CONNECTIVITY AMONG NORWAY RAT SUBPOPULATIONS (*Rattus norvegicus*) AT POULTRY FARMS IN EXALTACIÓN DE LA CRUZ, BUENOS AIRES, ARGENTINA

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ABSTRACT. Despite of the substantial economic and human health problems produced by rodents of the genus *Rattus* almost nothing is known about its actual dispersal. In the present study we analyze the genetic subdivision of *R. norvegicus* inhabiting poultry farms of Buenos Aires, Argentina, and the relation between geographic and genetic distances by means of variation in microsatellite loci. We genotyped 40 rats captured between April-06 and June-07 from nine poultry farms distributed in an area of 110 km². No genetic subdivision was found among different poultry farms. This result supports the hypothesis of high connectivity between *R. norvegicus* inhabiting poultry farms in Buenos Aires. Because this species is a known host of diverse zoonosis as leptospirosis and trichinosis, our results point out that there is a great risk of transmission for distances lower than 12 km. Health control and preventive measures should therefore be applied simultaneously in all nearby poultry farms.

RESUMEN. Conectividad entre las subpoblaciones de rata noruega (*Rattus norvegicus*) que habitan granjas avícolas de Exaltación de la Cruz, Buenos Aires, Argentina. A pesar de los problemas económicos y de salud producidos por roedores del género *Rattus*, se conoce muy poco sobre su dispersión efectiva. En el presente estudio analizamos la subdivisión genética de las subpoblaciones de *R. norvegicus* que habitan granjas avícolas de Buenos Aires, Argentina, y la relación entre las distancias geográficas y genéticas por medio de la variabilidad en loci de microsatélites. Se genotiparon 40 ratas capturadas entre abril-06 y junio-07 en nueve granjas avícolas distribuidas dentro de un área de 110 km². No se encontró subdivisión genética entre las diferentes granjas avícolas. Esto apoya la hipótesis de alta conectividad entre *R. norvegicus* que habitan granjas avícolas en Buenos Aires. Debido a que *R. norvegicus* es un conocido hospedador de diversas zoonosis como leptospirosis y trichirosis, nuestros resultados indican que existe un gran riesgo de transmisión para distancias menores a 12 km. Las medidas de control y prevención sanitarias deberían, por lo tanto, aplicarse en todas las granjas cercanas en forma simultánea.

Key words: dispersion, metapopulation, microsatellites, movements, rodents.

Palabras clave: dispersión, metapoblación, microsatélites, movimientos, roedores.
INTRODUCTION

Rats are distributed throughout the world, in close association with humans. They cause contamination by food consumption, damage in building components and equipment (Pratt 1991; Villa & Velasco 1994; Singleton et al. 2003) and they are primary transmitters or reservoirs of several human diseases such as salmonellosis, leptospirosis, hantavirus renal syndrome and trichinosis (Gómez Villafañe et al. 2004; Cueto et al. 2008; Lovera et al. 2017).

Despite of the substantial economic and human health problems produced by rodents of the genus Rattus, essentially nothing is known about their dispersal. However, the success in their control is based on the knowledge of the species’ population dynamics and the characteristics of the place where the control will be applied. Additionally, when control is applied only locally, rat movement from surrounding places may reinfect controlled areas (Leirs et al. 1997; Gómez Villafañe & Busch 2007). For management plans to be effective it is crucial to understand the ecological factors affecting population connectivity and the sources of reinvasions after control (Hinten et al. 2003).

The study of genetic differentiation among subpopulations was extensively used to detect effective dispersal patterns, under the hypothesis that an absence of differentiation implies that individuals are moving and reproducing freely among subpopulations (Hinten et al. 2003; León et al. 2010; Varudkar & Ramakrishnan 2015; Desvars-Larrive et al. 2017). The genetic markers most used to detect contemporary patterns of effective dispersal are microsatellites; given their great variability, these markers are more adequate than mitochondrial DNA to detect recent ecological processes (Hinten et al. 2003). This methodology will provide information that summarizes events occurred over many generations and covers a temporal and spatial scale that is difficult to achieve by classical capture-recapture methods. For example, a previous study carried out at the same spatial scale and in the same area studied here found that Mus musculus shows genetic differentiation between farms (León et al. 2010). Other studies at larger spatial scales suggested that R. norvegicus may move long distances transported by humans (Lack et al. 2013).

