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SHORT COMMUNICATION

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# Cross species transferability of G-SSR and EST-SSR markers to *Neltuma affinis* Spreng

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## Abstract

*Aim of study:* To examine the transferability of G-SSR (genomic simple sequence repeats) and EST-SSR (expressed sequence tag simple sequence repeats) markers developed for several *Neltuma* species to *N. affinis*, a species with no genomic data.

*Area of study:* West-Center of Entre Ríos province, Argentina. The set of molecular markers here proposed can be used to analyze samples from the entire species' distribution range.

*Material and methods:* Twenty-five genomic G-SSRs and eleven EST-SSRs from multiple species were amplified in thirty *N. affinis* genotypes. Polymorphism, discrimination power and possible deviations from Hardy-Weinberg equilibrium were assessed.

*Main results:* Seventeen highly polymorphic G-SSRs were successfully transferred to *N. affinis*, with a PIC (polymorphic information content) average value of 0.811 and a He (expected heterozygosity) average value of 0.694; thirteen were validated, showing very low frequencies of null alleles and no linkage disequilibrium. Additionally, seven polymorphic EST-SSRs were transferred. As expected, PIC and He average values were low. Six out of seven markers were validated, and very low frequencies of null alleles and no linkage disequilibrium were observed.

*Research highlights:* This work provides information on the levels of microsatellites' cross transferability to *N. affinis*, and its polymorphism degree. Two sets of polymorphic SSRs (genomic and expressed) to study the genetic status of the species are proposed.

**Additional key words:** microsatellites; genomic markers; functional markers; markers validation; ñandubay; espinal.

**Abbreviations used:** CTAB (cetyl trimethyl ammonium bromide*);* EST-SSR (expressed sequence tag simple sequence repeats); Fis (inbreeding coefficient); G-SSR (genomic simple sequence repeats); He (expected heterozygosity); Ho (observed heterozygosity); HWE (Hardy-Weinberg equilibrium); LD (linkage disequilibrium); Na (number of alleles); NA (frequency of null alleles); Ne (number of effective alleles); PCR *(*polymerase chain reaction); PIC (polymorphic information content).

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# Introduction

Molecular genetics provides several tools to study genetic diversity of tree species, their response to landscape fragmentation and their adaptation to changing environments (Neophytou et al., 2022). Among the molecular tools available, microsatellites or SSRs stand out for their wide distribution in eukaryote genomes in both coding and non-coding regions, as well as nuclear and organellar DNA. Microsatellites are a type of DNA sequence consisting of tandem repeats of 1 to 6 nucleotide motifs and are characterized by a low degree of repetition per marker (5 to 100) and a random distribution per genome ($10^4$-$10^5$) (Wu et al., 2020). These markers exhibit codominant inheritance, hypervariability, extensive genome coverage and can be transferred among phylogenetically close species (Wu et al., 2020). Additionally, are widely used for population genetic, as well as genetic diversity studies (Vinson et al., 2018).

SSRs can be categorized as G-SSRs, when obtained from a whole genome, and as EST-SSRs, when obtained from transcribed regions and consequently related to gene function (Ouyang et al., 2018). The development of markers based on transcriptome information has been effectively applied in numerous tree species, including several *Neltuma* species (Torales et al., 2013; George et al., 2017).

Recently, the traditional *Prosopis* genus has been split based on strong phylogenetic evidence (Hughes et al., 2022). Maintaining the unity of *Prosopis* sensu Burkart (1976) is no longer sustainable. Most representatives of this genus in the New World are now located in the resurrected genus *Neltuma* (Hughes et al., 2022). *Neltuma affinis* (Spreng.) C. Hughes et Lewis (= *Prosopis affinis* Spreng.; Fabaceae; Caesalpinioideae; Mimosoideae clade) (Hughes et al., 2022), also known as ñandubay, it's a tree species distributed in north and central-western Argentina, southern Brazil, Paraguay and Uruguay (Oyarzabal et al., 2018). This species has Chacoan lineage (Morales et al., 2019) and is one of the dominant species in some forests of the Espinal ecoregion, configuring an exclusive biogeographic district with epicenter in the province of Entre Ríos, in Argentina (Cabrera, 1976). It is severely exploited by local communities due to medicinal and chemical properties and as a source of fodder, fuel, shade, food and wood. In the present, only small relicts of the species remain immersed in a heterogeneous mosaic of crops, pasture, forest plantations, grazing and urban areas (Sabattini et al., 2016).

