**Genetic analyses and occurrence of diploid males in field and laboratory populations of
Mastrus ridens (Hymenoptera: Ichneumonidae), a parasitoid of the codling moth.**

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Abstract

The parasitoid wasp *Mastrus ridens* (Hymenoptera: Ichneumonidae) is a particularly well-suited biological model to document the history and evolution of populations used in classical biological control, repeatedly moved from laboratory to laboratory worldwide and introduced in various environments. This specialist ectoparasitoid of the codling moth was first imported from Kazakhstan to the USA in the 1990's, and then sent to Argentina, Chile and New Zealand. More recently, it was sent to Australia and France from other laboratory colonies, and imported again from field collections in Kazakhstan to Chile. Here, we used DNA sequencing to confirm the taxonomic identity of several populations used for biological control worldwide, and developed microsatellite markers for population genetics studies. A multiplex PCR amplifying 11 polymorphic markers was designed. These markers were used to compare the genetic diversity of laboratory and field populations and evaluate genetic differentiation between them. Results showed that laboratory populations with the longest rearing history had lower genetic diversity. Moreover, the genotyping of males with the markers revealed the occurrence of diploid males, which was further confirmed by flow cytometry, suggesting complementary sex determination (CSD) in this species. The percentage of diploid males in the populations ranged from 4% to 25% and were negatively correlated with diversity indices, which is consistent with a single-locus CSD and genetic bottlenecks in laboratory rearings. Molecular tools proved to be suitable and reliable for genetic diversity analyses in *M. ridens*, and should be implemented more frequently in classical biological control programs.

Key words: classical biological control, diploid males, laboratory rearing, *Mastrus ridens*, microsatellites

1. Introduction

When a promising natural enemy of a key pest is found, it is a common practice in classical biological control to transfer populations from laboratory to laboratory (e.g. Li et al., 2015). Each transfer is generally associated with the establishment of a new small founder colony under new rearing conditions. The new colonies thus undergo bottlenecks either because the number of transferred individuals is small or because their establishment under new rearing conditions temporarily causes high mortality rates. These repeated transfers can lead to a loss of genotypic variability, with strong founder effects and increased inbreeding, which can result in a failure of successfully rearing or augmenting a species under laboratory conditions (Francuski et al., 2014; Gilchrist et al., 2012; Reed et al., 2003; Woodworth et al., 2002). How this loss of genetic variability will ultimately affect establishment success and/or efficacy of the field-released individuals is a matter of debate, as very few data on population genetics is collected during most biological control programs (Hopper et al., 1993; Hufbauer and Roderick, 2005). Nevertheless, there is evidence that the low success of some biological control programs could be due to loss of genetic variability (Fauvergue et al., 2012; Fowler et al., 2015; Homchan et al., 2014; Stouthamer et al., 1992; Taylor et al. 2011). Thus, natural enemies are suitable biological models to investigate the biology of small populations, which can be useful for the development of mass-rearing protocols and introduction strategies (Fauvergue et al., 2012; Hufbauer and Roderick, 2005).

Microsatellite markers or simple sequence repeats (SSRs) have been used in population genetics and ecological studies in many groups (Guichoux et al., 2011), including insects (e.g. Behura, 2006; Putman and Carbone, 2014). They have also been developed for hymenopteran parasitoids, mostly braconids (e.g. Anton et al., 2007; Baker et al., 2003; Homchan et al., 2014; Lozier et al., 2006), to study host-parasitoid associations (e.g. Lozier et al., 2009), population

genetic structure (e.g. Grillenberger et al., 2008), population genetic diversity and differentiation (e.g. Khidr et al., 2014), and gene flow, dispersal (e.g. Lavandero et al., 2011) and foraging behavior in the field (Tentelier et al., 2008). Genetic markers also have the potential for quantifying genetic diversity of laboratory mass-reared natural enemies (e.g. Woodworth et al., 2002), and for the detection of diploid males (Fauvergue et al., 2015). In haplodiploid species with complementary sex determination (CSD), the presence of diploid males is an indication of low genetic diversity and high levels of inbreeding (Heimpel and de Boer, 2008). This could result in male biased sex ratio during laboratory rearing, a phenomenon that has been observed in hymenopteran parasitoids (e.g. De Boer et al., 2007; Sandanayaka et al., 2011b).

