Short Note

Development and characterization of twelve new polymorphic microsatellite loci in the Iberian Ribbed newt, *Pleurodeles waltl* (Caudata: Salamandridae), with data on cross-amplification in *P. nebulosus*

JORGE GUTIÉRREZ-RODRÍGUEZ¹, ELENA G. GONZALEZ¹, ÍÑIGO

MARTÍNEZ-SOLANO^{2,3,*}

¹ Museo Nacional de Ciencias Naturales, CSIC, c/ José Gutiérrez Abascal, 2, 28006

Madrid, Spain; ² Instituto de Investigación en Recursos Cinegéticos, CSIC-UCLM-

JCCM, Ronda de Toledo, s/n, 13005 Ciudad Real, Spain; ³ (present address: Centro de

Investigação em Biodiversidade e Recursos Genéticos (CIBIO), Rua Padre Armando

Quintas, s/n, 4485-661 Vairão, Portugal

Number of words: 3017; abstract: 138

(*) Corresponding author:

I. Martínez-Solano

Instituto de Investigación en Recursos Cinegéticos (CSIC-UCLM-JCCM)

Ronda de Toledo, s/n

13005 Ciudad Real, Spain

Phone: +34 926 295 450 ext. 6255

Fax: +34 926 295 451

Email: inigomsolano@gmail.com

Running title: Microsatellite loci in *Pleurodeles waltl*

Abstract

Twelve novel polymorphic microsatellite loci were isolated and characterized for the Iberian Ribbed Newt, *Pleurodeles waltl* (Caudata, Salamandridae). The distribution of this newt ranges from central and southern Iberia to northwestern Morocco. Polymorphism of these novel loci was tested in 40 individuals from two Iberian populations and compared with previously published markers. The number of alleles per locus ranged from two to eight. Observed and expected heterozygosity ranged from 0.13 to 0.57 and from 0.21 to 0.64, respectively. Cross-species amplification was tested in *Pleurodeles nebulosus*, which is listed as Vulnerable by the IUCN. Eight new and seven previously published loci amplified successfully in that species and thus represent a valuable conservation tool. The novel microsatellites will be useful for a better understanding of the population dynamics, demography, genetic structure, and evolutionary history of *Pleurodeles waltl* and *P. nebulosus*.

Keywords: microsatellites, polymorphism, amphibians, *Pleurodeles waltl*, *Pleurodeles nebulosus*, Iberian Peninsula, North Africa

The Iberian Ribbed Newt, *Pleurodeles waltl* Michahelles, 1830, is a large (up to 30 cm) salamandrid that is widely distributed throughout most of the Iberian Peninsula and northwestern Morocco (Beukema et al., 2013). Currently, it is listed as Near Threatened by the UICN, given that populations probably are in significant decline (Beja et al., 2009). The main threats are aquatic habitat loss, road mortality and the introduction of invasive species like the crayfish *Procambarus clarkii* (Montori et al., 2002). However, habitat fragmentation may also play a negative role in the Ribbed Newt's regional dynamics, especially at the southern end of the range. Potentially available conservation measures such as identification and protection of corridors connecting populations are limited by the lack of data on their biology and demography (Salvador, 2002).

Previous phylogeographic studies have identified two distinct mitochondrial lineages (Carranza and Arnold, 2004; Veith et al., 2004). A western lineage is distributed across the west and center of the Iberian Peninsula, while an eastern lineage spreads across eastern Iberia and northern Morocco. According to Carranza and Arnold (2004), the process of vicariance between the two lineages would have occurred between 3.2 and 2 million years ago, in the Late Pliocene or Early Pleistocene. Populations of Morocco and the Iberian Peninsula are genetically very similar and even share mtDNA haplotypes (Veith et al., 2004), which has led some authors to propose alternative hypotheses to explain the low genetic differentiation between populations on both sides of the Gibraltar Strait. The first hypothesis is a recent anthropogenic introduction in northern Morocco, and the second suggests the origin of Moroccan populations through passive transport on vegetation rafts carried by ocean currents (Busack, 1986; Veith et al., 2004). These hypotheses have not been tested with fast-evolving molecular markers, like microsatellites, which are well suited for the study of

such recent events. Microsatellites are ubiquitous, codominant, non-coding genetic markers consisting of repeats of one to six base pairs with high levels of length polymorphism due to higher rates of mutation than other genomic regions. Because of their high variability and ease of genotyping they are widely used to address questions in Evolutionary and Conservation Biology (Ellegren, 2004).

