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Significance of habitat type for the genetic population structure of *Panonychus citri* (Acari: Tetranychidae)

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Abstract. Restricted migration and habitat fragmentation promote genetic differentiation between populations. Because most of the hosts of *Panonychus citri* are woody plants, mainly citrus trees that are usually planted at intervals of several metres, this mite likely faces more risks (e.g., starvation) by dispersing between host plants, compared to other spider mite species that infest both herbaceous and woody plants, such as *Tetranychus urticae*. Such a limited gene flow between patches (host plants) can lead to differentiation of populations even within a small area. Therefore, we hypothesize that *P. citri* populations are genetically differentiated not only between distant populations but also within small areas, such as within a grove. To test this hypothesis, we investigated the divergence of *P. citri* populations in Japanese citrus groves according to a hierarchical arrangement of geographical distance, ranging from distant populations (10 groves distributed throughout different areas in two major Japanese islands; this level of analysis is referred to as 'geographic') to local populations (different trees in a specific grove; 'local'). Three molecular markers were used: an esterase locus, one microsatellite and a point mutation in the mitochondrial cytochrome oxidase subunit I. At a local level acaricide susceptibility tests were also performed using two acaricides: fenpyroximate (25 ppm) and etoxazole (3.33 ppm). At a broad geographic level the gene diversity decreased with decreasing area size and distance between populations. By contrast, at the local level, populations maintained a significant level of variation between trees within groves, and the divergence within groves was higher than between groves. Whereas no statistical difference of the mortalities was detected among groves for the two acaricides tested, the difference was statistically significant among trees within groves in fenpyroximate (ANOVA, $p < 0.025$) and marginal in etoxazole ($0.1 < p < 0.05$). We concluded that *P. citri* populations maintain a higher level of variation between trees (or patches of trees) within groves than between groves at the local level, though the gene diversity tended to be smaller with decreasing distance between populations at the geographical level. Results are discussed in relation to the dispersal behaviour of spider mites.

Introduction

Restricted migration and habitat fragmentation promote genetic differentiation between populations. Spider mites are tiny, wingless arthropods that

exhibit two major dispersal behaviours, crawling and aerial drift using threads (e.g., Jeppson et al. 1975; Kennedy and Smitley 1985). Crawling is assumed to be used over short distances and aerial drift is a means of longer-distance migration. Orchards with weeds might provide continuous habitats for some polyphagous species infesting many plant species, including herbaceous and woody plants, such as *Tetranychus urticae* Koch. In nature, Tsagkarakou et al. (1997) found that *T. urticae* populations collected from herbaceous and woody host plants within an area of 50 m^2 were panmictic, whereas populations separated by 150 m were not. According to that study, dispersal between adjacent plants was mostly accomplished by crawling.

However, orchards represent patchy habitats for species that specialized on a particular fruit tree. Patchy habitats might lead to restricted gene flow of spider mites in comparison with continuous habitats. *Panonychus citri* (McGregor) is a polyphagous species, yet most of its hosts are woody plants (Bolland et al. 1998). Citrus trees are the major host plants for *P. citri*. Because citrus trees are usually planted at intervals of several metres in Japanese groves, the habitat of *P. citri* is likely to be patchily distributed. After surveying a small citrus grove (130 m^2), Yamamoto et al. (1995) found strong spatial aggregation of the hexythiazox resistance gene in *P. citri* both between trees and within a single tree. Laboratory experiments also suggested that a breeding patch of *P. citri* occurs on several leaves or at most branches (Wanibuchi and Saitō 1983; Osakabe and Komazaki 1999). It is likely that *P. citri* faces higher risks (e.g., starvation) by dispersing between host plants compared to other polyphagous spider mite species such as *T. urticae*, which may encounter herbaceous species between woody patches. Thus, we hypothesized that *P. citri* populations are genetically differentiated not only between distant populations but also within small areas, such as within a grove.

In this study, we estimate gene flow between *P. citri* populations at both broad geographic level and at patch level using three molecular markers: esterase (α-*Est1*; Osakabe 1991), a microsatellite (*Pcms1*; Osakabe et al. 2000) and the mitochondrial cytochrome oxidase subunit I (COI; Toda et al. 2000, 2001). We also investigated the distribution of resistance genes against two acaricides, fenpyroximate and etoxazole, among patches in citrus groves.

Materials and methods

Sampling methods for the analysis of geographic diversity

Leaves of *Citrus unshiu* Marc. infested with *P. citri* were collected from 10 groves widely distributed over the agricultural areas on Honshu Island (HI) and Kyushu Island (KI), Japan, from October to November 1998 (Table 1). The leaves were randomly collected from 5 to 10 trees in each grove. Twenty-four adult *P. citri* females were randomly picked up from the leaves for preparation of DNA samples.

Table 1. Collection records of *P. citri* local populations used for the analysis of the geographical diversity.