Despite the economic value and threat condition of *N. affinis*, there are currently no molecular tools available to study this species. Therefore, microsatellites could provide a helpful tool to assess this species. These markers are still actively used due to its numerous advantages, including its transferability among species of the same genus or even among different genera (Ferreira-Ramos et al., 2014; Karci, 2023). Additionally, EST-SSRs are present in more conserved regions and, therefore, exhibit high transferability rates (Wu et al., 2020).

Since the ñandubay is a species with no genomic data, our objective was to examine the transferability to *N. affinis* of different G-SSR and EST-SSR markers, all developed in several *Neltuma* species. Our findings provide information on the levels of cross transferability of microsatellite markers among *Neltuma* species as well as the relative degrees of polymorphism. Two sets of polymorphic SSR (genomic and expressed) to study the genetic status of *N. affinis* are proposed.

# Material and methods

We analyzed thirty *N. affinis* individuals, from sixteen fragments of remaining native forest from Entre Ríos (Argentina) (Fig. 1). Voucher specimens were collected and deposited in the herbarium of Instituto de Recursos Biológicos (BAB) in order to confirm their taxonomic identity (Annex [suppl]). Total genomic DNA from dried leaves was extracted following Soldati et al. (2013).

Cross species transferability of G-SSRs and EST-SSRs markers was assessed using a sample of eight *N. affinis* individuals to examine twenty-five G-SSRs and eleven EST-SSRs from multiple source species (Table S1 [suppl]). To achieve a pre-selection of microsatellites we assessed transferability success, clearness of resolution patterns and polymorphism level (at least two alleles at any frequency). Different PCR conditions were tested on each primer pair to optimize the transferability (Table S2 [suppl]), following Mottura et al. (2005) and Torales et al. (2013) PCR protocols. PCR products were genotyped using a 6% standard denaturing polyacrylamide gel, silver stained following the protocol by Benbouza et al. (2006). Results were classified into three categories: polymorphic (P), monomorphic (M), and non-specific (NS). In order to assess the discrimination power of each polymorphic microsatellite, the pre-selected microsatellites were evaluated through thirty individuals. Several genetic diversity parameters (Na, Ne, Ho and He) for each polymorphic locus were estimated using GenAlEx 6.503 software (Peakall & Smouse, 2012). PIC was estimated using Cervus 3.0.3 software (Kalinowski et al., 2007). Finally, possible deviations from HWE were assessed: NA, Fis and LD were estimated using GENEPOP 4.0 (Rousset, 2008) software. SSRs were selected according to Soldati et al. (2013) criteria for these parameters.

# Results and discussion

Two set of SSR markers (genomic and expressed) were obtained to study *N. affinis*. Cross transferability of SSR markers to *N. affinis* was 100%, with polymorphism levels ranging from 58% to 68% (EST-SSR and G-SSR, respectively). Our results agree with studies that have reported higher transferability rates for G-SSRs and EST-SSRs, when the phylogenetic distance is low (Demdoum et al., 2012). Additionally, our work is similar with the literature highlighting differences between G-SSRs and EST-SSRs, particularly when the comparison is carried out within a plant species (Manco et al., 2020).

