest.

Author contribution statement

TK, MTK and HS conceived the study and planned study design. TK and HS collected materials from the field. MY grew nettles in the greenhouse and measured leaf characteristics. TK conducted the laboratory work and statistical analyses. HS wrote the early draft. All authors read, edited, and approved the final submitted version of the manuscript.

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Figures

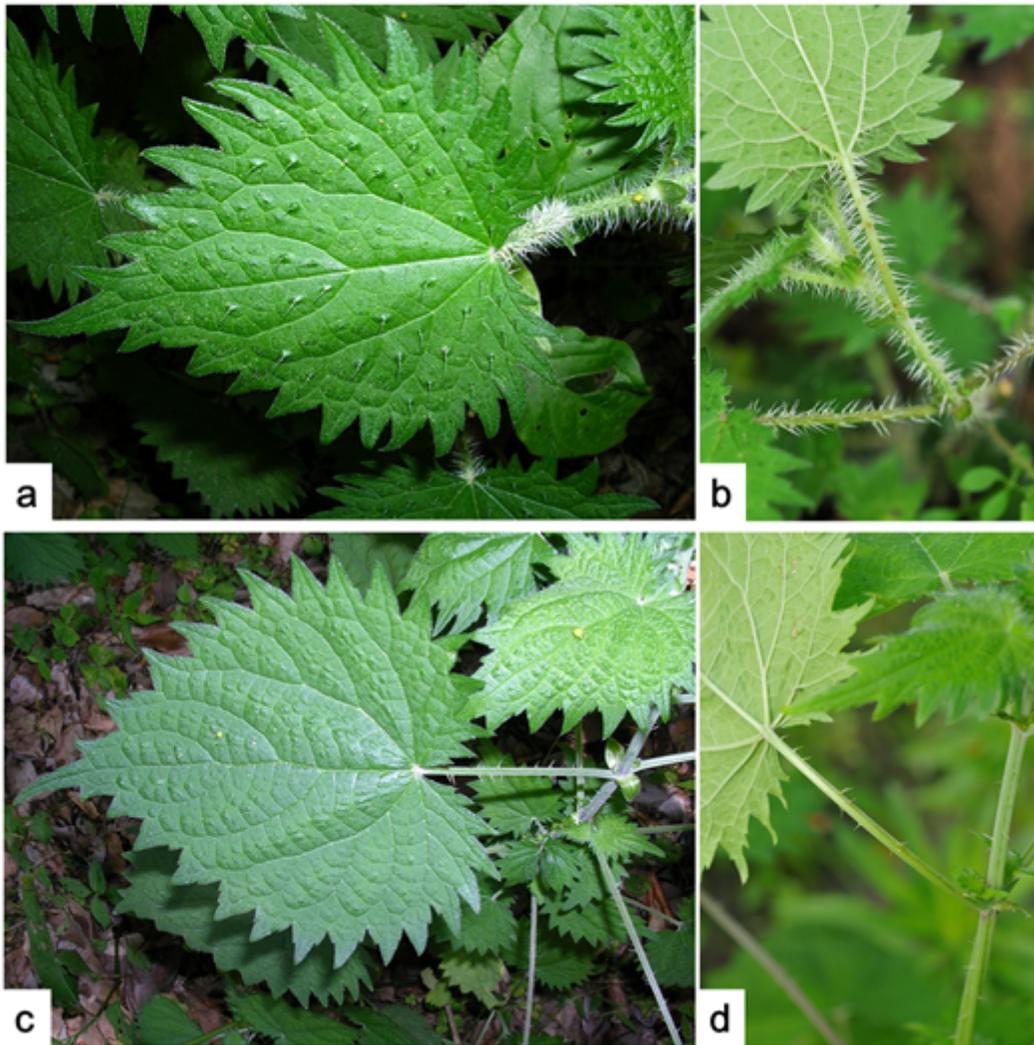


Figure 1

a, b Heavily haired nettle of Todaiji population (TO) in Nara Park (NP) and c, d lightly haired nettle of Takatori population (TA) in Takatori Castle Site