Population	Date	Locality	Latitude
From Honshu Island (HI)			
KgOw	October 1998	Odawara, Kanagawa Pref.	35°15′ N–139°9′ E
SoSm	November 1998	Shimizu, Shizuoka Pref.	35°1′ N–138°29′ E
AcGg	October 1998	Gamagôri, Aichi Pref.	34°50′ N–137°14′ E
MeMh	October 1998	Mihama, Mie Pref.	33°51′ N–136°3′ E
OkKu	October 1998	Kurashiki, Okayama Pref.	34°36′ N–133°17′ E
HsAk	October 1998	Akitsu, Hiroshima Pref.	34°20′ N–132°49′ E
From Kyushu Island (KI)			
OiKs	November 1998	Kunisaki, Ôita Pref.	33°34′ N–131°44′ E
SgSg	October 1998	Saga, Saga Pref.	33°17′ N–130°18′ E
NsKt	October 1998	Kuchinotsu, Nagasaki Pref.	32°36′ N–130°11′ E
KmMbl	November 1998	Matsubase, Kumamoto Pref.	32°39′ N–130°41′ E

Sampling methods for the analysis of diversity within localities and groves

In September 2000 we collected *P. citri* from a grove in Fuji City (35°9′ N–138°39′ E), Shizuoka Prefecture, on HI (population: SoFj), 2 groves in Matsubase Town (32°39′ N–130°41′ E), Kumamoto Prefecture, on KI (KmMb2 and KmMb3), and 1 grove in Kumamoto City (32°48′ N–130°43′ E), Kumamoto Prefecture (KmKm) (Figure 1). Hosts of these populations were

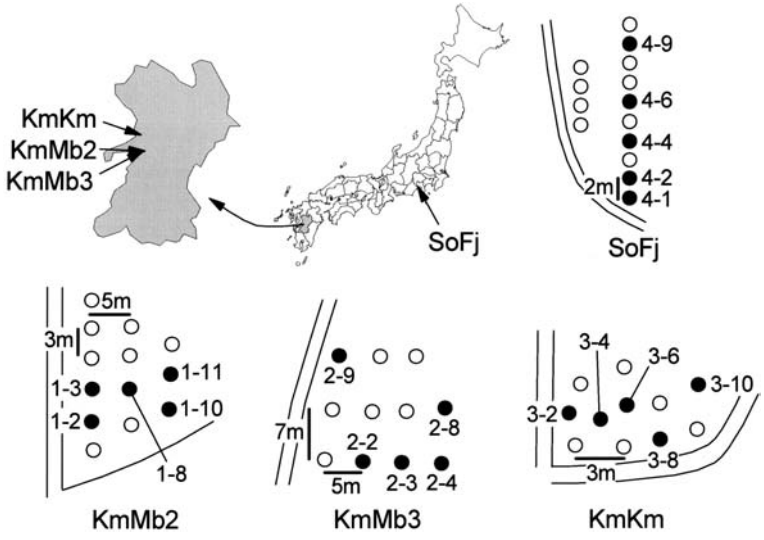

Figure 1. Maps of citrus groves used for the analysis of population structure. Circles in each population show the position of citrus trees. Solid circles show the trees from which the mites were collected.

all *C. unshiu*, except KmMb3 from Kawachi-bankan, which was a Japanese miscellaneous citrus variety. SoFj and KmMb2 had been sprayed with only mineral oil or no pesticides for at least 2 years before sampling, whereas KmMb3 and KmKm had been sprayed with agrochemicals on a conventional schedule for pest control.

Before collecting samples, we randomly chose 10–12 trees in a grove and checked the patchiness index (m^*/m) of *P. citri* between trees. This ratio was calculated from the mean crowding (m^*) and the mean density (m) according to Iwao (1968), from the number of adult females on 30 leaves in each tree. We also calculated m^*/m within a single tree for 1–3 trees per grove (9 total).

We selected 5 trees in each grove (except KmMb2, see below), excluding the ones infested with few mites. From each tree we collected 30 leaves from twigs on the same branch spatially distributed within about a 60-cm cube (patch). To analyse the diversity within trees, from several trees we sampled an additional one or two patches in the same manner. In KmMb2, the leaves were collected not from one branch but from various parts of the tree. The densities of adult females in this grove were too low (0.07–0.30 females/leaf) to sample in the above manner, except on one tree, which was infested at a relatively high density (1.0 females/leaf). Consequently, we collected 35 patches from these 4 groves: one in Shizuoka Prefecture and 3 in Kumamoto Prefecture.