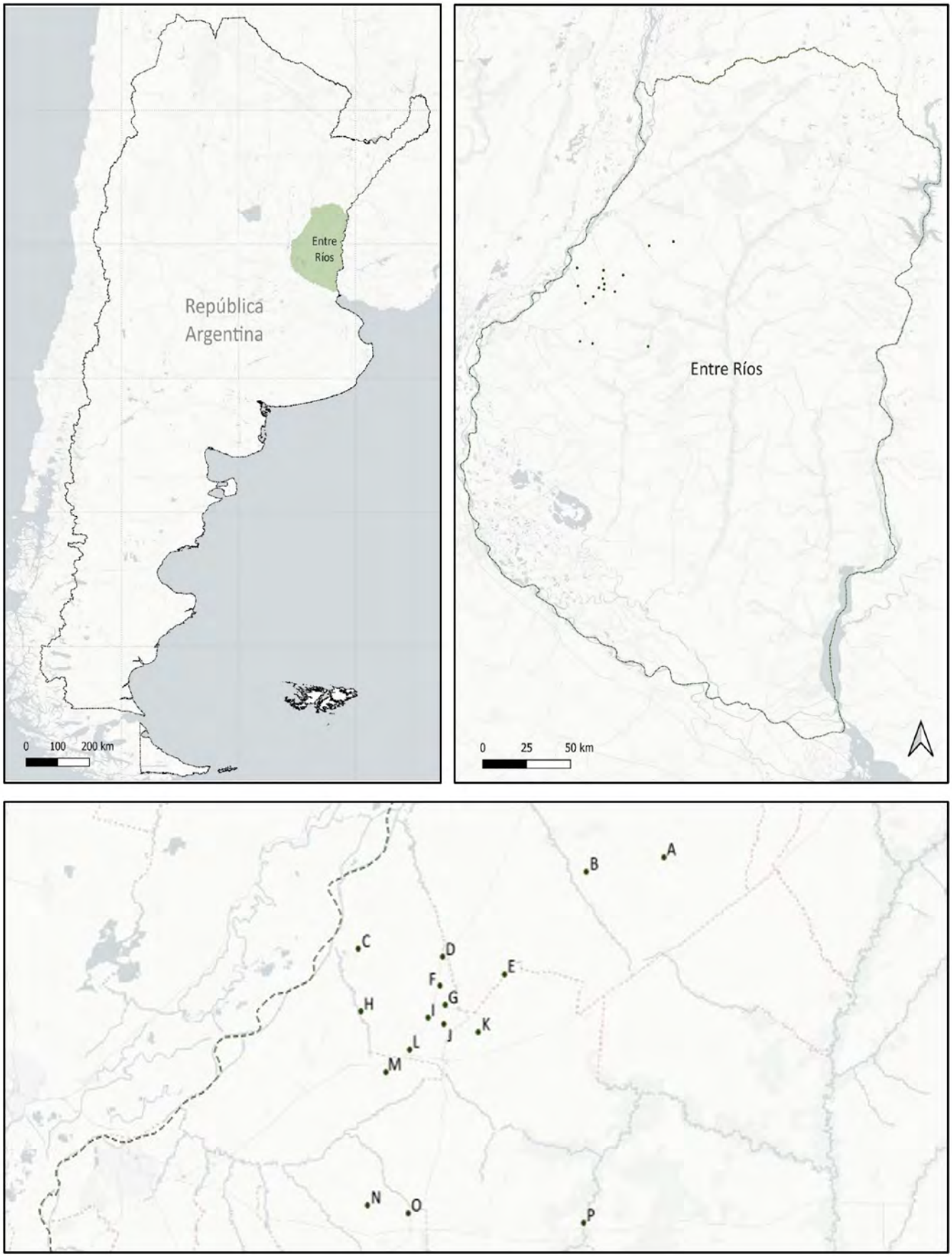


**Figure 1.** Fragments of remaining native forest (16, named from A to P) where the analyzed samples were collected.

All G-SSRs assessed showed successful amplification in *N. affinis*: seventeen were polymorphic (68%), one was monomorphic (4%) and seven loci (28%) showed non-specific amplification (Table S1 [suppl]). Additionally, all polymorphic markers produced fragments within the expected size range (≤ 100 bp larger or smaller than the original sequences, according to the Arnold et al. (2002) criteria; see Table S2 [suppl]. Our findings agree with studies showing higher success rates on cross-genera microsatellites transferability than cross-family microsatellites transferability (Contreras et al., 2019). This is particularly clear when analyzing the results of polymorphic markers, where those that were developed for phylogenetically closer species showed a higher success. A clear decrease in the percentage of polymorphic loci was particularly observed for *Neltuma ruscifolia* markers; this result is probably related to the phylogenetic distance between *N. affinis* and *N. ruscifolia* supporting our conclusions of higher success with lower phylogenetic distance (Catalano et al., 2008).

In *N. affinis*, 149 alleles were detected (average = 8.76) ranging from 4 to 18 for loci Mo08 and PRB04. The He and PIC values ranged from 0.214 to 0.906 and from 0.523 to 0.938 for loci GL18 and PRB04, respectively. Average values for those parameters were 0.694 and 0.811, demonstrating the presence of highly polymorphic markers for *N. affinis* (Table 1). Botstein et al. (1980) proposed that microsatellites with PIC values greater than 0.5 are considered highly informative, which supports these results. Moreover, these genetic diversity parameters estimated for *N. affinis* were comparable and higher that those reported for *Neltuma alba* and *Neltuma chilensis* using the same microsatellite sequences (Bessega et al., 2013).