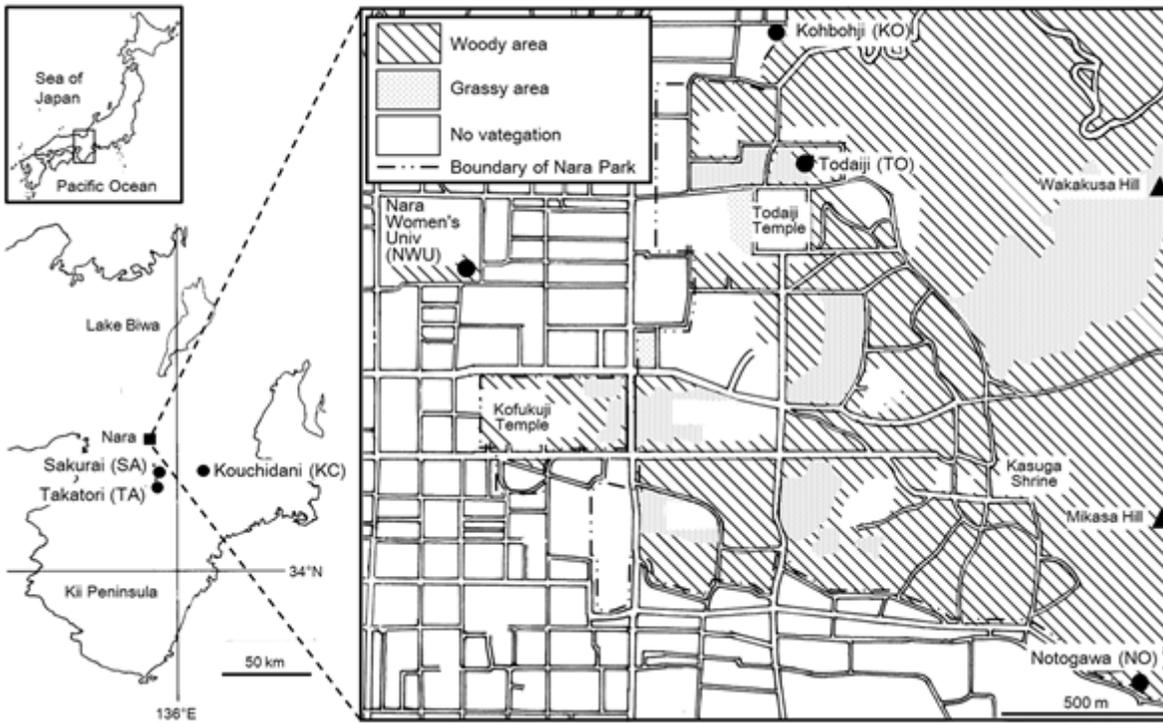


Figure 2

Map of seven *Urtica thunbergiana* populations sampled. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