After sampling, adult females were transferred to detached sour orange leaves placed on 0.5% agar gels including a small amount of Crystal Violet (Wako Pure Chemical Industries.) in Petri dishes and allowed to oviposit in the laboratory at 25 °C for 24 h. The adult females were used directly for polymerase chain reaction (PCR; SoFj population) or for α-*Est1* detection by PAGE (KmKm, KmMb2 and KmMb3), and the eggs deposited on the sour orange leaves were reared to adulthood. Newly emerged adult females were used for PCR (KmKm, KmMb2 and KmMb3) or for α-*Est1* detection (SoFj). The genetic variation of these new females should reflect the consequence of mating of their parents in the field because only the first mating is effective in spider mites in general (Takafuji 1986) and most adult females were inseminated immediately after the last molting. Offspring remaining on the leaves were continuously reared until the population increased enough to use for acaricide susceptibility tests.

Sample preparation for PCR

To analyse geographic diversity, DNA was extracted from individual adult females by using GenomicPrep Cells and a Tissue DNA Isolation Kit (Amersham Pharmacia Biotech) without RNA digestion, following the manufacturer's instructions. Isolated DNA was dissolved into 10 μl of TE buffer. For the analysis of diversity within localities, a newly established sample

preparation method (Osakabe et al. 2002) modified from Goka et al. (2001) was used because it was found that the new method was more efficient for preparation of a lot of DNA samples than that above. A single adult female was homogenized in 20 μl of lysis buffer [10 mM Tris–HCl (pH 8.0), 100 mM EDTA (pH 8.0), 0.5% Igepal CA-630 (Sigma, St., Louis, MO, USA), 10 mM NaCl, 1 mg/ml Protenase K], incubated at 65 °C for 15 min and then at 95 °C for 10 min and diluted to 400 μl with 0.1× TE buffer [1 mM Tris–HCl (pH 8.0), 0.1 mM EDTA]. Resulting DNA templates were stored at −20 °C. The difference in the sample preparation methods did not affect the following amplification by PCR.

Amplification of Pcms1

DNA templates were amplified by PC-OPB10-121F (forward, 5′-CTAAGCCAAACATAACTCAC-3′) labeled with a fluorescent dye, FITC, and PC-OPB10-403R (reverse, 5′-CAATGGTTCTGCAAATCGTA-3′) using AmpliTaq DNA polymerase (PE Applied Biosystems). These primers were based on a previously reported sequence of the Fukuoka strain of *P. citri* (Osakabe et al. 2000; accession number is AB030297 in DDBJ/EMBL/GenBank; numbers in the primer names before 'F' or 'R' show the site of the 5′-end of each primer in the original sequence). After incubation at 95 °C for 2 min, the reaction was cycled 30 times through the following temperature profile: 95 °C for 30 s, 55 °C for 30 s and 73 °C for 1 min. The reaction was then incubated at 73 °C for 30 min in a thermal cycler (GeneAmp PCR System 2400; PE Applied Biosystems). The fragment analysis was done with an automated sequencer (ALF DNA Sequencer-II; Pharimacia).

Detection of a point mutation in mitochondrial COI

The presence of a point mutation in the mitochondrial COI gene (Toda et al. 2000) was screened by the PCR amplification of specific alleles (PASA) method (ffrench-Constant et al. 1995; Zhu and Clark 1996). Allele-nonspecific primers and two primers specific for competitive alleles were the same as those used by Toda et al. (2001). The allele-nonspecific forward and reverse primers were 5′-ATATTTTAATTCTTCCTGGG-3′ and 5′-TACAGCTCCTATAGATAAAAC-3′, respectively, and the internal allele-specific forward primers were 5′-TCGCTATAATATCAATTGGT-3′ (PcT) for haplotype *T* and 5′-TCGCTATAATATCAATTGGG-3′ (PcG) for haplotype *G*. The PASA was carried out in 15 μl of reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.25 mM of each dNTPs, 15 pmoles of either PcT or PcG, 15 pmoles each of allele-nonspecific forward and reverse primers, 0.5 μl of template DNA, and 0.375 units of *Taq* polymerase (AmpliTaq Gold, PE Applied Biosystems). After preheating at 95 °C for 12 min, 40 amplification

cycles: 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, and 1 cycle of 7 min at 72 °C were performed. PCR products were visualized by using ethidium bromide after the agarose gel electrophoresis.

Detection of esterase allozyme

Nonspecific esterase alleles at the locus *α-Est1* (Osakabe 1991) were detected by PAGE. Electrophoresis and staining were carried out by the same method described by Osakabe and Sakagami (1993b), but using 7.5% precast polyacrylamide gels (PAGEL, ATTO). Adult females were homogenized in 10 μl of sample preparation buffer [32% sucrose, 2.8 mM EDTA, 0.05% 2-mercaptoethanol, 0.34% DTT, 0.1% Triton X-100 and a small amount of BPB dissolved in 0.1 M Tris–HCl (pH 7.5)] and stored at −20 °C until used for the electrophoresis.