Null alleles were found in sixteen out of seventeen polymorphic G-SSRs transferred; however, values greater than 0.05 were reached at only four loci (Mo05, Mo07, Mo13 and PRB08). These alleles are caused by mutations in the microsatellite flanking regions, resulting in erroneous PCR amplification. Higher frequencies of null alleles have been documented when transferring heterologous primers among species, as the phylogenetic distance increases (Jahnke at al., 2022). Our results support the hypothesis that the frequency of null allele increases with the phylogenetic distance among species (Chapuis & Estoup, 2007). Additionally, none of the polymorphic loci revealed significant LD ($p > 0.05$) and the average inbreeding coefficient showed no significant deviations from HWE genotypic proportions, except for loci Mo05, Mo07 and Mo13. For this parameter, values close to zero are expected under random mating. Considerable positive Fis values, as those shown in loci Mo05, Mo07 and Mo13, indicate a defect of heterozygosity and are associated with high frequencies of null alleles (Peyran et al., 2020).

Additionally, all twelve assessed EST-SSRs developed for *N. alba* (Torales et al., 2013), showed successful amplification in *N. affinis*. Among the amplified loci, seven were polymorphic (58.3%) and five were monomorphic (41.7%). All polymorphic loci produced reproducible and reliable amplicon patterns within the expected size range (Arnold et al., 2002). None of the loci showed non-specific amplification (Table S1 [suppl]). These results, regarding global transferability and polymorphism levels, are comparable to those obtained by Pomponio et al. (2015), who assessed the transferability of this EST-SSRs to *N. flexuosa*, *N. chilensis*, *Neltuma denudans* and *Neltuma hassleri*, supporting the relation between phylogenetic distance and transference levels. EST-SSRs usually have higher transferability rates than G-SSRs, due to be obtained from transcribed, more conserved regions (Demdoum et al., 2012). This characteristic is also the cause of higher levels of monomorphism within transferred loci, as was observed in our results and those for *Neltuma juliflora* (Freitas et al., 2019).

A total of 30 allelic variants were identified through the seven polymorphic loci, with 2 to 8 alleles per EST-SSR (average = 4.286). The He and PIC values ranged from 0.162 to 0.794 and from 0.103 to 0.756 for loci S-P1EPIV2and I-P06286b, respectively. Four out of seven makers were highly polymorphic according to Botstein et al. (1980) criteria (Table 1). Our results can be explained by the conserved nature of the EST-SSRs, which limits their polymorphism (Manco et al., 2020). However, it is important to note that the results here obtained for EST-SSRs are comparable with those obtained by Pomponio et al. (2015) and Freitas et al. (2019), using the same set of markers.

Null alleles were found in all seven EST-SSRs loci, but only reached frequencies greater than 0.05 for locus S-P1EPIV2. This is probably the cause of the extremely low Ho value observed for this marker (Table 1). However, EST-SSRs are expected to be less susceptible to null alleles, considering the lower mutation rates assumed in the coding portion of the genome (Kovach et al., 2010), as can be observed in our results. A positive and high Fis value was estimated also for marker S-P1EPIV2, likely because of the high frequency of null alleles showed by that locus (Peyran et al., 2020). Finally, no significant *LD* ($p > 0.05$) was observed.

Based on our results, a set of thirteen polymorphic and validated G-SSRs (GL08, GL12, GL15, GL16, GL18, GL21, GL23, GL24, Mo08, PRB01, PRB04, PRB05 and PRSC02) and a set of six polymorphic and validated EST-SSRs (I-P00930d, I-P03211, I-P03325a, I-P03408, I-P06286b and I-P10500) are proposed. The usefulness of the *Neltuma* microsatellites to assess genetic diversity and structure in several *Neltuma* and *Prosopis* species has been widely documented (Bessega et al, 2021). Therefore, markers here proposed are valuable tools for several genetic analyses in *N. affinis* and could be implemented in studies of genetic diversity and structure, genetic relationships and functional genomics. Both sets of microsatellites will help to better understand genetic erosion processes by overexploitation and/or habitat anthropization and to guide appropriate management and conservation plans for *N. affinis*.

