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Forest fragmentation may endanger a plant-insect interaction: the case of the highly specialist native aphid *Neuquenaphis staryi* in Chile

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Abstract. 1. The diversity and distribution of the genetic variation in forest phytophagous insect populations can be highly sensitive to forest fragmentation. This should be particularly evident for monophagous insects living on endangered host plants.

2. The aphid *Neuquenaphis staryi* uses the tree *Nothofagus alessandrii*, commonly named Ruil, exclusively as a host. *Nothofagus alessandrii* is an endemic and endangered species whose distribution is highly fragmented in the unique Maulino temperate forests of the coastal range of Central Chile.

3. Here, we provide proof of the specialist status of *N. staryi* and provide evidence on the genetic diversity and structure of their populations sampled on distinct remnant fragments of the Ruil forest. A sample representing 480 individuals collected from five fragments of Ruil forests revealed 147 distinct multilocus genotypes at six microsatellite loci. Clonal diversity and other genetic parameters were consistent with aphids reproducing by cyclic parthenogenesis and showed few signs of diversity loss.

4. The genetic differentiation among populations was significant as evidenced by the presence of at least three genetic clusters, which are mostly explained by low migration due to geographic barriers that restrict aphid dispersion.

5. Our results suggest that forest fragmentation imposes genetic discontinuities on a highly specialised phytophagous insect, which may have important implications for the conservation status of this ancient aphid-plant interaction.

Key words. Endangered aphid, fragmented populations, genetic diversity, *Neuquenaphis staryi*, *Nothofagus alessandrii*.

Introduction

Habitat fragmentation and habitat loss due to human activities is currently one of the major threats to biodiversity at a global scale (Chapin *et al*., 2000). Anthropogenic forest fragmentation can produce serious disruptions in the biological processes that maintain biodiversity and ecosystem functioning (Fahrig, 2003; Dobson *et al*., 2006; Peh *et al*., 2014); among them, significant alterations of nutrient-cycling, community composition, food webs and species interactions are likely to ultimately lead the destabilisation of ecosystems, thus intensifying species extinctions (Harrington *et al*., 2010).

Chile has the largest temperate forests in the southern hemisphere; these are classified as a biodiversity hotspot for conservation (Myers *et al*., 2000; Echeverria *et al*.,

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2006). Many forests in Chile however have been seriously affected and fragmented for forestry and agricultural activities since the mid-1970s. One of the most striking examples of fragmentation is the Maulino temperate forest, a mesic forest located in the coastal range of central Chile and presently covering an area of just 314 ha with a strongly skewed size distribution; this figure should be compared to the 9841 ha that the Maulino forest occupied in the past, and which is currently mostly used for forestry (Santelices *et al*., 2012). In spite of the small size of the remnant Maulino forest, only about the 30% of its present distribution is under governmental or private protection programs. A dominant species of the Maulino forest is the endemic long-lived caducifolious species of Southern beech, *Nothofagus alessandrii* Espinosa (commonly named Ruil); it is accompanied by two other species of *Nothofagus*, *N. glauca* (Phil.) Krasser and *N. dombeyi* (Mirb.) Oerst. (Bustamante & Castor, 1998; Grez *et al*., 1998). The present distribution of *N. alessandrii* consists of a mosaic of some 180 small patches scattered within pine (*Pinus radiata* D. Don) plantations (Bustamante & Castor, 1998).

Despite the reduced and fragmented distribution of *N. alessandrii*, the genetic diversity of these populations is still relatively high, possibly due to historical processes since fragmentation is rather recent and trees are long-lived (Torres-Díaz *et al*., 2007) and/or to high gene flow among fragments (Lowe *et al*., 2015). The fragmentation of *N. alessandrii* forests and the reduction in their surface cover, however, is expected to negatively impact other species in the forest, but mostly those that live exclusively associated with this tree species.

Insects are among the most diverse group of living organisms, playing different roles in the functioning of forest ecosystems (e.g. energy flow, pollination, etc.). Furthermore, insect herbivores constitute eco-evolutionary drivers that shape plant communities and explain the evolution of plant biodiversity (Forest *et al*., 2007). Hence, the understanding of the consequences of forest fragmentation on phytophagous insects seems relevant as they are part of the evolutionary history and key players in the functioning of present-day ecosystems (Didham *et al*., 1996; Tscharntke & Brandl, 2004). This should be particularly relevant for populations of specialist insects, since the interruption of habitat continuity may result in habitat loss and eventually in extinction (Steffan-Dewenter & Tscharntke, 2000; Kondoh, 2003).

Aphids are phloem-feeding insects frequently specialised on a small number of plant species (Blackman & Eastop, 2000). They show a variety of polyphenisms in response to environmental changes, including winged/wingless and sexual/asexual individuals in the same population (Simon *et al*., 2002). The genus *Neuquenaphis* Blanchard (Hemiptera: Aphididae: Neuquenaphidinae) is endemic of South American temperate forests, grouping 11 Gondwanan relict species that mainly feed on *Nothofagus* trees. This aphid group reproduces by cyclical parthenogenesis; sexual, parthenogenetic, winged and wingless morphs have been described (Hille Ris Lambers, 1968; Quednau & Remaudiere, 1994; Gaete-Eastman *et al*., 2004). *Neuquenaphis staryi* Quednau and Remaudière, has been previously reported to feed and reproduce exclusively on *N. alessandrii* (Quednau & Remaudiere, 1994; Fuentes-Contreras *et al*., 1997). Furthermore, leaf volatiles of *N. alessandrii* were attractive to *N. staryi* but not to other more generalist *Neuquenaphis* species while leaf volatiles of other *Nothofagus* species were attractive to more generalist *Neuquenaphis* species but not to *N. staryi* (Russell *et al*., 2004). These facts strongly suggest that *N. staryi* is a specialist species, and hence should be particularly sensitive to environmental changes (Gilman *et al*., 2010).