Hierarchic analysis of gene diversity

Gene diversity was analysed at two geographic scales: on HI and KI (see Figure 2a; this level of analysis is referred to as 'geographic') and within pre-

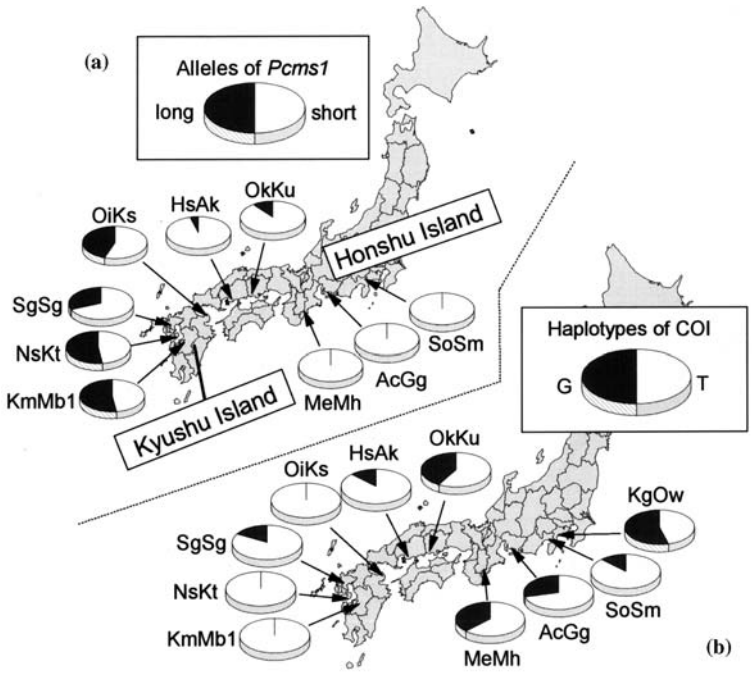

Figure 2. Geographical distribution of *Pcms1* alleles (a) and COI haplotypes (b). *Pcms1* alleles were classified as shorter (205–228 bp) and longer (266–330 bp).

Table 2. Geographic gene diversity analysis of *P. citri* using unbiased estimators[a].

Gene diversity (D_{ST})[b]	*Pcms1*	CO1	α-*Est1*[c]	Average	SE
H_P (within populations)	0.5931	0.2693	0.1543	0.3389	0.0299
D_{PA} (between populations, within islands)	0.0657	0.0318	0.0049	0.0341	0.0005
D_{AT} (between islands)	0.0511	0.0352	0.0651	0.0505	0.0001
H_T (total gene diversity)	0.7099	0.3362	0.2242	0.4235	0.0373
Coefficient of gene differentiation (G_{ST})[b]					
H_P/H_T (within populations)	0.8355	0.8008	0.6879	0.7747	0.0019
G_{PA} (between populations, within islands)	0.0926	0.0945	0.0217	0.0696	0.0003
G_{AT} (between islands)	0.0719	0.1047	0.2903	0.1556	0.0072

[a]See Nei (1987).
[b]Parameters were computed by the method of Chakraborty (1980) in hierarchic analysis.
[c]Data are from Osakabe and Sakagami (1993a, b).

fectures ('local'; see Figure 1). According to Nei (1973, 1987) and Chakraborty (1980), the gene diversity in the total population (H_T) was divided into the absolute gene diversity within the smallest unit of subpopulation and between subpopulations at each hierarchic level. Thus, H_T values for the geographic and local gene diversity analyses were divided as $H_T = H_P + D_{PA} + D_{AT}$ (variables are defined in Table 2), $H_T = H_B + D_{BR} + D_{RP} + D_{PC} + D_{CT}$ (in Kumamoto populations: KmKm, KmMb2 and KmMb3) and $H_T = H_B + D_{BR} + D_{RP}$ (in SoFj) (Table 4), respectively. Measures of the relative gene diversity (divided by the coefficient of gene differentiation, G_{ST}; Nei 1973) were computed from those components. We computed these parameters using unbiased estimators of the expected heterozygosity under Hardy–Weinberg equilibrium and the gene diversity in the total population (Nei 1987). The standard errors of the estimated average gene diversity measures were computed by the sampling variance formula described by Chakraborty et al. (1982).

To test allele frequency homogeneity (Chakraborty and Leimar 1987), the $R\times C$ test of independence (Sokal and Rohlf 1995) was performed by using a G-test in the hierarchic fashion following Smouse and Ward (1978). For this test, unique populations (or subpopulations) were omitted from the calculation. For *Pcms1*, which was a multi-allelic locus with many absent alleles in some populations, we combined independently shorter (205–240 bp) and longer (256–346 bp) alleles in each population (Figure 2).