**Supplementary material** (Tables S1, S2 and Annex) accompanies the paper on *Forest System´*s website

**Data availability:** Not applicable

**Competing interests:** The authors have declared that no competing interests exist.

**Table 1.** Microsatellite characterization in *Neltuma affinis (= Prosopis affinis)*

| | Na | Ne | Ho | He | PIC | NA | DL | Fis |
|---|---|---|---|---|---|---|---|---|
| **G-SSRs** | | | | | | | | |
| GL08 | 13 | 6.070 | 0.902 | 0.835 | 0.877 | 0.001 | 0.353 | -0.044 |
| GL12 | 6 | 3.634 | 0.483 | 0.721 | 0.817 | 0.050 | 0.362 | 0.036 |
| GL15 | 10 | 2.304 | 0.652 | 0.565 | 0.682 | 0.024 | 0.316 | -0.012 |
| GL16 | 10 | 5.863 | 0.321 | 0.829 | 0.909 | 0.030 | 0.101 | 0.063 |
| GL18 | 6 | 1.272 | 0.232 | 0.214 | 0.523 | 0.001 | 0.216 | -0.052 |
| GL21 | 10 | 6.184 | 0.726 | 0.836 | 0.891 | 0.041 | 0.149 | 0.017 |
| GL23 | 7 | 4.091 | 0.551 | 0.755 | 0.881 | 0.033 | 0.221 | 0.032 |
| GL24 | 9 | 5.294 | 0.695 | 0.808 | 0.882 | 0.046 | 0.215 | 0.018 |
| Mo05 | 7 | 3.131 | 0.067 | 0.671 | 0.842 | **0.409** | 0.077 | **0.907** |
| Mo07 | 9 | 3.791 | 0.358 | 0.736 | 0.851 | **0.401** | 0.060 | **0.541** |
| Mo08 | 4 | 1.382 | 0.310 | 0.276 | 0.565 | 0.001 | 0.292 | -0.088 |
| Mo13 | 5 | 3.349 | 0.271 | 0.696 | 0.831 | **0.275** | 0.051 | **0.610** |
| PRB01 | 10 | 4.005 | 0.625 | 0.748 | 0.849 | 0.047 | 0.199 | 0.018 |
| PRB04 | 18 | 10.669 | 0.964 | 0.906 | 0.938 | 0.000 | 0.241 | -0.032 |
| PRB05 | 9 | 3.807 | 0.528 | 0.733 | 0.798 | 0.042 | 0.730 | 0.034 |
| PRB08 | 8 | 3.703 | 0.802 | 0.729 | 0.841 | **0.437** | 0.793 | -0.038 |
| PRSC02 | 8 | 3.782 | 0.790 | 0.734 | 0.802 | 0.001 | 0.674 | -0.054 |
| Average | 8.765 | 4.255 | 0.546 | 0.694 | 0.811 | 0.108 | 0.297 | 0.115 |
| sd | 3.251 | 2.183 | 0.256 | 0.186 | 0.115 | 0.160 | 0.229 | 0.284 |
| **EST-SSRs** | | | | | | | | |
| I-P00930d | 3 | 1.460 | 0.233 | 0.320 | 0.291 | 0.016 | 0.284 | 0.034 |
| I-P03211 | 2 | 1.578 | 0.207 | 0.373 | 0.299 | 0.049 | 0.565 | 0.027 |
| I-P03325a | 4 | 2.754 | 0.923 | 0.649 | 0.582 | 0.001 | 0.225 | 0.002 |
| I-P03408 | 5 | 2.675 | 0.200 | 0.637 | 0.561 | 0.028 | 0.372 | 0.000 |
| I-P06286b | 8 | 4.532 | 0.786 | 0.794 | 0.756 | 0.049 | 0.375 | 0.001 |
| I-P10500 | 4 | 2.203 | 0.433 | 0.555 | 0.501 | 0.016 | 0.433 | 0.000 |
| S-P1EPIV2 | 4 | 2.859 | 0.001 | 0.162 | 0.103 | **0.521** | 0.304 | **0.204** |
| Average | 4.286 | 2.580 | 0.398 | 0.499 | 0.442 | 0.097 | 0.365 | 0.038 |
| sd | 1.890 | 1.027 | 0.339 | 0.221 | 0.221 | 0.188 | 0.112 | 0.074 |

Na: number of alleles; Ne: number of effective alleles; Ho: observed heterozygosity; He; expected heterozygosity; PIC: polymorphic information content; NA: frequency of null alleles; DL: linkage disequilibrium; Fis: Inbreeding coefficient.

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