Mastrus ridens Horstmann (originally identified as *Mastrus ridibundus* or *M. castaneus*) (Hymenoptera: Ichneumonidae) is one of the main parasitoids of codling moth (*Cydia pomonella* (L.)) in its area of origin, showing up to 44% parasitism of cocooned larvae in the bark of apple trees in Kazakhstan (Horstmann, 2009; Makarov, 1983; Mills, 2005). It is a gregarious idiobiont ectoparasitoid, with females laying three to five eggs on the cuticle of the last instar larvae. Because *M. ridens* is a specialist parasitoid of the codling moth, it has been used in classical biological control programs against this pest in apple and walnut orchards in several countries (Charles and Dugdale, 2011; Devotto et al., 2010; Mills, 2005; Sandanayaka et al., 2011a; Tortosa et al., 2014), with insects being transferred from laboratory to laboratory over the years (Figure 1). *Mastrus ridens* was first collected in Kazakhstan in 1993 and later released in the western United States (California and Washington). There it was reported as established with up to 70% parasitism of codling moth in orchards without pesticide applications (Mills, 2005). Then *M. ridens* from the California US laboratory colony, initiated from several collections in Kazakhstan from 1993-1998 with a total of around 400 females (N. Mills, University of California – Berkeley, personal communication), was sent to Argentina in 2005 and from this

laboratory colony to Chile in 2006, where it was mass reared and later released (Devotto et al., 2010; Tortosa et al., 2014). In 2009, individuals from the Argentina laboratory colony were sent to New Zealand (Sandanayaka et al., 2011a, Tortosa et al., 2014) and kept in rearing until their release in 2012. More recently, insects from the New Zealand colony were sent to Australia (2010-2013) and to France (2015) where they are currently maintained under laboratory conditions and planned to release when permits are granted. Thus, all released insects in the different countries so far are descendants from one founding laboratory colony initiated over 20 years ago (Figure 1).

Besides the long history of exchanges between laboratories, there are two facts that makes *M. ridens* interesting as a model to investigate the biology of small populations in the context of biological control. First, in Chile *M. ridens* was released in two distant regions, but successful establishment apparently only occurred in one of these locations (T. Zaviezo, personal observation), with no apparent cause for this failure, and thus it could have been due to genetic factors. Second, there has been a shift from female biased sex ratio in earlier laboratory colonies in California (Bezemer and Mills, 2003) to male biased sex ratio in more recent colonies in New Zealand (Sandanayaka et al., 2011b). In this work we report on the development of microsatellite markers for *M. ridens* and assess their usefulness in detecting genetic diversity in field-collected and laboratory populations with a known history. Additionally, using these microsatellite markers, we tested whether male-biased sex ratios observed in laboratory populations could be caused by the occurrence of diploid males.

2. Materials and Methods

2.1. Insects origin and populations

We used four main types of populations (Table 1): (1) wild insects, field collected within the native range in Kazakhstan near Kaskelen (43°9'53" N, 76°34'59" E) and Almaty (43°10'8.8"N, 76°53'29.8"E) by T. Zaviezo and K. Toleubayev in September 2013, corresponding to populations KZ1 and KZ2 (Zaviezo et al. 2014); (2) laboratory reared descendants of the 2013 field collected insects, populations CL2 and CL3; (3) laboratory reared insects from different countries, but all descendants from the collections made in the 1990's by N. Mills and T. Unruh near Almaty, Kazakhstan (see introduction), populations CL1, USA, NZ and FR; (4) mixed populations of descendants of the 1990's collections and those collected in 2013, populations CL4 and CL5. Historical information of these populations provided by the researchers involved in the introductions (see acknowledgements) are presented in Table 1 and Figure 1. We estimated the number of founding females for each population, that in the case of multiple introductions was the sum of all single introductions, and when sex of individuals was not noted we assumed a 1:1 sex ratio. We also recorded when (year and month) the colony was started and when insects were sampled for genotyping (year and month) and estimated the number of generations in rearing up to the sampling. For this, we assumed 15 generations per year (25 days to from oviposition to adult emergence; Devotto et al., 2010; Mills, 2005).

To check if individuals collected in 1990's and 2013, and their descendants corresponded to the same species, besides morphological identification following Horstmann (2009) and Torrens and Tortosa (2008), DNA of 46 individuals from seven populations (2 from KZ1, 13 from KZ2, 6 from CL1, 6 from CL2, 6 from CL3, 6 from NZ and 7 from USA) was extracted using the prepGEM Insect kit (ZyGEM, Hamilton, New Zealand), following standard protocols. DNA was then amplified by PCR reaction at two regions: Cytochrome oxidase I (COI) and 28S using the universal primers Forward 5' – GGTCAACAAATCATAAAGATATTGG – 3' and Reverse 5'TAAACTTCAGGGTGACCAAAAAATCA-3' for COI (Folmer et al. 1994) and

Forward 5'-AGAGAGAGTTCAAGAGTACGTG-3' and Reverse
5'TTGGTCCGTGTTTCAAGACGGG-3' for 28S (Dowton and Austin 2001). PCR products
were sequenced by Beckman Coulters Genomics (France), and comparisons for similar
sequences in Genbank (<http://www.ncbi.nlm.nih.gov/genbank>) were carried out with BLAST
(Basic Local Alignment Search Tool, <http://blast.ncbi.nlm.nih.gov/>).