Acaricide susceptibility test

Adult females of SoFj, KmKm, KmMb2 and KmMb3 reared in the laboratory during one to three months were transferred to fresh detached sour orange leaves (2×3 cm; 10 per leaf) and were allowed to lay eggs in the laboratory at 25 °C for 24 h. After removing the females, the leaves carring eggs were dipped into fenpyroximate (25 ppm) or etoxazole (3.33 ppm) solution for 5 s. As a

control, leaves with eggs deposited by adult females from the same patch were prepared in the same way and dipped into water for 5 s on the same day. The leaves were allowed to dry and were then placed on agar gel in Petri dishes. After 10 days, hatchability of the eggs was checked under a binocular microscope. Mortalities of eggs treated with acaricides were corrected by Abbott's method (Abbott 1925) using the hatchability of eggs that had been from the same patch and simultaneously treated with water. After arcsine transformation of the mortalities, variation between and within groves was evaluated by two-level nested ANOVA (Sokal and Rohlf 1995).

Results

Geographic gene diversity

In the geographic analysis, 19 alleles of *Pcms1* were found in total, ranging in length from 205 to 330 bp. The shorter alleles (205–228 bp) were more common (78.7%) than the longer alleles (266–330 bp). Three out of the five populations from HI carried the shorter alleles only and frequencies of the longer alleles in the remaining three populations were almost lower than 0.1 (Figure 2a). However, 31.1–78.0% of the alleles detected in KI belonged to the longer alleles. Allelic diversity was higher on KI [10.3 ± 1.7 (SD) alleles per population] than on HI (3.0 ± 1.3). Differentiation between islands was also found in the mitochondrial COI (Figure 2b). Whereas mites on HI carried both *T* and *G* haplotypes, although *T* was more frequent than *G*, most populations on KI were fixed for *T* (*G* was found only in SgSg).

For the hierarchic analysis of geographic gene diversity, we used the data on gene frequencies of *Pcms1* and COI from this study (Figure 2) and α-*Est1* frequencies from Osakabe and Sakagami (1993b). Total absolute gene diversity (H_T) analysis showed that the 3 loci were highly polymorphic (Table 2). The relative gene diversity (coefficient of gene differentiation) between populations in total [$= 1-(H_P/H_{T)}$] exceeded 0.15 for *Pcms1* and COI, and exceeded 0.25 for α-*Est1*, corresponding to great divergence and very great divergence, respectively (*sensu* Hartl 2000). Absolute gene diversity components between populations within islands (D_{PA}) were smaller than those between islands (D_{AT}) for both COI and α-*Est1*. For α-*Est1*, D_{PA} was smaller than D_{AT} owing to fixation of the allele on HI. This may result in a higher G_{AT} value. This resulted in larger G_{AT} than G_{PA} on average.

According to the $R \times C$ test of independence, homogeneity was rejected in many cases (Table 3), meaning that most of the relative gene diversity (G_{ST}) values listed in Table 2 were significantly different from zero. In two cases, between populations within islands for *Pcms1* and between islands for COI (both, $0.5 > p > 0.1$), homogeneity was not rejected. In the test for *Pcms1*, three of the five populations on HI were omitted because only the shorter alleles were present (see Figure 2a), However, 215- and 228-bp alleles were recovered at all

Table 3. $R \times C$ test of independence using G-test[a] in geographical diversity.

	df	G-statistic	
Pcsm1			
Between population in the total area	5	54.819	$p < 0.001$
Between islands	1	47.683	$p < 0.001$
Between populations, within islands	4	7.136	$0.1 < p < 0.5$
COI			
Between population in the total area	6	18.590	$0.001 < p < 0.005$
Between islands	1	2.322	$0.1 < p < 0.5$
Between populations, within islands	5	16.268	$0.005 < p < 0.01$
α-*Est1*			
Between populations, within Kyushu Island	2	15.016	$p < 0.001$

[a]Test was performed following Sokal and Rohlf (1995). Method of the hierarchical analysis was following Smouse and Ward (1978). Monomorphic populations were omitted.

populations, and 78.3% of all alleles were these two alleles. Thus, we also performed the $R \times C$ test of independence with the frequencies of the 215- and 228-bp alleles. Homogeneity was rejected at all hierarchic levels: between populations across the total area (df = 8, G-statistic = 85.5089, $p < 0.001$), between islands (df = 1, G-statistic = 22.4955, $p < 0.001$) and between populations within islands (df = 7, G-statistic = 63.0134, $p < 0.001$). For COI, because most populations on KI were monomorphic, only the SgSg data was considered for the test. We performed the test between islands with a COI dataset pooled over all KI populations and the hypothesis of homogeneity was rejected (df = 1, G-statistic = 30.4393, $p < 0.001$). From these, G_{PA} for *Pcms1* and G_{AT} for COI were also considered significantly different from zero. Owing to the fixation for α-*Est1* on HI (Osakabe and Sakagami 1993b), homogeneity between islands could not be tested. However, G_{AT} for this locus was high (Table 2).