In this study, we evaluate the population status of *N. staryi* using microsatellite markers, the genetic diversity and differentiation among *N. staryi* populations sampled from small patches of *N. alessandrii* trees was surveyed along its current distributional range, and the biological and physical features assessed in a system which may indicate the possible fate of this endangered plant-insect interaction.

Methods

Host choice, survival and reproduction of Neuquenaphis staryi

In order to confirm the specialist status of *N. staryi*, laboratory and field experiments were conducted to assess its capacity to select and successfully survive and reproduce on other *Nothofagus* species that naturally coexist with *N. alessandrii*. Host selection experiments were performed using winged parthenogenetic females collected at Los Ruiles National Forest Reserve (from now on, LR) maintained on excised leaves of *N. alessandrii* at 4 °C until used for bioassays in the laboratory. Branches with leaves of *N. alessandrii*, *N. dombeyi* and *N. glauca* were transferred to the laboratory to provide leaves for the bioassays. One single winged individual *N. staryi* was set at the centre of a host choice arena (10 cm diam. Petri dish) containing three excised leaves of similar size of each host species placed equidistantly from each. The tips of the leaves were oriented towards the centre of the Petri dish, while their petioles were oriented towards its outside. Petioles were wrapped in wet cotton to prevent wilting. Light sources were placed above the arena and provided homogeneous illumination. Six hours thereafter, the choice of the focal individual was recorded. Three independent trials on about the same days were conducted, each comprising 10 biological replicates (n = 30).

In addition, survival and reproduction tests were performed at LR by enclosing one single winged parthenogenetic female from a pool of randomly selected individuals in a 2 cm diameter clip-cage using one cage per tree (n = 18 per host), and the number of surviving aphids and nymphs produced in each clip-cage 4 days later recorded.

Statistical significance for host preference assays were tested using the extended McNemar's test for high order tables. Survival and the number of nymphs produced on each host tree were compared using a proportion test and a generalised linear model, respectively, as implemented in the R package (R Core Team, 2014).

Aphid sampling

Wingless individuals of *N. staryi* (n = 480) were sampled from *N. alessandrii* trees in forest fragments of different sizes at five localities and located both north and south of the Maule river (Fig. 1; Table 1): Alto Huelón [AH; 35°04′S, 72°04′W; 312 m a.s.l.], Los Ruiles (LR; 35°50′S, 72°30′W; 528 m a.s.l.), Polhuín (PH; 35°49′S, 72°30′W; 449 m a.s.l.), Porvenir (PV; 35°41′S, 72°22′W; 192 m a.s.l.) and Quivolgo (QV; 35°23′S, 72°13′W; 432 m a.s.l.). Abundance of trees was extremely low in some of the localities sampled (only a few mature and a few young individuals in some cases); thus, a sampling that is nearly representative of tree scarcity was conducted. A minimum of 29 aphid individuals per population were collected (Table 1), a sample size that provides a reasonable chance of detecting alleles at a frequency >0.05 within each population sample (Sjörgren & Wyöni, 1994). Aphids were collected by performing a transect line crossing the whole patch. To avoid 'edge effect', the first five and last five trees were excluded from sampling. To limit the chance of sampling individuals from the same parthenogenetic lineage, aphids were taken from trees separated by at least 25 m from one another and from three different branches in each tree (Figueroa *et al*., 2005). All samples were preserved in 95% ethanol.

Microsatellite amplification

Since no microsatellite markers have been developed from any *Neuquenaphis* species yet, patterns of allelic diversity in populations of *N. staryi* were studied using available markers isolated from other aphid species. Indeed, several microsatellite loci are reported as showing cross-species amplification between aphid taxa, including *Neuquenaphis* species (Wilson *et al*., 2004). Of those available, 19 loci were evaluated, 12 cloned from *Myzus*