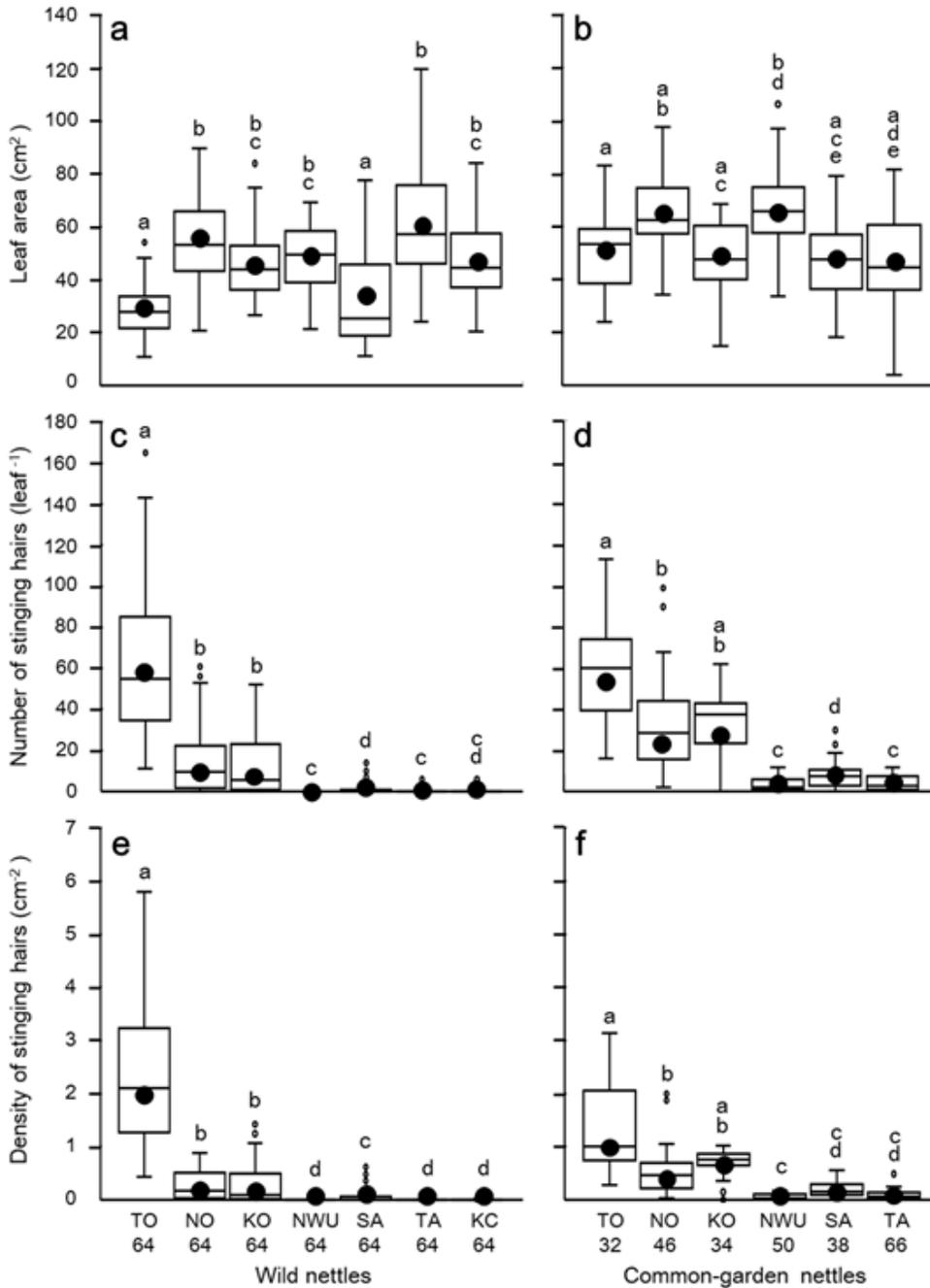


Figure 3

Box-whisker plots of a, b leaf area, c, d stinging hair number (leaf⁻¹) and (e, f) stinging hair density (cm⁻²) of a, c, e wild and b, d, f common-garden nettles from seven or six *Urtica thunbergiana* populations. Mean values (solid circles; expected by GLMMs) with the same letters are not different significantly at $\alpha = 0.05$ according to sequential Šidák multiple comparison post hoc tests. The upper and lower levels of the box represent the third and first quartiles respectively, the solid horizontal line the median, open circles

outliers (over 1.5 times the interquartile range), and the upper and lower tips of the whisker the maximum and minimum values, respectively, when outliers are excluded. Numbers under abbreviations of populations indicate sample sizes. See Fig. 2 for population abbreviations

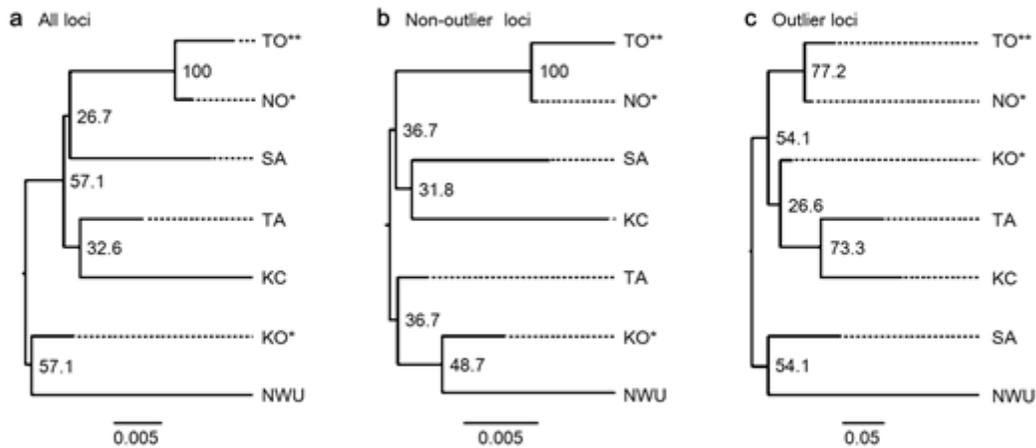


Figure 4

Neighbour-joining trees based on Nei’s genetic distances using datasets of a all loci, b non-outlier loci and c outlier loci. Bootstrap values (%) based on 10,000 replicates are shown by blanch points. See Fig. 2 for abbreviations of populations. **: heavily haired; *: moderately haired; no asterisks: lightly haired