Local gene diversity

The m^*/m values among trees were equivalent in each population (1.38–1.67), despite the density in KmMb2 [0.27 ± 0.07 (SE) females/leaf], KmMb3 (0.59 ± 0.12), KmKm (3.95 ± 0.83) and SoFj (2.15 ± 0.50). The m^*/m values within a single tree (1.39–1.67) were equivalent to those among trees in six of nine trees, although values outside this range were found in one tree in KmMb3 (2.81), one in KmKm (1.09) and one in SoFj (1.07). The relationship of m^* to m of adult females was fitted to a linear regression model for the distribution patterns both among trees ($m^* = 2.0622 + 1.3897m$; $R^2 = 0.9985$) and among leaves within a single tree ($m^* = 0.2154 + 1.4349m$; $R^2 = 0.9770$). The models suggest that adult females formed contagiously distributed colonies complying with the negative binomial distribution with a

common k (Iwao 1968) both among trees ($k = 2.57$) and within a single tree ($k = 2.30$).

Some field-collected adult females died in transit to the laboratory, and the rearing lines from some patches did not increase enough to use in analyses. Consequently, we analysed 26 ($n = 411$ females) and 21 ($n = 404$) patches for *α-Est1* and *Pcms1*, respectively, in KmKm, KmMb2 and KmMb3, and 9 patches for *Pcms1* ($n = 114$) and COI ($n = 116$) in SoFj. The genotypes of *α-Est1* of all adult females ($n = 55$) in SoFj were all A_2 homozygotes, corresponding to previously published geographic data (Osakabe and Sakagami 1993b), and COI was inferred to be monomorphic in KmKm, KmMb2 and KmMb3 from the geographic data in this study (see Figure 2). Thus, these were excluded from local analyses.

Measures of the relative gene diversities between patches in total [$= 1-(H_B/H_T)$] in the local analysis (Table 4) were smaller than those in the geographic analyses [$= 1-(H_P/H_T)$; Table 2]. Values in the local analysis did not exceed

Table 4. Gene diversity analysis among and within *P. citri* local populations using unbiased estimators[a].

Among and within Kumamoto populations: KmMb2, KmMb3 and KmKmb[b]	*Pcms1*	*α-Est1*	Average	SE
Gene diversity (D_{ST})[c]				
H_B (within patches)	0.8008	0.4472	0.6240	0.0442
D_{BR} (between patches, within trees)	0.0391	0.0232	0.0312	<0.0001
D_{RP} (between trees, within populations)	0.0441	0.0081	0.0261	0.0005
D_{PC} (between populations, within cities)	0.0039	<0.0001	0.0020	<0.0001
D_{CT} (between cities)	0.0150	0.0178	0.0164	<0.0001
H_T (total gene diversity)	0.9029	0.4963	0.6996	0.0584
Coefficient of gene differentiation (G_{ST})[c]				
H_B/H_T (with in patches)	0.8869	0.9011	0.8940	<0.0001
G_{BR} (between patches, within trees)	0.0433	0.0468	0.0451	<0.0001
G_{RP} (between trees, within populations)	0.0488	0.0162	0.0325	0.0002
G_{PC} (between populations, within cities)	0.0044	<0.0001	0.0022	<0.0001
G_{CT} (between cities)	0.0167	0.0358	0.0262	0.0001
Within SoFj[b]	***Pcms1***	***CO1***	**Average**	**SE**
Gene diversity (D_{ST})[c]				
H_B (within patches)	0.5437	0.1383	0.3410	0.0581
D_{BR} (between patches, within trees)	0.0449	0.0022	0.0235	0.0006
D_{RP} (between trees, within populations)	0.0252	0.0202	0.0227	<0.0001
H_T (total gene diversity)	0.6138	0.1607	0.3872	0.0726
Coefficient of gene differentiation (G_{ST})[c]				
H_B/H_T (with in patches)	0.8859	0.8604	0.8732	0.0001
G_{BR} (between patches, within trees)	0.0731	0.0138	0.0434	0.0003
G_{RP} (between trees, within populations)	0.0410	0.1258	0.0834	0.0022

[a]See Nei (1987).
[b]See Figure 1.
[c]Parameters were computed by the method of Chakraborty (1980) in hierarchic analysis.