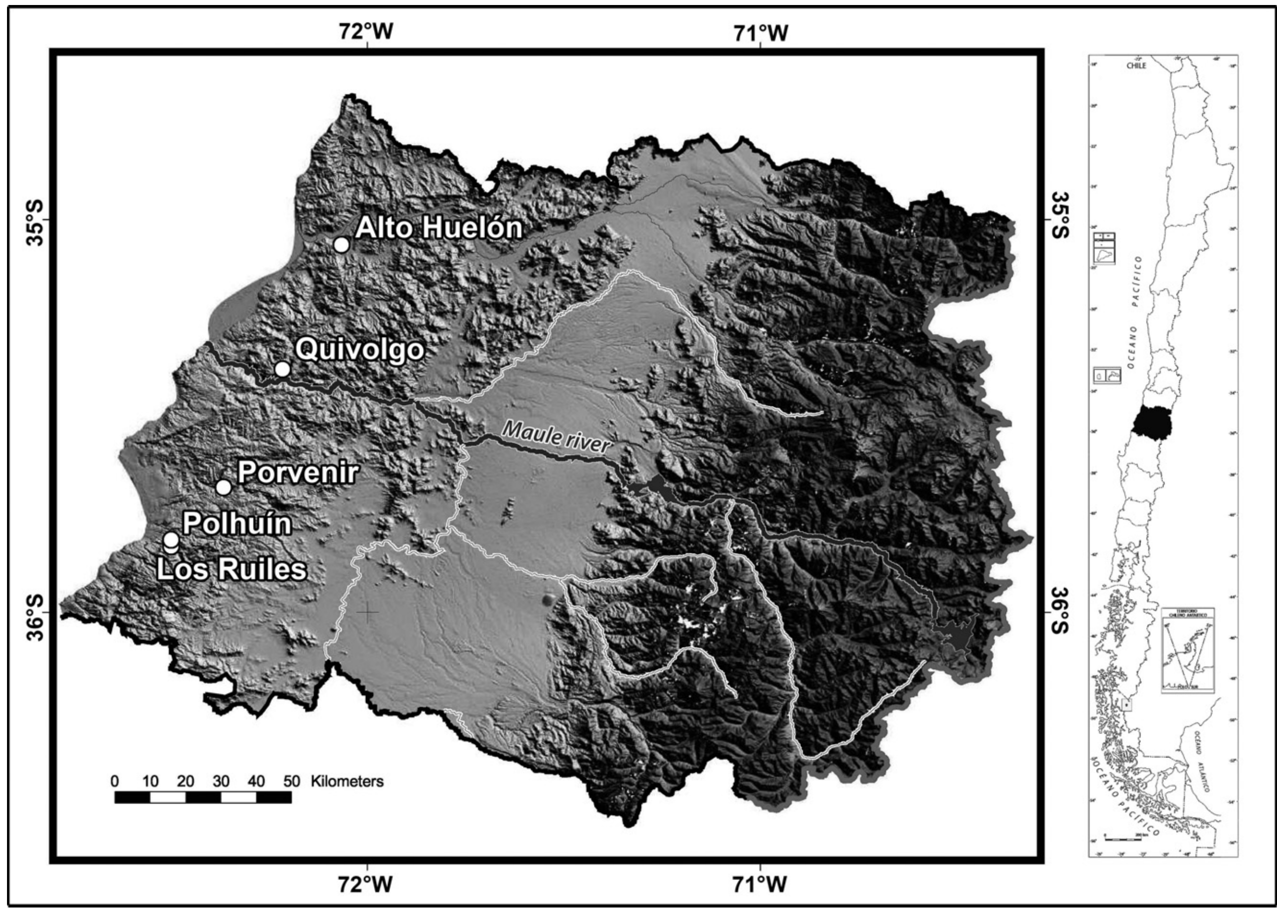

Fig. 1. Collection sites for *Neuquenaphis staryi* aphids. The main physical components of the Maule river system are emphasised.

Table 1. Number of *Nothofagus alessandrii* host trees sampled and number of *Neuquenaphis staryi* aphids collected at five localities in Central Chile.

Locality	Surface (ha)*	Trees sampled	Aphids collected per tree	Aphids collected per site
Alto Huelón	7.6	13	2–4	38
Quivolgo	5.2	15	2–4	47
Porvenir†	0.7	9	3–4	29
Polhuín	2.7	22	2–4	66
Los Ruiles	45.0	100	2–7	300
Total sampled		159		480

*Santelices *et al.* (2012).
†The whole tree population was sampled.

persicae (Sulzer) (*Myz2*, *Myz3*, *Myz9*, *Myz25*, *M35*, *M37*, *M49*, *M62*, *M63*, *M77*, *M86* and *M107*), four from *Sitobion miscanthi* (Takahashi) (*Sm10*, *Sm11*, *Sm12* and *Sm17*), and three from *Sitobion avenae* (F.) (*S3.R*, *S.4Σ* and *S5.L*) (Simon *et al.*, 1999; Sloane *et al.*, 2001; Wilson *et al.*, 2004). A minimum of 10 *N. staryi* individuals were used to test amplifications at each locus, repeating each PCR three times, and using DNA from *M. persicae* and *S. avenae* as positive controls. Amplicons were sized after polyacrylamide gel electrophoresis using the sequence of pGEM®−3Zf(+) vector (Promega, Madison, Wisconsin, USA) as reference (Figueroa *et al.*, 2005).

Data analysis

Multilocus genotypes (MLGs). To characterise the clonal diversity and genotypic composition in the whole sample as well as to compare within and between populations, *N. staryi* aphids were analysed as MLGs (i.e. the genotype resulting from the combination of the six microsatellite loci that showed a polymorphic and reproducible amplification). This method assumes that individuals with the same genotype have a good chance to have evolved from a predominantly genetically identical asexual ancestor (i.e. they are clones), but with some differences due to ongoing mutations within asexual lineages (Loxdale, 2008).