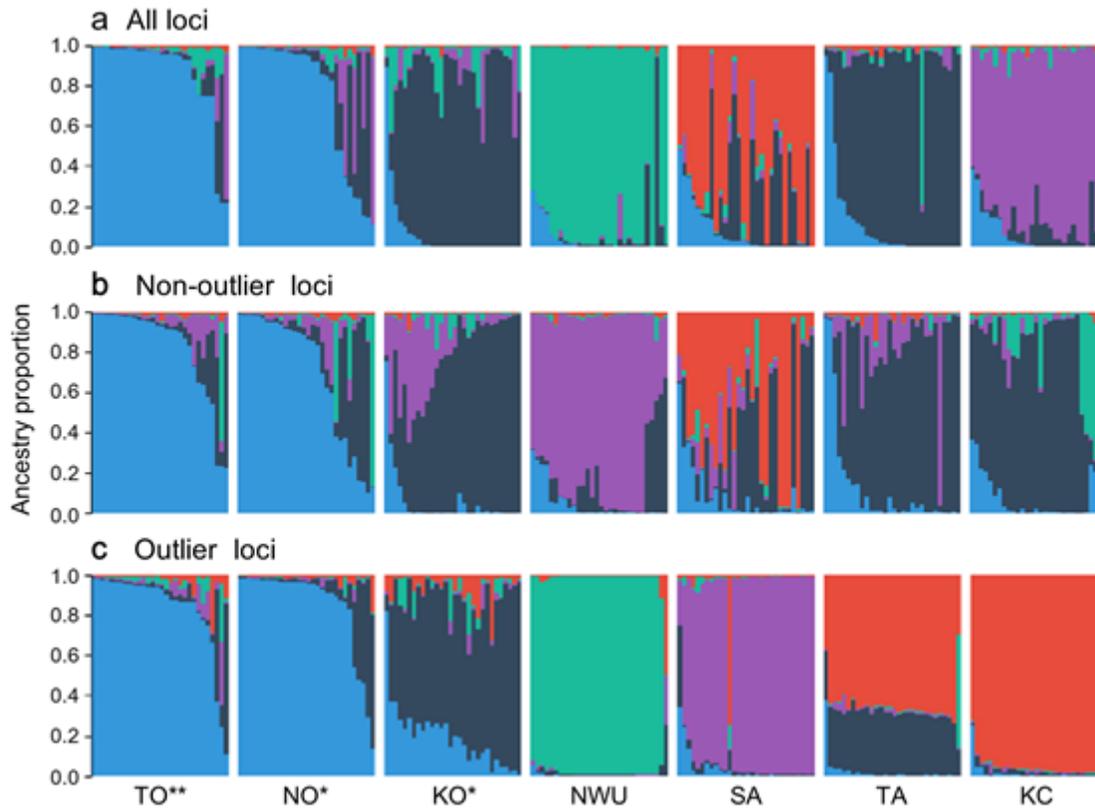


Figure 5

Results of STRUCTURE at $k = 5$ using a all-loci, b non-outlier loci and c outlier loci datasets. Each vertical coloured bar represents one individual. Fractional proportions of five coloured segments in a bar indicate assignment probability of the individual to each of five genetic clusters. See Fig. 2 for abbreviations of populations and Supp 3 for results at $k = 2$ to 7. **: heavily haired; *: moderately haired; no asterisks: lightly haired

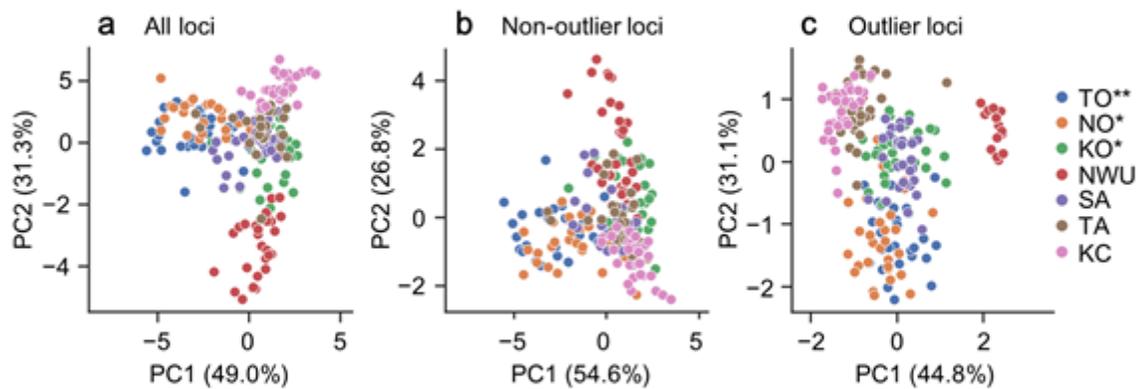


Figure 6

PCoA biplot diagrams represented by the first two principal components based on Euclidian distance using a all-loci, b non-outlier loci and c outlier loci datasets. Percentages in parentheses indicate the proportion of total variance for each component. See Fig. 2 for abbreviations of populations and Suppl 4 for biplot matrix with three principal components. **: heavily haired; *: moderately haired; no asterisks: lightly haired

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