In the Department of Exaltación de la Cruz, Buenos Aires province, Argentina, commensal rodents are mainly present on poultry farms (Gómez Villafañe & Busch 2007; León et al. 2007; Gómez Villafañe et al. 2008). Poultry farms are favorable habitats that are sparsely distributed within a matrix of cultivated fields and pastures where commensal rodents are rarely found (León et al. 2013). Besides, rats are mainly associated to human modified habitats. Nevertheless, in many countries, rats have colonised rural habitats, which may function as population sources for reinfection of human buildings after control measures. In farms, both the domestic mouse (Mus musculus) and rats (Rattus norvegicus and Rattus rattus) are considered pests because they consume and contaminate poultry feed and also can directly affect humans (Singleton et al. 2003). Rodent control is mainly carried out through the application of anticoagulants, which are often not applied simultaneously on all farms in the region. In a previous work, Gómez Villafañe & Busch (2007) did not find differences in rat abundance between farms –within an area of 4 700 km²– with and without application of rodenticide. The persistence of rat populations in farms may be the result of a metapopulation dynamic process (Hanski et al. 1997) where local treated populations are recolonized from untreated areas.

In the present study we analyze the genetic subdivision of R. norvegicus inhabiting poultry farms of Buenos Aires (Argentina) as an indirect method for estimating movements of rats. Based on previous ecological (Gómez Villafañe & Busch 2007) and genetic studies (Lack et al. 2013) we propose that R. norvegicus inhabiting poultry farms of Buenos Aires are tightly connected, conforming a whole system without genetic structure, and therefore no relationship between geographic and genetic distance is expected.

The evaluation of the genetic connectivity among R. norvegicus will help us to assess the degree of isolation between different subpopulations and to provide direction in the application of adequate control measurements.

MATERIALS AND METHODS

Study area, habitat description and sample collection

The study area is located in Exaltación de la Cruz, Buenos Aires Province, Pampean region, Argentina, 34°S 59°W. Nowadays the grassland vegetation has been mostly replaced by implanted crops and the area is intensely cultivated with soybean, maize and wheat. Other activities include extensive cattle and intensive poultry farming, which has strongly increased during the past 30 years. Poultry farms in this area are interspersed within a matrix of crop fields and pastures. Other habitats, such as railways, crop field edges and river embankments function as corridors for wildlife but its role for rats is not known.
Rodent samplings were conducted in nine poultry farms (one time on each poultry farm) included in an area of 110 km$^2$, between April 2006 and June 2007 (Fig. 1). Live traps of 15x16x31 cm (specific for rodents > 30 g) baited with meat and carrot, were placed around sheds in each poultry farm for three consecutive nights. All *R. norvegicus* captured were sacrificed and a tissue sample of about one centimeter of the tip of the tails was collected. Tissues were preserved in 90% alcohol for later analysis in the laboratory. Sample sizes of each farm are indicated in Table 1. Because only one individual was captured in poultry farms 7, 8 and 9, they were omitted from population-based analyses. All rats were manipulated according to regulations complying with National Law 14 346 and recommendations in Sikes (2016).

**Microsatellite genotyping**

Total DNA was extracted using the procedure described in Sambrook et al. (1989). In a preliminary analysis we amplified 7 loci of microsatellites, but based on the polymorphism information content (PIC) for each locus, following Botstein et al. (1980) we chose these three loci: Rno1, Rno2 and Rno8. They were taken from the database of the Whitehead Institute for Biomedical Research (http://www.genome.wi.mit.edu).