0.15, suggesting moderate divergence (*sensu* Hartl 2000). The smallest absolute gene diversity was found between populations within cities, D_{PC}, for both *Pcms1* and α-*Est1* in Kumamoto populations (KmKm, KmMb2 and KmMb3). The largest absolute gene diversity was found between patches within trees for α-*Est1* and between trees within populations for *Pcms1* in Kumamoto populations. Consequently, the average of the relative gene diversities within groves in Kumamoto populations ($G_{BP} = G_{BR} + G_{RP} = 0.0776$) and SoFj ($G_{BP} = 0.1268$) showed 'moderate divergence,' whereas the measure between populations suggested 'little divergence' in Kumamoto populations ($G_{PT} = 0.0211$ for *Pcms1* and 0.0358 for α-*Est1*; *sensu* Hartl 2000). The measures of G_{BR} were larger than G_{RP} for α-*Est1* in Kumamoto populations and for *Pcms1* in the SoFj population, whereas G_{RP} was larger than G_{BR} for the remaining two loci. Therefore, the contribution to the total diversity (H_T) of the divergence between trees and that between patches within trees cannot be clearly established. Most results of the $R \times C$ test of independence rejected homogeneity, although it was not rejected between patches within populations for α-*Est1* of Kumamoto populations in spite of a larger G_{BR} (Table 5). The lack of significance for α-*Est1* in Kumamoto populations might be due to a small harmonic mean of the sample sizes, leading to an increased D_{BR} and a larger G_{BR} (Chakraborty and Leimar 1987). However, owing to very small D_{PC} in Kumamoto populations for both *Pcms1* and α-*Est1* and the measures of larger absolute gene diversity within populations in Kumamoto ($D_{BP} = D_{BR} + D_{RP} = 0.0832$ for *Pcms1* and 0.0313 for α-*Est1*) and SoFj ($D_{BP} = 0.0701$ for *Pcms1* and 0.0224 for COI; Table 4), the divergence within groves (populations) was likely to be larger than that between groves in a locality.

Table 5. $R \times C$ test of independence using *G*-test[a] in local diversity.

	df[b]	*G*-statistic	
In Kumamoto populations			
Pcms1			
Between patches in the total population	20	57.738	$p < 0.001$
Between populations	2	10.650	$0.001 < p < 0.005$
Between patches, within populations	18	47.088	$p < 0.001$
α-*Est1*			
Between patches in the total population	22	59.605	$p < 0.001$
Between populations	2	42.186	$p < 0.001$
Between patches, within populations	20	17.418	$0.5 < p < 0.9$
In Fuji population			
Pcms1			
Between patches, within population	5	18.447	$0.001 < p < 0.005$
COI			
Between patches, within population	3	11.136	$0.01 < p < 0.025$

[a]Test was performed following Sokal and Rohlf (1995). Method of the hierarchical analysis was following Smouse and Ward (1978).
[b]In the connection of handling unique branches and *Pcms1* alleles, see text.

Table 6. Two-level nested ANOVA with unequal sample sizes for the test of acaricide susceptibility variation in population levels.

Source of variation	df	SS	MS	F	P
Fenpyroximate (25 ppm)					
Among orchards	3	0.5428	0.1809	1.5017	$0.25 < p < 0.5$
Among trees within orchards	11	1.3254	0.1205	5.1487	$0.01 < p < 0.025$
Among patches within trees	7	0.1638	0.0234		
Total	21	2.0320			
Etoxazole (3.33 ppm)					
Among orchards	3	0.0156	0.0052	0.3259	$0.75 < p$
Among trees within orchards	10	0.1599	0.0160	3.9456	$0.05 < p < 0.1$
Among patches within trees	5	0.0203	0.0041		
Total	18	0.1958			

Distribution of acaricide resistance gene in groves

In KmMb3 and KmKm, which were sprayed with conventional pesticides, the mortality due to fenpyroximate was low in every patch, in spite of the fact that the tested concentration was the recommended concentration (Appendix 1). The mortalities were around 20% in patch B tree 2-2, patch A tree 2-8 and patch A tree 3-6. A low susceptibility against fenpyroximate was also observed in half of the trees in KmMb2 and SoFj (1-2,1-11, 4-1 and 4-4), although no pesticides or only mineral oil had been applied in these groves. High mortalities were observed in the remaining 4 trees in KmMb2 and SoFj. Variation of fenpyroximate susceptibility was also found among patches within foliage. The differences in egg mortalities among patches on the same tree ranged from 8.4 to 28.7%. Although no statistical difference of the mortalities by fenpyroximate was detected among groves (two-level nested ANOVA, $p > 0.05$), mortality was significantly different among trees within a grove ($p < 0.01$; Table 6).

No apparent variation was observed in mortalities due to etoxazole, although the concentration used for screening was almost one-tenth of the recommended concentration (Appendix 1). More than 95% of the eggs did not hatch in 17 out of the 19 patches analysed, and the mortalities were around 90% in the remaining two patches. The difference in mortality among trees was marginal in the two-level nested ANOVA ($0.1 > p > 0.05$), and no difference was detected among orchards ($p > 0.75$; Table 6).

Discussion

The genetic divergence between Japanese *P. citri* population on HI and KI was established in this study. The gene diversity of *P. citri* tended to be smaller with decreasing area size and distance between populations. This geographic trend is

consistent with the results of protein analysis in *P. citri* (Osakabe and Sakagami 1993a). However, the diversity of *P. citri* populations within a local population and/or a microhabitat was different from that of *T. urticae*, which maintains panmixia over short distances (Tsagkarakou et al. 1997; Navajas et al. 2002). The divergence within a grove and/or a tree clearly accounted for the total diversity of Japanese *P. citri* populations and was larger than the divergence between groves. The larger variation of fenpyroximate susceptibility between trees within groves than between groves, in spite of the fact that both the nonsprayed and conventionally controlled groves were included in this investigation, also reveals the genetic divergence of this mite within groves.