As sampling was performed in January (Austral summer), aphids from *N. staryi* were then reproducing asexually. Indeed, no sexual morphs were found on any sampled tree; rather, parthenogenetic colonies only were found. As a consequence of their reproductive mode, aphids within a colony and sometimes populations can be more mutually similar than in the case of other diploid organisms (but see Loxdale, 2008). To avoid the over representation of some asexual lineages ('clones' *sensu* Loxdale, 2008), data analyses were done considering one single copy per genotype and the whole sample. Frequencies of each MLG were computed rather than allelic frequencies, and used Hardy–Weinberg equilibrium (HWE) in order to assess the expected frequencies for each genotype in every population (Figueroa *et al.*, 2005).

The clonal diversity (i.e. the diversity of MLGs) was used to determine the genetic variability in *N. staryi* in the whole sample as well as within forest fragments. The Shannon–Weaver diversity index (H), expressed as $H = -\Sigma_i p_i \log_e p_i$, where p_i is the relative frequency of the ith genotype (Shannon & Weaver, 1949) was also computed. This algorithm can be expressed as e^H to obtain an index proportional to the actual genotypic richness (Llewellyn *et al.*, 2003).

Standard population genetic analysis. To avoid distortions on estimates of deviations from the HWE due to differential clonal amplification among *N. staryi* genotypes, the data were analysed considering a single copy per genotype as well as the whole sample (Sunnucks *et al.*, 1997). The genetic frequencies were computed using GenAlEx v.6.5 (Peakall & Smouse, 2012), analysing departures from the HWE, linkage disequilibrium (LD), and genetic heterogeneity among the entire dataset and among pairwise fragments using exact tests available in the Genepop package v.3.2a (Raymond & Rousset, 1995), and tested for significance of multiple pairwise comparisons using Fisher's method.

The degree of population differentiation was assessed by calculating F_{ST} values on allelic and genotypic frequencies considering one copy per genotype. This violation of the island-model assumptions can be accepted when F_{ST} values are only used to quantify differences among populations (Llewellyn *et al.*, 2003). Hence, F-statistics were computed according to Weir and Cockerham (1984) using the Genepop package, with bootstrapping (Weir, 1990). Furthermore, the occurrence of isolation by distance (IBD) indices were tested by performing a regression of $F_{ST}/(1-F_{ST})$ estimates for pairs of subpopulations (Rousset, 1997). To estimate genetic similarities, the shared allele distance $(1-D_{PS})$ was computed in the software Microsat (Minch, 1997), then running a Mantel test as implemented in the software Genstat (Genstat Committee, 1993) using 10 000 permutations to test IBD. A neighbour-joining tree was constructed using software Mega v.4.0 (Tamura *et al.*, 2007).

Bayesian population analysis. To determine the level of genetic substructure in the dataset independently of sampling locations, the Bayesian methodology as implemented in Structure v.2.0 was used (Pritchard *et al.*, 2000). This approach uses MLGs to assign individuals to the distinct K subpopulations (where K may be unknown). To estimate the number of subpopulations, one single copy per genotype as well as the whole sample was used. Five independent runs of K = 1–10 were performed with iteration parameters set to a burning-in period of 60 000 iterations followed by 600 000 iterations assuming correlated allele frequencies and admixture. Subsequently, the most likely number of populations was determined using the log-likelihood of K, and individuals assigned to each subpopulation, based on the highest percentage membership (q). Although the model-clustering algorithm assumes

panmixia, this approach is robust to some deviations from these assumptions (Falush *et al.*, 2003), as expected for individuals reproducing asexually (Halkett *et al.*, 2005). The optimal value of K depending on ΔK value (Evanno *et al.*, 2005) was determined using Structure Harvester v.0.6.94 (Earl & von Holdt, 2012). The number of genetic clusters was estimated using the aggregation Bayesian algorithm implemented in Tess v.2.3 (Chen *et al.*, 2007) by running the algorithm with 10 000 sweeps and discarding the first 5000 with 20 independent iterations for each model for maximum clusters (K_{max}) varying from 2 to 10. The highest likelihood runs were selected based on the deviance information criterion and plotted graphically against K_{max} (Chen *et al.*, 2007), thus allowing selection of the number of hypothetical clusters (K) after the program was run 100 times for the selected K_{max} with 50 000 sweeps, discarding the first 10 000 and averaging the 10 highest likelihood runs. In addition, the migration rate among populations was estimated as implemented in the program Bayesass v.1.2 (Wilson & Rannala, 2003); this software estimates the number of migrants based on the proportion of genotypes sampled in each fragment but also found in other fragments, and it uses a Bayesian approach without depending on potentially unrealistic assumptions such as migration-drift equilibrium.

Results

Use of alternative hosts by Neuquenaphis staryi

The three-choice experiment consistently showed that winged individuals of *N. staryi* significantly selected more leaves of *N. alessandrii* than of the other two *Nothofagus* species offered. Thus, of the 30 individuals assayed, 20 selected *N. alessandri*, while 5 and 4 selected *N. dombeyi* and *N. glauca*, respectively; only one individual failed to select any host (McNemar's χ^2 = 19.8, d.f. = 3, P < 0.01). Similarly, survival (χ^2 = 36.5, d.f. = 2, P < 0.01) and reproduction ($F_{2,51}$ = 25.5, P < 0.01) were significantly higher on *N. alessandrii* (Fig. 2).