2.2. Microsatellite library construction and molecular marker development

A microsatellite library for *M. ridens* was constructed following Malausa et al. (2011).
DNA was extracted and isolated from 20 individuals from the NZ population, with the extraction
Kit prepGEM® Insect (ZyGEM). Library enrichment and sequencing were carried out by
GenoScreen (Lille, France) on a Roche 454 GsFLX Titanium system (Brandford, USA). The
biotin labeled oligonucleotides had the following motifs: (AG)₁₀, (AC)₁₀, (AAC)₈, (AAG)₈,
(AGG)₈, (ACG)₈, (ACAT)₆ and (ATCT)₆. Both, primer design and sequence analyses, were
performed using the QDD software (Méglec et al., 2010). In total, 112 primer pairs were
selected, which were chosen based on the following criteria: target PCR product size between 80
and 450 bp with at least 10 repetitions, perfect microsatellite motifs, primer annealing
temperature close to 60°C and amplification of a variety of motifs. Five parasitoids (two from NZ
and three from KZ2) were used to test effective amplification without fluorescence. PCR was
performed with a 10µL reaction mixture and 2µl of diluted DNA (1–20 ng). Reagent
concentrations were 5µL of 1X QIAGEN Multiplex PCR buffer and 0.2µM of each primer. PCR
was carried out as follows: initial denaturation at 95°C for 15 minutes, followed by 35 cycles of
denaturation at 94°C for 30s, annealing for 90s at a temperature of 60°C, elongation at 72°C for
60s, followed by a final extension for 30 minutes at 60°C. The final products were separated by
electrophoresis with the QIAxcel Advanced System (QIAGEN, Hilden, Germany) for quality

checking. The absence of amplification for one or more individuals led us to discard the tested primer pair.

For all primer pairs that successfully amplified all five tested individuals, simplex PCR assays with fluorescent primers were carried out with the same method as that used for non-fluorescent primers. Two microliters of PCR products were analyzed by capillary electrophoresis with laser-induced fluorescence in a 9 μ L mix of formamide:size-standard-fluorescent-dye (500 LIZ GeneScanTM, Applied Biosystems, Woolston, UK) (25:1000). Amplification was successful with clearly readable patterns for 24 markers. Microsatellite patterns and sizes were scored with GeneMarkerTM version 1.75 software (Softgenetics LLC). For multiplex PCR assays, primer concentration and number of PCR cycles were modified until fluorescent intensity was satisfactory for all markers. The PCR multiplex kit was tested for all populations and validation of the kit was based on two populations: NZ and CL3. Linkage disequilibrium (LD) between pairs of loci and deviation from Hardy–Weinberg equilibrium (HWE) for each locus were tested, using Fisher’s exact tests implemented in Genepop 3.4 software (Raymond and Rousset, 1995). *P*-values were corrected using the False Discovery Rate approach and Bonferroni correction, for HWE and LD tests respectively. After these tests, 11 markers were kept for the final kit which was used in the analyses below.

2.3 Genetic diversity and differentiation estimates among populations

Females were used to estimate genetic diversity and differentiation considering all populations, except for KZ1 and CL1 from which we did not have enough females. Genetic diversity estimates were: number of alleles (N_a) calculated as the mean number of alleles considering the eleven microsatellites, allelic richness ($\hat{A}$) estimated with the R package StandArich v1.02 (<http://www.ccmар.ualg.pt/maree/software.php?soft=sarich>) and standardized

for a sample size of eight (which was the population with the smallest sample size, KZ2) to correct for the effect of sample size on the estimation of richness, observed and expected heterozygosities (H_o and H_e) and the inbreeding coefficient (F_{is}) calculated with GeneAEx v.6.502. Genetic differentiation between populations were described by pairwise F_{st} (Weir, 1996) calculated with Genepop. Additionally, we calculated the proportion of heterozygous females (number of females that were heterozygous at one or more loci of the total number of females genotyped) and mean number of heterozygous loci.