The forward primer of each locus was labeled with a fluorescent dye before amplification by polymerase chain reaction (PCR) (Schuelke 2000). The reaction was performed in 20 µl volume, containing 50-100 ng DNA 1X reaction buffer, 2.5 mM Cl$_2$Mg, 0.06 mM of each dNTPs, 10 ng/µl for the forward primer, 40 ng/µl for the reverse primer and 0.45 µM for the label with 1 of 3 fluorescent dyes (6-FAM, VIC and NED), 1 unit of Taq polymerase (Invitrogen). DNA amplification was performed in a TECHNE GENIUS (Techne, Cambridge, UK) thermal cycler. PCR amplification conditions were: an initial period at 94°C for 5 min, then 30 cycles at 94°C (30 s) / 53°C (45 s) / 72°C (45 s), and a final extension at 72°C for 10 min. PCR products were run on an ABI PRISM 3130 Genotyper Analyzer (Applied Biosystems) from the Unidad de Secuenciación y Genotipifico of the Buenos Aires University. Amplification size was scored using GeneMapper v3.7 (Applied Biosystems).

**Statistical analyses**

Allele frequencies were calculated using the program FSTAT version 2.9.3.2 (Goudet 2002), which was also used to estimate the average number of alleles per locus (*A*), and allele richness (*r*), which estimates the number of alleles independently of the sample size. Expected (*H*$_e$) and observed (*H*$_o$) heterozygosities for each farm were calculated with program TFPGA, version 1.3 (Miller 2000). Differences between *H*$_e$ and *H*$_o$ were assessed by means of a test of scores using the *U* statistic (Raymond & Rousset 1995).

Wright’s *F*-statistics (Wright 1965) were estimated according to Weir & Cockerham (1984), where *θ* is an estimator of *F*$_{ST}$, *F* estimates *F*$_{IT}$ and *f* estimates *F*$_{IS}$. The index *F*$_{ST}$ was used as a measure of population subdivision at the farm level, while *F*$_{IT}$ estimates the deficiency or excess of heterozygotes in the whole population, and *F*$_{IS}$ the degree of inbreeding within subpopulations. For these calculations we used the program TFPGA. Each parameter was estimated for each locus and then averaged through all loci.

We performed a spatial PCA (henceforth sPCA, Jombart et al. 2008) analysis through “adegenet” R package. This model relies on Moran’s *I* (Moran 1948) and compares the allelic frequency of individuals to the allelic frequencies of their neighbours. Following the methods in Jombart et al. (2008), we tested for global (neighbouring individuals are more similar than expected) and local (neighbouring individuals are more dissimilar than expected) spatial structures using permutation tests.
Table 1
Allele frequencies, expected and observed heterozygosity ($H_e$ and $H_o$, respectively), mean number of alleles per locus, and allele richness for each poultry farm with at least 2 individuals of Exaltación de la Cruz, Buenos Aires, Argentina.

| Farms and sample size | 1   | 2   | 3   | 4   | 5   | 6   |
|-----------------------|-----|-----|-----|-----|-----|-----|
| Allele                | (14)| (4) | (9) | (4) | (4) | (2) |
| $Rno1$                |     |     |     |     |     |     |
| 1                     | 0.036 | -   | -   | -   | -   | -   |
| 2                     | 0.714 | 0.625 | 0.722 | 0.5 | 0.625 | 1   |
| 3                     | 0.179 | 0.250 | -   | 0.250 | 0.125 | -   |
| 4                     | -   | -   | 0.111 | -   | -   | -   |
| 5                     | -   | 0.125 | 0.167 | 0.250 | 0.125 | -   |
| 6                     | -   | -   | -   | -   | 0.125 | -   |
| 7                     | 0.071 | -   | -   | -   | -   | -   |
| $Rno2$                |     |     |     |     |     |     |
| 1                     | 0.346 | 0.125 | 0.688 | 0.375 | 0.275 | 0.750 |
| 2                     | 0.462 | 0.875 | 0.125 | 0.625 | 0.625 | 0.250 |
| 3                     | 0.038 | -   | -   | -   | -   | -   |
| 4                     | 0.038 | -   | -   | -   | -   | -   |
| 5                     | 0.077 | -   | -   | -   | -   | -   |
| 6                     | 0.038 | -   | 0.188 | -   | -   | -   |
| 7                     | -   | -   | -   | -   | -   | -   |
| $Rno8$                |     |     |     |     |     |     |
| 1                     | 0.045 | -   | 0.167 | -   | -   | -   |
| 2                     | 0.091 | -   | -   | -   | -   | -   |
| 3                     | 0.864 | 0.875 | 0.833 | 1   | 1   | 1   |
| 4                     | -   | 0.125 | -   | -   | -   | -   |

$H_e$: 0.47 0.37 0.43 0.42 0.39 0.17
$H_o$: 0.36 0.42 0.28 0.42 0.5 0.17
Mean no. alleles: 4.33 2.33 2.67 2.0 2.33 1.33
Allele richness: 1.47 1.37 1.43 1.42 1.4 1.17

A minimum Spanning Network with Nei’s distance matrix among all microsatellite genotypes was performed in R (version 3.1.2, R Core Team 2014) using the function ‘msn’ of package pegas v 0.6 (Paradis 2010).