Cross-species amplification of microsatellite loci in Neuquenaphis staryi

Of the 19 microsatellites tested, six loci which were polymorphic and showed reproducible amplifications without null-alleles (data not shown) were chosen for further studies. All sampled aphids were genotyped using these six loci, five cloned from *M. persicae* (*Myz2, M49, M62, M63* and *M77*), and one from *S. miscanthi* (*Sm11*). *Sm11* is an X-linked locus, while the other loci are autosomal (Sloane *et al.*, 2001; Wilson *et al.*, 2004). Considering the whole sample, locus *M63* was the most polymorphic (11 alleles), while loci *M49* and *M77* the least (5 alleles).

Multilocus genotypic diversity and within population structure

One hundred and forty-seven MLGs were distinguished among the 480 individuals of *N. staryi* sampled. Clonal diversity was variable among populations of *N. staryi*, with LR as the most diverse population (e^H = 51.27) and PV as the least diverse (e^H = 9.29) (Table 2). Deviations from the HWE due to excess of heterozygosity as revealed by the F_{IS} index and were observed mainly for the entire dataset (i.e. considering all populations) and for AH population (Table 2); no differences were detected when the HWE was

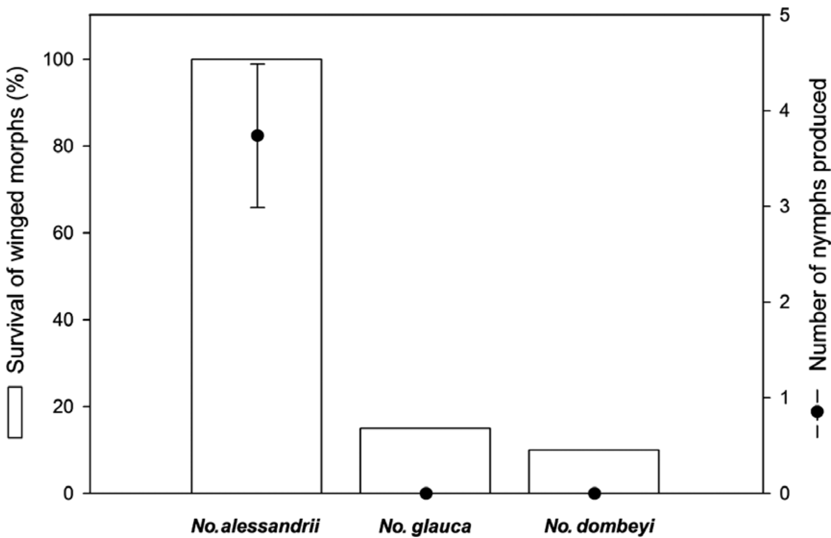

Fig. 2. Survival and reproduction of *Neuquenaphis staryi* individuals after transference from *Nothofagus alessandrii* to *Nothofagus glauca*, *Nothofagus dombeyi* and *Nothofagus alessandrii*. Mean values and standard errors are shown.

Table 2. Frequency and distribution of all multilocus genotypes (MLG) of *Neuquenaphis staryi* identified at sampled sites.[†]

Genotype	Population					Total	Frequency
	AH	LR	PH	PV	QV		
Nst1	8.000	25.000	16.000	0.000	7.000	56.000	0.12
Nst2	2.000	30.000	0.000	8.000	8.000	48.000	0.10
Nst3	4.000	11.000	0.000	4.000	10.000	29.000	0.06
Nst4	0.000	23.000	0.000	0.000	0.000	23.000	0.05
Nst5	0.000	18.000	0.000	0.000	0.000	18.000	0.04
Nst6	3.000	11.000	0.000	0.000	0.000	14.000	0.03
Nst7	5.000	7.000	0.000	0.000	0.000	12.000	0.03
Nst8	0.000	4.000	0.000	5.000	1.000	10.000	0.02
Nst9 to Nst147	16.000	171.000	50.000	12.000	21.000	270.000	0.56
N° genotypes	17.000	104.000	27.000	13.000	16.000	147.000	
MLG sampled more than once	8.000	37.000	11.000	4.000	9.000	69.000	
N	38.000	300.000	66.000	29.000	47.000	480.000	
G/N	0.447	0.347	0.409	0.448	0.340	0.306	
e^H	12.930	51.270	17.230	9.290	11.140	62.220	
F_{IS}[‡]	−0.183*	0.002[NS]	−0.046[NS]	−0.005[NS]	−0.049[NS]	−0.183*	
F_{IS}[§]	−0.005[NS]	−0.022[NS]	0.038[NS]	−0.076[NS]	−0.062[NS]	−0.159*	

A homogeneity test for each population (F_{IS}) was performed.
eH, diversity index proportional to the actual genotypic richness in each population; G/N, ratio of the number of multilocus genotypes and the sample size; NS, non-significant; AH, Alto Huelón; LR, Los Ruiles; PH, Polhuín; PV, Porvenir; QV, Quivolgo.
*$P < 0.05$.
[†]The number of individuals collected per genotype is indicated for each population, as well as the entire sample (N). [‡]HWE computed considering one single aphid per genotype or [§]the whole sample.

computed considering one single copy per genotype (i.e. supposing that populations are made by distinct not repeated genotypes) or the whole sample (i.e. all the individuals genotyped irrespective of its frequency) (Table 2). Most loci pairs were in linkage equilibrium, with the exception of loci pairs *M63/M77*, *M77/Sm11* and *Myz2/M77* which showed a strong LD ($P < 0.001$) both using one single individual per genotype or the whole sample.