2.4. Detection of diploid males

To detect the presence of diploid males, 205 males from the eight different populations were genotyped using the microsatellites. For each population, we estimated the number of diploid males based on (1) the number of males heterozygous at one or more loci, Mh , and hence unambiguously diploid, and (2) the proportion of similarly heterozygous females in the population, which gives an approximation of the probability p of diploids being heterozygous at one or more loci. This defined, $Mh = p \times Md$, where Md is the number of diploid males. We thus used the rounded value of $Md = Mh / p$ to estimate the true number of diploid males in each population, this estimation being higher than the number of heterozygous males in populations where heterozygosity was lower than 1. The proportion of diploid males was computed as Md / M where M is the number of males genotyped in each population. We compared the proportion of diploid males in field versus the combined laboratory populations using a Fisher's exact test. To investigate if the proportion of diploid males related to the estimated genetic diversity, we correlated the proportion of diploid males with allelic richness and heterozygosity using Spearman rank correlations.

To confirm the capacity to detect diploid males through microsatellite genotyping, seven females and 29 males from the FR laboratory rearing, a highly inbred population, were analyzed by flow cytometry, which evaluates the quantity of DNA in cell nuclei by binding DNA to a fluorescent dye (De Boer et al. 2007). Briefly, heads of individuals were ground in a 0.5 mL ice-cold Galbraith buffer (Galbraith et al. 1983) with a Dounce tissue grinder. Homogenate was sieved (40 μ m) and stained with 25 μ g propidium iodide per sample. The DNA content of 10,000 nuclei from each individual was analyzed on a LSRII Fortessa flow cytometer and FACSDiva Version 6.1.3 (BD Biosciences, San Jose, USA) at 561 nm excitation wavelength. The results are presented as cell number versus fluorescence intensity, which is proportional to DNA content. Male DNA profiles were compared to those of females, which we expected to be diploid. For each individual the rest of the insect body was genotyped to compare the results from microsatellites genotyping and flow cytometry.

3. Results

3.1. Insect molecular identification

Of the 46 insects from seven different populations sequenced for identification, only one haplotype was found for 28S (Genbank accession number KU881764) and two for COI (Genbank accession numbers: KU881765 for haplotype 1; KU881766 for haplotype 2), the second haplotype being found in only one individual and differing by one substitution. This, along with the morphological identification, confirms that the insects sampled in 1994-1998 belong to the same species of those collected in 2013. The best Blast hit for the 28S haplotype was for a sequence reported for *Mastrus* sp. (Laurenne et al., 2006, GenBank accession number AY604411), with 99% identity. The best Blast hit for the COI haplotypes was a sequence from

an unidentified species of Ichneumonidae (Genbank accession number HQ972398.1) with only 90% similarity.

3.2. Mastrus ridens microsatellite library and markers

The final microsatellite DNA library contained 27,135 sequences. In total, 2,736 microsatellite loci were identified and satisfactory PCR primers could be designed for 942 loci. Primers for a set of 112 loci that displayed a variety of fragments (di-nucleotides, tri-nucleotides, tetra-nucleotides, with a range of repeat numbers) were selected for further use. After testing these primers on five DNA extracts, 59 led to clear amplification patterns for all DNA extracts, 21 led to amplification of only some of the DNA extracts and 32 did not result in any amplification. After PCR assays with the fluorescent primers, 24 markers amplified correctly and sufficiently with fluorescence (Appendix Table A.1). Of these, seven were not polymorphic (MR003, MR063, MR066, MR088, MR090, MR093 and MR101), and five did not show enough intensity of fluorescence in multiplex tests (MR038, MR089, MR094, MR104 and MR106). Only seven of the markers had more than two alleles, suggesting poor genetic diversity among the parasitoid individuals used. Finally, 12 markers amplified correctly at 30 cycles of PCR and different concentrations per reaction: MR002, MR006 and MR034 at 0.19 μ M; MR010 at 0.125 μ M; MR011 at 0.0875 μ M; MR018, MR035, MR056 and MR080 at 0.31 μ M; MR030 and MR044 at 0.25 μ M; and MR051 at 0.5 μ M. These 12 markers were tested on all ten populations, and showed polymorphism. For the kit validation using the NZ and CL3 populations, none of the markers showed deviation from HWE, and 16 marker pairs showed LD, 14 in CL3 and two in NZ. One pair of markers presented LD in both populations (MR056 and MR010), and thus MR010 was excluded from the kit because it provided less information than MR056. The 11 microsatellite markers selected for the kit are shown in Table 2.

3.3. Genetic diversity and differentiation among populations of different origin and rearing history

A total of 202 females from eight populations were tested to compare different measures of genetic diversity using 11 microsatellites (Table 3). The mean number of alleles ranged from 2.9 (KZ2) to 1.7 (FR). The mixed laboratory populations CL2 and CL5 showed the highest allelic richness and heterozygosity, while the FR population had many of the lowest diversity indices. Inbreeding coefficients ranged from -0.185 to 0.165, with the mixed population (CL5) having the lowest coefficient and the NZ laboratory population the highest. The proportion of heterozygous females varied from 0.64 for the USA population to 1.0 in KZ2, CL4, NZ and FR populations, while the mean number of heterozygous loci in females declined from 5.6 in KZ2 to 2.6 in CL4 (from a total of 11). The CL2, CL3 and CL5 populations had on average 4.4 heterozygous loci, while USA, FR and NZ had on average 2.9 heterozygous loci.