Owing to the limited crawling dispersion of *P. citri*, even among leaves on the small citrus seedling, Osakabe and Komazaki (1999) emphasized that interbreeding between patches depended on the interpatch distance. The m^*/m and the spatial distribution in our preliminary investigation were consistent with the laboratory measurements. Therefore, we concluded that *P. citri* populations maintain a significant level of variation between trees (or patches) within groves, and the level of divergence within groves is higher than between groves.

Hosts of *P. citri* are patchily distributed in citrus groves; thus it is likely that mites migrate mainly by aerial drift between groves (Fleschner et al. 1956; Furuhashi and Nishino 1978), Because *P. citri* does not enter diapause, mites cannot overwinter in Japanese pear orchards (Takafuji and Fujimoto 1986; Gotoh and Kubota 1997). Nevertheless, every year *P. citri* migrates to Japanese pear orchards from surrounding evergreen hosts such as Japanese holly trees, *Ilex crenata* Thunb. (Kunimoto et al. 1993; Gotoh and Kubota 1997), and even from distant citrus groves (Takafuji and Morimoto 1983), suggesting that this mite frequently uses airborne dispersal. The impact of the dispersal behavior and type of habitat on the genetic structure have been revealed in many species of animals (e.g., a migratory bat, Miller-Butterworth et al. 2003), and the significance of microhabitat for the genetic population structure, as we found for *P. citri*, has been indicated in marine organisms with reduced dispersal, such as polychaetes, which disperse only in the planktonic larval stage (Beckwitt 1980).

After a period of passive aerial dispersion on ground, the number of arrivals per unit area is generally expected to decline steeply with the increasing distance from the point of departure (e.g., Janzen 1970). Kennedy and Smitley (1985) estimated that most aerially dispersing mites fall from the air stream fairly soon after they are carried aloft. From the spatial distribution and frequency of the adult females caught by sticky traps in citrus groves, Furuhashi and Nishino (1978) inferred that migration of *P. citri* occurred more often between adjacent trees than between distant trees. Moreover, in Japanese pear orchards, patches of immigrants established in the new habitats are distributed concentrically and extend in two-dimensions owing to the mites' short-distance dispersal ability (Kunimoto et al. 1993; Gotoh and Kubota 1997). Such short-

distance migration between the adjacent hosts might increase the level of variation within groves.

The genetic similarity between groves within localities found in this study suggests that *P. citri* does not use only adjacent hosts. Considerable aerial migration of *P. citri* between adjacent citrus groves was observed by Fleschner et al. (1956), and the migration from distant citrus groves to Japanese pear orchards (Takafuji and Morimoto 1983) implies longer-distance migration of this mite. Our data suggest that *P. citri* frequently migrate between citrus groves within localities but not across wider areas. Aside from the significant decrease of mite density on the host plants after the peak of airborne dispersal (Fleschner et al. 1956; Furuhashi and Nishino 1978), the physical mechanism of aerial drift of *P. citri* is not clearly understood. To better understand genetic structure of *P. citri* population within a locality, we need to further investigate the aerial dispersal of this mite.

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Appendix 1. Among patch and among tree variation in fenpyroximate and etoxazole susceptibility of eggs in four local *P. citri* populations.

Population	Tree	Patch	Fenpyroximate (25 ppm)		Etoxazole (3.33 ppm)	
			No. of eggs tested	Mortality (%)	No. of eggs tested	Mortality (%)
KmMb2	1–2	A	95	67.7	186	100
		B	85	43.9	235	100
	1–3	A	133	95.9	–	–
	1–10	A	147	98.6	123	100
	1–11	A	200	35.4	63	90.4
KmMb3	2–2	A	57	50.5	–	–
		B	189	21.8	–	–
	2–3	A	62	47.4	109	88.8
	2–4	A	63	33.8	209	98.6
	2–8	A	65	23.6	43	7.4
		B	–	–	51	100
		C	115	32.0	–	–
	2–9	A	47	56.5	310	99.0
KmKm	3–2	A	–	–	75	96 8
	3–6	A	128	19.3	–	–
		B	79	37.0	94	100
		C	157	41.1	121	99.0
	3–10	B	122	32.6	134	100
SoFj	4–1	A	133	25.9	252	99.6
		B	81	44.3	204	99.5
		C	82	15.9	69	98.4
	4–2	A	–	–	42	100
	4–4	A	61	38.6	54	98.0
	4–6	A	83	98.6	107	97.1
	4–9	A	122	89.7	–	–

Mortality was corrected by Abbott's method (1925) using hatchability of eggs of the same patch treated by water on the same day as those treated by the acaricides.