Genetic differentiation and isolation by distance

All multiple pairwise comparisons between pairs of populations showed a significant genetic differentiation (Table 3). The lowest differentiations were found between AH and LR ($F_{ST} = 0.05$) and between AH and PH ($F_{ST} = 0.06$); the sites of these two pairs are the mutually most distant sites (93.9 and 92.2 km, respectively; Fig. 1). The highest differentiation between subpopulations were found between QV and PH ($F_{ST} = 0.19$) and between PV and PH ($F_{ST} = 0.17$); two pairs of sites relatively close to one another (54.6 and 19.1 km, respectively; Fig. 1). Both the Mantel test ($r = -0.478$, $P = 0.242$) and the linear regression analyses ($r^2 = 0.345$, $P = 0.07$) revealed no significant association between geographic distances and genetic differentiation, thus discarding the possibility of IBD. In addition, a neighbour-joining tree constructed using one single copy of each of the 147 MLGs found, separated all populations of *N. staryi* (Fig. 3). Furthermore, the genetic distance between populations as measured by the length of the branches in the tree, was not in agreement with the geographic distances between populations, giving additional support for the notion of the absence of IBD.

Bayesian population analysis

The Bayesian assignation analysis of individuals to subpopulations revealed that the peak distribution of ΔK is

Table 3. Genetic differentiation (F_{ST}) between populations of *Neuquenaphis staryi* (lower triangle) and statistical significance computed by the Fisher's method (upper triangle).*

Population	Population[†]				
	AH	LR	PH	PV	QV
AH	—	<0.001	<0.001	<0.001	<0.001
LR	0.05[§]	—	<0.001	<0.001	<0.001
PH	0.06[§]	0.080[§]	—	<0.001	<0.001
PV	0.12[§]	0.060[§]	0.170[¶]	—	<0.040
QV	0.10[§]	0.090[§]	0.190[¶]	0.060[§]	—

*AH, Alto Huelón; LR, Los Ruiles; PH, Polhuín; PV, Porvenir; QV, Quivolgo.
[†]The significance level was adjusted by the sequential Bonferroni method (Sokal and Rohlf, 1995). Since this procedure however made little difference on the overall pattern of significant results, the non-adjusted results are shown.
[‡]According to Hartl and Clark (1997).
[§]0.05–0.15, moderate genetic differentiation.
[¶]0.15–0.25, large genetic differentiation.

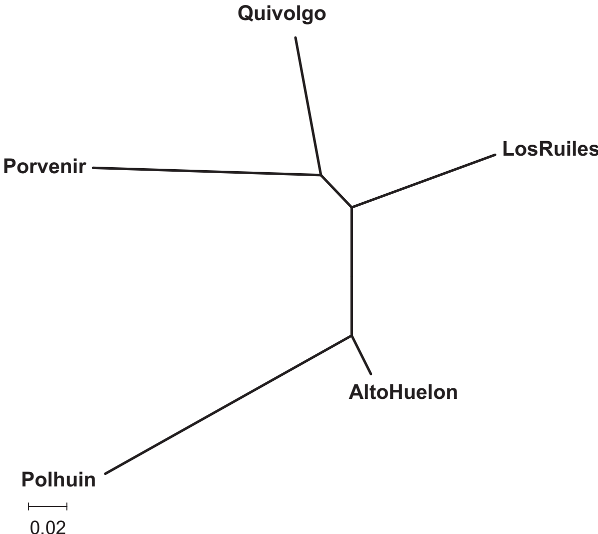

Fig. 3. Neighbour-joining tree relating populations of *Neuquenaphis staryi* based on allele shared distance ($1-D_{PS}$) calculated at 6 microsatellite loci for 147 multilocus genotypes. The scale of distances between genetic groups is noted as the proportion of shared alleles between each branch in the tree.

highest at $K = 3$, indicating that three is the most likely number of genetic clusters, grouping AH and QV in a northern cluster, PV in a central cluster, and PH and LR in a southern cluster. Similarly, the aggregation Bayesian algorithm resulted in a posterior distribution analysis among clusters that was best explained at $K = 3$ [Log P (X/K) = −4424.3 and P(K) ~ 1], thus also estimating the number of hypothetical genetic clusters as three. When the estimated membership coefficient for each individual was plotted (Fig. 4), AH and QV mostly consisted of genotypes from cluster 1 (grey), PV exhibited most genotypes assigned to a separated cluster 2 (light grey), whereas LR and PH formed a single but separated subpopulation with most genotypes from cluster 3 (dark grey) (Fig. 4). This analysis, conducted on Tess, further provided a spatial arrangement of clusters based on Voronoi tessellations which was coherent with the estimated membership fraction plots (Fig. 4). Lastly, the mean posterior probabilities analysis estimated that the migration rates between subpopulations were lower than the migration within subpopulations (data not shown), showing that most individuals were native to the site where they were sampled from, thus corroborating the genetic structuring observed.