The genetic differentiation (F_{st}) found between populations was large (≥ 0.1 ; Table 4), except for KZ2 with CL2 and CL3 and for NZ with FR, which is consistent with the fact that CL2 was formed by mixing KZ1 and KZ2, CL3 were the laboratory reared descendants of the wild collected KZ2, and FR was formed with insects from the NZ laboratory. In all these cases no more than three generations had passed since the colony was initiated (Table 1). The highest differentiation was between the laboratory colonies from USA with NZ and FR, which both come from the 1990's collections but diverted from the beginning, almost 20 years (300 generations approximately) before we sampled them (Figure 1, Table 1). NZ and FR showed very high genetic differentiation with most of the populations ($F_{st} \geq 0.32$).

3.4. Occurrence of diploid males

Samples of the 205 males analyzed with the microsatellites showed an overall proportion of heterozygotes of 0.17 with 95% confidence intervals of [0.12, 0.23], indicating the presence of diploid males (Table 5). After correcting for the probability of misclassifying homozygous diploid males as haploids, we estimated the overall proportion of diploid males at 0.19 [0.14, 0.25]. The proportion of diploid males in the laboratory-reared populations was high (0.21 [0.15, 0.27]), while the field sampled populations had the lowest percentage of heterozygous males (0.04 [0.0001, 0.196]). The proportion of diploid males in laboratory populations was significantly larger than in the field population (Fisher's exact test $p = 0.026$). Using Spearman rank correlations to account for small sample sizes ($N = 6$), we found marginally significant negative correlations between the proportion of diploid males and either allelic richness (A: $r = -0.76$, $p = 0.077$) or expected heterozygosity (He: $r = -0.75$, $p = 0.084$).

Of the 29 males analyzed by flow cytometry and microsatellites, five heterozygous individuals showed a DNA profile from the flow cytometry that was similar to that of females with a first peak at 400 fluorescent units (Figure 2A and 2B). The DNA profile of the other hemizygous males showed a first peak at half the number (200) of fluorescent units, thus having half of DNA content (Figure 1C). Interestingly, all male DNA profiles showed a second peak at twice the number the fluorescent units as the first peak, indicating endoreduplication in head tissues (Figure 1A and 1C). All males with a female-like DNA profile were identified as heterozygous genotypes from the microsatellite analysis.

4. Discussion

In this study we developed molecular markers for *M. ridens*, a specialist parasitoid of the codling moth imported to several countries for classical biological control programs, and carried

out genetic analyses with the aim of exploring how neutral genetic diversity varied with rearing history and how it related with the proportion of diploid males.

Morphological identification, DNA sequencing at two loci (COI, 28S) and microsatellite genotyping confirmed that the insects collected from the field in Kazakhstan (its area of origin) 20 years apart, and from different regions, all belonged to the same species. The partial 28S and COI sequences of descendants of the 1993-98 collected individuals and those collected in 2013 and their descendants were all identical. The partial 28S sequence displayed high similarity with a specimen identified as *Mastrus* sp. Laurene et al. (2006) in a phylogenetic study. The availability of these sequences could be used for quick preliminary identification of this species in many circumstances, like exploration for codling moth natural enemies in its area of origin, or carrying out surveys and detecting non-target effects in released areas (Hoddle et al., 2015).

The development of the microsatellite library and selection of markers for *M. ridens* did not present any particular difficulty: the library obtained was above average quality, standard success rates were observed at each step of the development, and the final number of available markers was superior to initial expectations. In this study, we used 11 microsatellite markers amplified in a single multiplex PCR, which is a sufficient amount of markers for basic analyses of population genetics. Previous studies of microsatellite development with parasitoid wasps produced on average 11.8 markers (Couchoux et al., 2015; Fauvergue et al., 2005; Jourdie et al., 2009; Lozier et al., 2006; Nyabuga et al., 2009), similar to the number developed in the current kit. However, the mean number of alleles per marker was lower in this kit (2.31 alleles/marker vs 5.86 alleles/marker).