We examined the genetic structure using the Bayesian algorithm implemented in Structure 2.3 (Falush et al. 2007) assuming a model of population admixture and correlated allele frequencies among groups; we used the spatial population membership of each individual as prior information. We performed preliminary analyses with K ranging from 1 to 10 to detect the most representative number of groups and final analyses for a number of K ranging from 1 to 5, with a burn-in of 4 000 000 iterations and 12 000 000 final MCMC iterations. The parameter ALPHAPROPSD was set to 0.125 to improve mixing of MCMC chains. The procedure was repeated five times for each K to check for consistency. The optimal value of K was calculated according to the ΔK method described by Evanno et al. (2005) using Structure Harvester 0.6.7 (available at http://taylor0.biology.ucla.edu/structureHarvester). Replicated runs of Structure were analyzed using CLUMPP 1.1.2 (Jakobsson & Rosenberg 2007) to summarize each individual’s membership across runs using the Greedy algorithm. We used these averaged results to build summary bar plots for each individual using the program DISTRUCT v 1.1 (Rosenberg 2004).

Relatedness ($r$) between pairs of individuals was calculated according to Queller & Goodnight (1989) with GENALEX 6.5 (Peakall & Smouse 2012). Mean values of $r$ were calculated using InfoStat with 5 000 bootstrap replicates to obtain a 95% confidence interval around each mean. We calculated: (1) The mean relatedness of individuals of all sampling places without subdivision of the farms; (2) the mean relatedness between pairs of individuals within each poultry farm; (3) the mean relatedness coefficient between pairs of poultry farms. Positive mean values indicate that individuals are significantly related. Negative mean values or confidence intervals for the mean including zero indicates that individuals are not significantly related. Only calculations (2) and (3) were represented graphically with their confidence 95% interval.
We estimated the levels of symmetric and asymmetric migration rates in the last generations between pairs of poultry farms using BayesAss 1.3 (Wilson & Rannala 2003). Analyses were performed for a total of 20,000,000 MCMC iterations, including a burn-in of 2,000,000 iterations. Convergence was checked with Tracer 1.6. (Rambaut et al. 2014). The program uses individual multilocus genotypes to estimate rates of recent immigration (over the last several generations) and to calculate two types of posterior probability distributions: population-based ancestries and individual immigrant ancestries. At the population level, it calculates the proportion of the genotypes of each subpopulation that is considered non-migrant and then it estimates the proportion of the genotypes that comes from other subpopulations. In addition, it calculates the probability that an individual is a resident, a first generation migrant or a second generation migrant and it also identifies the subpopulation of origin. Migration rates that exceeded 0.2 were considered important (see Wilson & Rannala 2003).

Given the uneven sample size of the different poultry farms (Table 1), we decided only to analyze results based on individuals.

RESULTS

We found a total of 18 alleles for the three microsatellite loci analyzed in 40 R. norvegicus from nine poultry farms (Table 1). All loci were polymorphic. The PIC values were moderately informative for locus Rno1 and Rno2 and were the less informative for Rno8 (Table 2). For all poultry farms with at least 3 individual, the mean $H_o$ varied between 0.37 and 0.47, whereas the mean $H_e$ varied between 0.28 and 0.50 (Table 1). The mean number of alleles per locus varied between 2 and 4.33 and the allele richness varied between 1.37 and 1.47 (Table 1). In terms of mean number of alleles, farm 6 was the least variable, although in terms of allele richness all farms had a low variability (Table 1).