Discussion

Genetic diversity and clonal composition of the aphid Neuquenaphis staryi

Using a population genetic approach, the fragmentation of *N. alessandrii*, the main tree species of Maulino forests in the coast of central Chile, was studied as driver of the

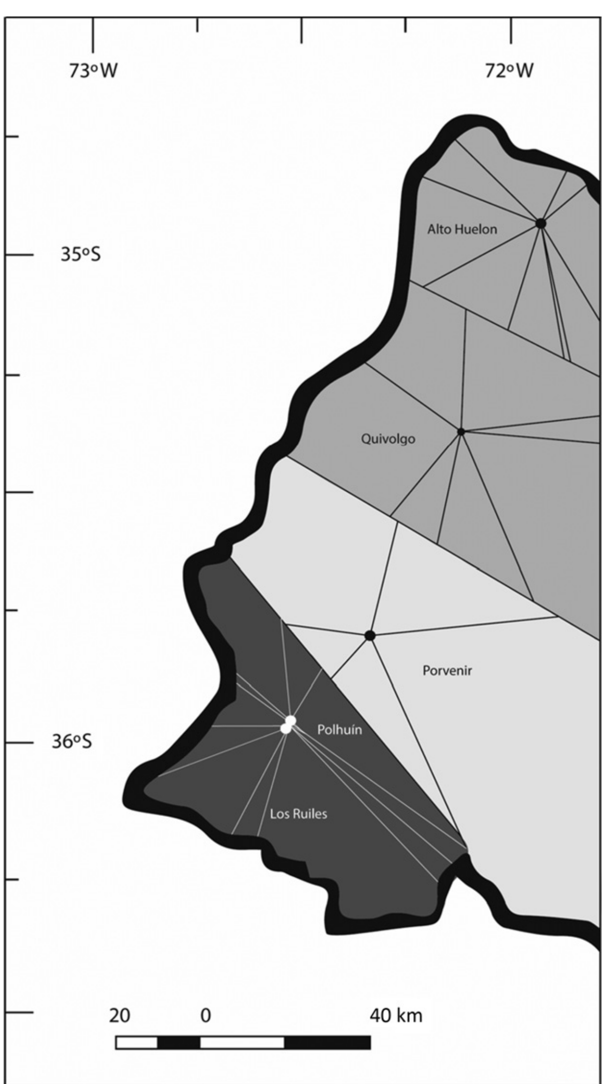

Fig. 4. Spatial clustering based on Voronoi tessellation analysis for *Neuquenaphis staryi* subpopulations as inferred using TESS. A cell of the tessellation corresponds to the physical neighbourhood of an observed data point, and is filled according to the cluster membership.

genetic diversity of its specialised aphid *N. staryi*. In a preliminary study on a restricted sample of *N. staryi* using low-reproducibility RAPD-PCR markers, low genetic diversity and high differentiation among populations were found (Gaete-Eastman *et al*., 2004). A similar pattern was then expected in the present work, that is, habitat fragmentation should lead to a loss of genetic diversity and increased differentiation among aphid populations as a consequence of habitat loss and geographic isolation followed by random drift (Braschler *et al*., 2003). However, we found that overall, the genetic diversity in *N. staryi* was rather high, clonal diversity (as computed by the number of MLGs, G/N and e^H) being comparable to that of other aphid species that reproduce by cyclical

parthenogenesis (Llewellyn *et al.*, 2003; Halkett *et al.*, 2005; Loxdale *et al.*, 2010). In fact, some MLGs were overrepresented and persisted in certain forest patches. As aphids were sampled during summer, several MLGs were found in multiple clonal copies due to asexual amplification, with eight MLGs grouping 44% of the sample (Table 2). This is because aphids mostly reproduce by female-only asexual reproduction during spring and summer, and they quickly multiply and spread genotypes through winged morphs ['genetic inflation' sensu Loxdale *et al.*, 2017] and thus can display temporal specialisation through disruptive selection (Halkett *et al.*, 2006). Our results clearly show and indeed emphasise that some life-history traits such as the reproduction mode in aphids can and do increase population growth through asexual multiplication, thus overcoming the negative effects of population bottlenecks and genetic drift on genetic diversity (Tomiuk & Köhler, 2007).

In addition, the relatively high genetic diversity detected in *N. staryi* may be suggested to result from phylogeographic events related to the time since fragmentation. Given that the aphid host is a long-lived tree and since fragmentation is relatively recent (mid-1970s) (Torres-Díaz *et al.*, 2007), the genetic erosion through drift and selection can be delayed due to temporal and spatial stochasticity (Lefèvre *et al.*, 2004). This assumption is supported by the high genetic diversity observed in *N. alessandrii*, despite the dramatic fragmentation suffered in the last decades (Torres-Díaz *et al.*, 2007), and by the high within population aphid gene flow estimated and the high genetic relatedness computed between nearby patches, as revealed by the clustering analyses.

Genetic differentiation among populations of Neuquenaphis staryi

High genetic diversity and differentiation were especially evident between relatively nearby populations (e.g. between QV and PH, and between PV and PH), while low differentiation was detected between AH and LR and between AH and PH, the most distant populations (Fig. 1). In addition, the analysis of clonal structures and clonal abundances in *N. staryi* populations grouped MLGs into three genetic clusters with a north-south geographic correspondence, suggesting a limited gene flow between populations due to biological and/or landscape features.