The microsatellite markers were used to estimate genetic diversity within populations, differentiation between populations, and to detect the presence of diploid males in *M. ridens*. Individuals analyzed were collected from the region of origin and from eight laboratory

populations for which the rearing history was known. Allelic richness of these populations was on average 2.2 alleles/marker and heterozygosity (He) was 35%. Other studies with parasitoids have shown a higher average allelic richness and heterozygosity: 4.5 alleles/marker and 38% heterozygotes for *Neotypus melanocephalus* Gmelin (Hymenoptera: Ichneumonidae) (Anton et al., 2007), and 5.2 alleles/marker and 63% heterozygotes for *Aphidius ervi* (Haliday) (Hymenoptera: Braconidae) (Zepeda-Paulo et al., 2015). The highest estimated diversity was from the laboratory mixed population CL2, which was formed by crossing insects collected in two different regions in the area of origin. The next most diverse population was CL5, which was somewhat surprising since it was initiated with only four field-collected females that were crossed with males from CL4, a not very diverse population (Table 3). This supports the idea that multiple introduction or adding wild individuals to laboratory colonies can increase the diversity of the population, at least temporarily (Francuski et al., 2014). These practices are common in commercial biological control laboratories (Gujar et al., 2006). Nevertheless, there are examples where adding individuals that differ genetically from those in rearing or established can be detrimental, for example when hybrid depression occurs and there is a reduction in fitness of locally adapted parasitoids (Vorsino et al., 2012).

The diversity of the studied populations, after CL2 and CL5, followed an expected pattern of decreasing diversity, according to the length of time in rearing and bottlenecks suffered during the transfer of individuals when initiating new laboratory colonies: the field-collected KZ2, its descendants CL3, a mixed of laboratory populations CL4, and finally the three populations that have the longest rearing history USA, NZ and FR. The estimated diversity of the field population in Kazakhstan was lower than the diversity estimated for other parasitoids, such as *N. melanocephalus* and *A. ervi* (Anton et al., 2007, Zepeda-Paulo et al., 2015). This could be because our collection was made in a small isolated patch of an old abandoned apple orchard,

which still remains in the outskirts of Almaty, a city that has grown rapidly in the last ten years. Additionally, trees in unmanaged apple orchards typically suffer alternate fruit bearing simultaneously, which means that every other year *M. ridens* host, codling moth larvae, suffers large population variations. Thus as unmanaged orchards in this area are progressively lost and become more isolated, *M. ridens* could be at risk of becoming locally extinct in some orchards.

The low diversity of the NZ and FR laboratory populations was expected, given they are comprised by descendants of a laboratory rearing begun over 20 years ago (California) and has suffered several bottlenecks of variable degree, including one after 165 generations in rearing in USA when a sample of 850 females was moved to begin the Argentina colony in 2005 (Castelar-Buenos Aires), then after 3 generations 50 females were used to begin a new colony in Argentina (Mendoza), four years later (60 generations) 90 females were sent from Argentina to New Zealand and six years later 500 females were sent from New Zealand to France (Figure 1, Sandanayaka et al., 2011a; Tortosa et al., 2014). Thus, it is surprising that NZ and FR populations do not show a greater loss of genetic diversity over time, and also that the change in allelic richness (A) has been less than in expected heterozygosity (He) (15% loss in A vs 26% loss in He for NZ, and 29% loss in A vs 65% loss in He for FR, in comparison with KZ1), given that theory predicts that bottlenecks should have a greater reduction in allelic richness (Bock et al., 2015). Two factors might have helped in maintaining diversity. First, the California colony was formed by eight collection campaigns in its area of origin, and new wild individuals were added to the colony on each occasion. Second, in the path to New Zealand and France colonies increased its numbers substantially, given that their objective was to quickly mass rear them for release. It has long been recognized that rapid population increase after a bottleneck event can prevent the loss or restore genetic diversity (Nei et al., 1975), and this could be a desirable management procedure for laboratory populations, even if mass releases are not the objective (Hufbauer and

Roderik, 2005). Other practices suggested for maintaining genetic diversity of captive populations that are planned to be released, are to fragment the laboratory population or generate isofemale lines and then mix them before release (Hoddle et al., 2015; Roush and Hopper, 1995; Woodworth et al., 2002).

From the microsatellite analysis, we also estimated the level of genetic differentiation among the populations, and as expected the lowest differentiations were found between the field population and its descendants in the laboratory (KZ2 with CL2 and CL3) and between NZ and FR. The high levels of genetic differentiation found between NZ and FR laboratory populations with the rest of the populations suggest strong founder effects, genetic drift or a combination of both, during the transfer of individuals and subsequent rearing. As expected, also low levels of genetic differentiation are found between CL laboratory populations, that have a short rearing history, but it is interesting that among them CL4, a population that had direct input from the NZ population, is the more distinct.