$H_e$ per locus varied between 0.20 and 0.52 while $H_o$ per locus values varied between 0.17 and 0.58. The number of alleles per locus ranged between four and seven (Table 2), being Rno8 the locus that had the lowest values (Table 2). $\Theta$ values for each locus and for the global analysis indicate that subpopulation of R. norvegicus did not present genetic differences among poultry farms (Table 2). The parameter $f$, which estimates the $F_{IS}$ index, was not significant for each locus or for the global analysis, indicating random breeding ($f = 0.141 \ p > 0.05$; Table 2). There were not departures form Hardy–Weinberg equilibrium in the allele frequencies of loci Rno1, Rno2 and Rno3 (Table 2).

Allelic frequencies did not suggest a significant global or local (nper = 9999) spatial structure of genotypes. The distribution of the permuted test statistic includes the observed statistic, suggesting the absence of global and local spacial structure (Fig. 2). In addition, the different poultry farms did not represent independent genetic units (Fig. 3). Analyses with program Structure also did not show genetic structure (Fig. S1). The genotypes of individuals within each farm and among farms was quite similar, indicating a lack of genetic structuring among R. norvegicus from different poultry farms.

Almost all mean $r$ values of the individuals within poultry farms were not significantly different from 0 (Fig. 4), except for the two individuals from poultry farm 6 which were strongly related. The comparisons among individuals from different poultry farms (Fig. 4), show that almost none individuals were related. The only inter-poultry farm comparison with significant relatedness corresponds to that involving the two individuals from farm 6 with the one from farm 7.

According to the results of BayesAss, 47.5% of the individuals were recovered as first- or second-generation migrants (Table 3). Eighteen individuals collected on poultry farms 2, 3, 4, 5, 6 and 7 would have part of their genome coming from farm 1. Only one individual collected on poultry farm 1 was a first generation migrant coming from farm 3 (Fig. 1). Values of posterior probability are given in supplementary material (Supplementary material S2).
Polymorphism information content (PIC) and estimates of genetic variability ($\overline{\Pi}_\alpha$, $\overline{\Pi}_e$, and mean values) in *Rattus norvegicus* per locus and of genetic differentiation ($f$, $\theta$, $F$, and mean values) among six poultry farms of Exaltación de la Cruz, Buenos Aires, Argentina (farms with two or more individuals) based on the analysis of three microsatellite loci. ns= not significant genetic differentiation, $\alpha = 0.05$.

| Locus | PIC   | n  | $\overline{\Pi}_\alpha$ | $\overline{\Pi}_e$ | $f$     | $\theta$   | $F$  |
|-------|-------|----|-------------------------|---------------------|---------|------------|------|
| Rno1  | 0.4492| 7  | 0.41                     | 0.48                | 0.162 ns| -0.040 ns  | 0.128 ns|
| Rno2  | 0.5468| 7  | 0.58                     | 0.52                | 0.067 ns| 0.121 ns   | 0.179 ns|
| Rno8  | 0.2545| 4  | 0.17                     | 0.20                | 0.284 ns| -0.075 ns  | 0.230 ns|
| Mean  | 0.36  | 0.40 |                         | 0.141 ns            | 0.032 ns| 0.169 ns   |      |

**DISCUSSION**

This is the first research assessing dispersal levels in the genus *Rattus* using genetic markers in Argentina, and one of the few reports of this type worldwide (Kajdakci et al. 2013; Lack et al. 2013; Varudkar & Ramakrishnan 2015; Desvars-Larrive et al. 2017). We were not able to detect geographic subdivision among poultry farms, suggesting that individuals move among them and that matings occur randomly, at least within the area included in our study (110 km$^2$, **Fig. 3**). This result is in agreement with the findings of Latch et al. (2006), who reported that the Structure software was unable to correctly identify the number of subpopulations or to properly assign individuals to a certain genetic cluster until levels of $F_{ST}$ reached around 0.05. All $\theta$ values were very low, indicating that there is no population differentiation (see **Table 2**); with all individuals with similar genotypes; so that Structure could not differentiate among subpopulations. In agreement with a scenario of random matings, half of the individuals had signatures of migration in their genotypes, involving always farm 1 (**Fig. 1, Table 3**). The subpopulation of this farm had the highest sample size. Subpopulation 3 had also a high sample size, but it was only related with one case of high migration. So, we think these differences are explained by the central position of poultry farm 1. Additional sampling in nearby farms would help clarify this contrast.