In terms of aphid features, the genetic structuring observed may be the result of selection on traits other than diet breadth (Loxdale & Harvey, 2016), which can promote differential reproduction rates for certain aphid genotypes that eventually specialise (Loxdale *et al.*, 2011a). Aphid populations living in highly sub-divided habitats (as those formed after forest fragmentation) may have had to face very singular local conditions. For instance, different predation pressures by parasitoid wasps in different sites could modify the frequency in which protective bacterial endosymbionts are harboured by aphids (even in the same MLG) (Sepúlveda *et al.*, 2017). Similarly, different microclimate conditions in *N. alessandrii* patches could lead to physiological/behavioural specialism in aphids harbouring endosymbionts that confer thermal tolerance or those having different antipredator behaviours at higher temperatures (Loxdale & Balog, 2017). Finally, aphid dispersal polymorphism, due to differences in flight behaviour among genotypes, and the landscape heterogeneity may also explain the genetic differentiation described here (Loxdale *et al.*, 2011a).

On the other hand, landscape features may also explain the lack of gene flow between contiguous sites and the connectivity between separate populations. Firstly, the pine plantation matrix surrounding the *N. alessandrii* patches may isolate aphid populations (Santelices *et al.*, 2012). As pasture lands are scarce in the studied areas (San Martın & Donoso, 1996), the understory structure may also function as a barrier between nearby populations of *N. staryi*. Indeed, differences in the composition and diversity of other plant life beneath the forest canopy are known to be structurally less complex than that in the original forest and represent an unsuitable habitat preventing the dispersion of different flying species that live in forests, such as insects (Schmuki *et al.*, 2006; Hedin *et al.*, 2008). This explanation seems to fit particularly well to *N. staryi* because of the further natural tendency of specialist aphids to avoid flying through semi-natural habitats (Loxdale *et al.*, 2011b). Secondly, the complexity of the mountainous chains in the Maulino forest ecosystems (Armesto *et al.*, 2007) can limit the availability of host corridors (Fig. 1); frequent ravines and rivers that cross the area in the east-west direction may also entail strong restrictions to aphid dispersion (Loxdale *et al.*, 1993). This seems particularly evident between aphids sampled in PV and QV (Figs 1 and 4), as the Maule river should represent an almost impassable low-altitude barrier to migration (Whitlock & McCauley, 1999; Chapman *et al.*, 2010), impeding the connectivity between populations at both sides of the river, particularly due to strong wind flows along the river bed.

Oppositely, wind currents may act as important high-altitude mixing factors for winged insects (Chapman *et al.*, 2010). Indeed, the genetic variation among insect herbivore populations can reflect the response of individual species to dispersal opportunities more than the effect of habitat quality (Brouat *et al.*, 2004). Coastal winds in the Maule Region frequently flow from the southwest (Saavedra *et al.*, 2010) at about 2.73 m s^{-1} on average (Agroclima, 2017), which may maintain the connectivity between aphid populations even across hundreds of kilometres, as reported for other specialist aphids (Massonnet *et al.*, 2002; Loxdale *et al.*, 2011b). Altogether, the dispersion of *N. staryi* may be governed by both abiotic and biotic factors.

Contributions to current conservation programs

The present work highlights the delicate relationship between the fragmentation of an endemic forest and the

biology of a specialised insect herbivore, and provides essential information to decision makers about where current conservation biology efforts should be directed to protect species interactions. This situation seems particularly critical for *N. staryi*, since this aphid species behaves as a strict specialist unable to prefer, survive and reproduce on alternative *Nothofagus* species. Such a strict specialisation may increase the vulnerability to extinction of such an insect herbivore due to habitat loss. Specialist insects can monopolise restricted resources on fragmented habitats as long as the latter are composed by large host populations (Dapporto & Dennis, 2013); this does not seem to be the case of the Maulino forest ecosystems.

It is known that the disappearance or significant suppression of one species can have detrimental effects through the food-web (Scheffer *et al*., 2001), and may even cause other species to disappear due to extinction cascades (Sahasrabudhe & Motter, 2011). In the particular case of *N. staryi*, this aphid is parasitised by the highly specialised parasitoid wasp *Pseudephedrus longivalvus* (Starý, 1995); hence, extinction of the aphid prey should likely result in the extinction of its predator. On the other hand, aphids during their feeding exude large amounts of honeydew that after being washed by rainfall reaches the ground and may provide nutrients to the ground and belowground community thus upsetting original equilibria, similarly to scale insects in *Nothofagus* forests (Wardle *et al*., 2010).

Hence, some interactions between species can be so specific that the extinction of one may involve the extinction on the other, as may be the particular case of this endangered and highly specific plant-aphid interaction. In order to preserve such species interactions, conservation efforts should be focused on increasing or at least maintaining the number of suitable forest patches currently under private or governmental administration, in order to preserve the connectivity of the species living in this forest. A request for considering *N. staryi* as endangered species was recently submitted to the Chilean Wildlife Species Inventory, managed by the Ministry of Environment (MMA, 2017). Such request included information gathered during the development of the present work. Preliminary, *N. staryi* was included in the category of 'Endangered' and awaits the official designation. This category of threat represents a useful tool for designing protection programs and for encouraging studies on the responses of forest ecosystems to natural and/or anthropic perturbations.

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