The microsatellites developed for *M. ridens* also proved useful as a tool for detecting diploid males. A high proportion of diploid males was found in laboratory colonies (20% overall, much higher than the 4% observed in the field), suggesting low diversity in the founding individuals and/or a loss of genetic diversity under these conditions. High proportions of diploid males were found even after short periods in rearing, as evidenced by the CL3 and CL4 populations. In *M. ridens*, this high proportion of diploid males is congruent with observed male-biased sex ratios found in colonies with longer rearing history (as in New Zealand; Sandanayaka et al., 2011b). These observations provide some of the few data available on the occurrence and frequency of diploid males under natural conditions (De Boer et al., 2012; Ruf et al., 2013). Further studies are underway to assess the fitness of diploid males for *M. ridens* and its population consequences.

The patterns of genetic diversity among the populations studied and the occurrence of diploid males show that genetic diversity can be reduced over time in laboratory colonies and after the importation and establishment of new colonies. How this low genetic diversity found using putatively neutral markers are likely to affect the establishment and success of biological control agents is not known. This highlights one of the weaknesses of past and current classical biological control programs, a failure to keep track of population genetic diversity and performance during the successive steps in an importation program. Molecular techniques, such as the analysis of microsatellite markers, proved to be suitable tools for this purpose for *M. ridens*, suggesting that they should be implemented more frequently when carrying out classical biological control programs.

In the context of invasive species biology, the role of genetic diversity (neutral or additive) in invasion success is currently highly debated, with many studies pointing to a weak effect (Bock et al., 2015; Dlugosch et al., 2015). Nevertheless, natural enemies used for biological control usually differ from invasive species in many important biological and ecological characteristics, and thus research specifically targeting natural enemies is needed (Hufbauer and Roderick, 2005; Roderick et al., 2012). A particular case being species of hymenopteran parasitoids with complementary sex determination, where successive founding events and long time in rearing favors sampling effects and genetic drift, which leads to an increase in the proportion of diploid males with potentially important negative effects on population growth and persistence (De Boer et al., 2007, 2012; Fauvergue et al., 2015).

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Figure legends.

Fig. 1. Representation of the history of *Mastrus ridens* collections, exchanges between laboratories and field releases. Grey boxes represent field populations or releases (year in the grey boxes indicate when releases were done). White boxes are the different laboratories to where *M. ridens* was imported and reared. Lines indicate the source and destination of populations and years when this happened, and number of founding females. Different types of lines represent different strains. Codes in boxes correspond to the populations genotyped (Table 1).

Fig. 2. Flow cytometry histograms of number of nuclei counts in relation to fluorescence intensity (DNA content) in a sample of laboratory reared *Mastrus ridens*. A: representative male with heterozygous genotype (diploid male); B: representative female (diploid); C: representative male with hemizygous genotype (haploid).

Figure 1

Figure 2

1 **Tables**

2

3 **Table 1.** *Mastrus ridens* populations used in this study: code, country and city of collection, type of population (collected in the field
4 or from a laboratory colony), year colony was started, number of founding females (total through the years), year individuals were
5 sampled for genotyping, approximate number of generations in rearing before sampled for genotyping (assuming 15 generations per
6 year), and number of females and males that were genotyped.

Code	Country (City)	Type and source	Year colony was started	Number of founding females	Year collected or sampled	Generations in rearing	Number genotyped	
							females	males
KZ1	Kazakhstan (Kaskelen)	Field	-	-	2013	0	6	2
KZ2	Kazakhstan (Almaty)	Field	-	-	2013	0	8	24
CL1	Chile (Chillán)	Laboratory: from Argentina	2006	50	2014	120	0	10
CL2	Chile (Santiago)	Laboratory: KZ1 x KZ2 progeny	2013	9	2013	2	16	14
CL3	Chile (Santiago)	Laboratory: KZ2 progeny	2013	14	2013	3	60	103
CL4	Chile (Santiago)	Laboratory: mix of NZ, CL2, CL3	2014	14	2014	4	10	5
CL5	Chile (Santiago)	Laboratory: mix of CL4 & KZ2	2014	4	2014	4	24	0

USA	USA (Wapato)	Laboratory	1993	50-100	2013	300	14	15
NZ	New Zealand (Aukland)	Laboratory: from Argentina	2009	90	2014	75	56	0
FR	France (Antibes)	Laboratory: NZ progeny	2015	500	2015	2	14	32

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Table 2. Characteristics of the 11 microsatellite markers amplified by the designed multiplex PCR and tested in the two populations of *Mastrus ridens* with N > 30. Ho/He and HWE *P* values were based in laboratory populations from New Zealand (NZ) and Chile (CL3).