The genetic results support previous ecological studies in the area (Gómez Villafañe & Busch 2007), and are in agreement with the hypothesis of high connectivity among *R. norvegicus* inhabiting poultry farms of Buenos Aires and with the idea that the whole system of poultry farms could be represented as a source-sink metapopulation (see Hanski et al. 1997). This is consistent with a study about this species by Lack et al. (2013), who analyzed microsatellite genotypes from 23 localities of the United States, separated by less than 7 000 km, and did not observe a pattern of migration correlated with geographic proximity. However, other studies about dispersion of *R. norvegicus* based on capture-mark-recapture, radiotracking and spool-and-line techniques indicated that rats move short distances, generally inside the farms (Gómez Villafañe et al. 2008; Montes De Oca et al. 2017), and their home range vary between 0.19-0.78 ha (Stroud 1982; Macdonald et al. 1999), although it may vary depending on habitat conditions (Taylor & Quy 1978; Stroud 1982).
This possible inconsistency could be overcome if the dispersion to farms is carried out by the trucks that transport chicken feed (Lack et al. 2013). This hypothesis was tested, and not supported by León et al. (2010) for *Mus musculus* in the same area as this study. In addition, as ecological methods use measurements of direct dispersion they disregard sporadic events of high dispersion which are unlikely to be detected with direct methods. However, indirect methods (e.g., genetic assessment of spatial structure), are able to detect effective long-term dispersal and not only movement (Slatkin 1987). An alternative explanation, proposed by Haniza et al. (2015) for a rural rat population from England, is that Norway rats have undergone a recent expansion homogenizing the genetic composition of all populations.

Because the expansion of different viral infections has been related to the dispersal of the host (Mills & Childs 1998), knowledge about gene flow patterns and the degree of isolation between subpopulations is of particular interest in species that act as hosts of infectious agents, like *R. norvegicus* in the study area (Lovera et al. 2017).

Our results indicate that individuals are moving among different farms, but not limited to nearby ones, being a great risk for zoonotic transmissions across long distances. Large-scale health control and preventive measures should be applied involving all poultry farms in the area simultaneously.

Since the number of loci used in this study is likely to be rather low to make robust estimates of small-scale spatial gene flow, future estimates should include additional microsatellite loci as well as samples in nearby crops and other farms. However, these estimates constitute a starting point for studying dispersion in rats using genetic tools in Argentina and serve as reference for future studies on the subject.

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**Table 3**

Individuals of *Rattus norvegicus* collected on poultry farms (in parenthesis) with important (more than 0.2) posterior probability of being first- or second-generation migrants from other poultry farms.

| Individual / poultry (p) | Putative origin | Generation migrant |
|--------------------------|-----------------|--------------------|
| S (1)                    | 3               | First              |
| 70 (2)                   | 1               | Second             |
| 71 (2)                   | 1               | Second             |
| 75 (2)                   | 1               | First              |
| 76 (2)                   | 1               | First              |
| 65 (3)                   | 1               | First              |
| 81 (3)                   | 1               | First              |
| 90 (3)                   | 1               | Second             |
| 91 (3)                   | 1               | First              |
| 16 (4)                   | 1               | Second             |
| 18 (4)                   | 1               | Second             |
| 78 (4)                   | 1               | First              |
| 9 (5)                    | 1               | First              |
| 39 (5)                   | 1               | Second             |
| 83 (5)                   | 1               | Second             |
| 84 (5)                   | 1               | First              |
| N (6)                    | 1               | First              |
| M (6)                    | 1               | First              |
| G (7)                    | 1               | Second             |

and serve as reference for future studies on the subject.
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ONLINE SUPPLEMENTARY MATERIAL

Supplement 1

Fig. S1. Mean log posterior probability of each K with its 95% confidence interval calculated with Structure Harvester 0.6.7.

Supplementary material S2. Output for program BayesAss. Posterior probability values of each individual of being resident, first or second generation migrant according to BayesAss 1.3. Migration rates that exceed 0.2 are considered important according to Wilson & Rannala (2003).