Marker (GenBank accession numbers)	NZ population					CL3 population				
	N	Na	Ho / He		HWE	N	Na	Ho / He		HWE
MR002 (KU880699)	56	2	0.429	/ 0.378	0.921	79	2	0.443	/ 0.399	0.904
MR006 (KU880700)	55	1	0	/ 0	-	70	3	0.686	/ 0.645	0.728
MR011 (KU880701)	56	2	0.054	/ 0.053	1	79	2	0.405	/ 0.426	0.428
MR018 (KU880702)	56	1	0	/ 0.250	-	80	3	0	/ 0.267	0.282
MR030 (KU880703)	56	4	0.339	/ 0.530	0.011 ^a	79	2	0.203	/ 0.223	0.348
MR034 (KU880704)	56	2	0.107	/ 0.102	1	80	3	0.050	/ 0.073	0.044 ^a
MR035 (KU880705)	56	3	0.750	/ 0.612	0.994	80	3	0.338	/ 0.379	0.007 ^a
MR044 (KU880706)	56	3	0.714	/ 0.611	0.908	80	2	0.638	/ 0.499	0.997
MR051 (KU880707)	55	1	0	/ 0	-	74	4	0.432	/ 0.403	0.731
MR056 (KU880708)	56	3	0.429	/ 0.405	0.738	80	4	0.600	/ 0.615	0.079
MR080 (KU880709)	56	2	0.107	/ 0.102	1	77	2	0.429	/ 0.482	0.231

N: number of individuals, Na: number of alleles; Ho: observed heterozygosity; He: expected heterozygosity; HWE: *P* values from Hardy Weinberg Equilibrium test; ^a Non-significant after FDR correction.

17 **Table 3.** Number of females genotyped and genetic diversity indices in the sampled populations
18 (populations details in Table 1).

Population	N	Na	$\hat{A}$	Ho (SE)	He (SE)	Fis	Proportion heterozygous	N Het loci
KZ2	8	2.73	2.35	0.411(0.06)	0.413(0.05)	0.037	1.000	5.63
CL2	16	2.91	2.69	0.430 (0.05)	0.517 (0.03)	0.153	0.813	4.69
CL3	60	2.36	2.16	0.367(0.06)	0.369(0.06)	0.007	0.883	4.40
CL4	10	1.91	1.89	0.236 (0.06)	0.275 (0.07)	0.075	1.000	2.60
CL5	24	2.46	2.64	0.523(0.08)	0.446(0.05)	-0.185	0.958	4.04
USA	14	2.18	1.89	0.266 (0.09)	0.252(0.08)	-0.052	0.643	2.71
NZ	56	2.18	1.98	0.247(0.05)	0.307 (0.06)	0.165	1.000	2.93
FR	14	1.73	1.66	0.266 (0.10)	0.228 (0.08)	-0.153	1.000	2.93

19 N: number of individuals; Na: mean number of alleles; $\hat{A}$: allelic richness; Ho: observed heterozygosity;
20 He: expected heterozygosity; SE: standard error; Fis: inbreeding coefficient; Proportion heterozygous
21 females: number of heterozygous females in one or more loci /total number of genotyped females; N Het
22 loci: the mean number of heterozygous loci (out of 11) in females.

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26 **Table 4. Genetic differentiation (Fst) between populations of *Mastrus ridens* based on**
27 **females.**

Population	KZ2	CL2	CL3	CL4	CL5	USA	NZ
CL2	0.066						
CL3	-0.013	0.118					
CL4	0.194	0.257	0.230				
CL5	0.100	0.130	0.125	0.251			
USA	0.264	0.293	0.261	0.408	0.444		
NZ	0.339	0.435	0.351	0.146	0.376	0.529	
FR	0.323	0.396	0.329	0.139	0.366	0.510	0.019

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Table 5. Detection of diploid males in field and laboratory populations of *Mastrus ridens*. Population (code), type of population (field collected or laboratory reared), total number of genotyped males (N), proportion of diploid males (i.e., proportion of heterozygous males corrected for the probability of misclassifying homozygous males as haploid, see text for details, except for CL1, with 95% CI estimated from a binomial distribution), and mean number of heterozygous loci (out of 11) for those males.

Population	Type	N	Proportion of diploid males [95% confidence intervals]	Mean number of heterozygous loci in heterozygous males
KZ1	field	2	0.000 [0.000-0.842]	-
KZ2	field	24	0.042 [0.001-0.211]	5
CL1	laboratory	10	0.100 [0.003-0.445]	1
CL2	laboratory	14	0.071 [0.002-0.339]	4
CL3	laboratory	103	0.233 [0.155-0.327]	4.2
CL4	laboratory	5	0.200 [0.005-0.716]	3
USA	laboratory	15	0.133 [0.017-0.405]	3.5
FR	laboratory	32	0.250 [0.115-0.434]	2.6
Laboratory pops		179	0.207 [0.150-0.273]	3.6
Total		205	0.185 [0.135-0.245]	3.7