In this study, we report the development of twelve new polymorphic microsatellite loci in *P. waltl*. Cross-species amplification was also tested in samples of the closely related, north African endemic species *P. nebulosus*, which is listed as Vulnerable by the IUCN. We compare levels of polymorphism in the newly developed loci with those previously published (Vliet et al., 2009) and discuss potential applications.

An enriched partial genomic library was generated from DNA of a single individual of *Pleurodeles waltl* collected from Valdemanco, Madrid, Spain. The library was prepared at the Sequencing Genotyping Facility, Cornell Life Sciences Core Laboratory Center (CLC). Genomic DNA was extracted with Qiagen DNeasy Blood and Tissue Kits, digested with the restriction enzymes BsaA I and Hinc II, and ligated to SNX linkers (Hamilton et al., 1999). The cut DNA was enriched by hybridization with a mix of biotin-labelled oligonucleotide probes (motifs: GT₈, TC_{9.5}, TA₁₅, TTA₁₁, GTT_{6.33}, TTC₇, GCT_{4.33}, GAT₇, GTA_{8.33}, GTG_{8.33}, GTG_{4.67}, GTC_{4.67}, TCC₅, TTTA_{8.5}, TTTG_{5.25}, TTTC₆, GATA₇, GTAT_{6.25}, GAAT_{5.5}, GATT_{5.5}, GTTA_{6.25}, TTAC_{6.75}, GATG_{4.25}, GGTT₄, GCTT_{3.75}, GTAG_{4.25}, GTCA_{4.25}, GTTC₄, TCAC_{4.25}, TTCC_{4.25}) and incubated at 56 °C for 20 minutes. The enriched product was carefully isolated from the rest of the genomic DNA fragments using streptavidin-coated magnetic beads (NEB) and amplified by PCR using the SNX linkers. PCR products were purified and ligated with vector pUC19 (NEB) and transformed into *Escherichia coli* electrocompetent DH5α-E cells (Invitrogen). The recombinant clones obtained were directly transferred onto nylon filter membranes (Osmonics), and once fixed, they were hybridized at 50 °C overnight with ³³P-labelled probes taken from the same motifs used previously. The nylon membranes were transferred to film plastic wrap and exposed to film for chemiluminiscent signal

detection. Positive colonies containing microsatellites were cultivated, amplified with M13 forward or reverse primers and sequenced using an ABI PRISM 3100 automated sequencer (Applied Biosystems).

A total of 34 clones containing microsatellite motifs were selected for further screening. Primer pairs were designed with the program Primer3plus (Untergasser et al., 2007). Genomic DNA was extracted from tail tips of larvae with NucleoSpin Tissue-Kits (Macherey-Nagel). PCR reactions were performed in a total volume of 15 µl, including 25 ng of template DNA, 5x GoTaq Flexi buffer (PROMEGA), 1.5 mM MgCl₂, 0.1 mM dNTP, 0.1 µM fluorescent primer, 0.1 µM primer and 0.5 U GoTaq Flexi DNA polymerase (PROMEGA). The PCR cycling profile consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing between 57- 62°C for 45 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. PCR products were visualized on 2.5% agarose gels. PCR primer fail rate (65%) was similar to that reported by Vliet et al. (2009) (60%).

Of the 34 initially selected loci, 26 showed unambiguous bands and were chosen to score genetic variation in samples from two populations of *P. waltl* (Valdemanco: Madrid, n: 20; and Chiclana: Cádiz, n: 20) and one of *P. nebulosus* (Jendouba, Tunisia, n: 8). The two populations of *P. waltl* represent the two major mtDNA lineages previously described (eastern: Chiclana; western: Valdemanco), although in Valdemanco there are eastern mtDNA haplotypes in low frequencies, too (around 10%, unpublished data). Fluorescent dyes 6-FAM, PET, NED, and VIC were used to label forward primers for use in multiplex reactions, which were designed with MULTIPLEX MANAGER v1.2 (Holleley and Geerts, 2009). PCR reactions were performed using Type-it Microsatellite PCR kits (Qiagen). All reactions were performed in a total volume of 15 µl, containing 7.5 µl of Master Mix, 1.2 µl of primer mix (0.2 µM of each primer) and 25 ng of template DNA. The PCR cycling profile consisted of an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing between 57- 62°C for 90 seconds, and extension at 72°C for 30 seconds, with a final extension at 60°C for 10 minutes. PCR products were genotyped on an ABI PRISM 3730 sequencer with the GeneScan 500 LIZ size standard (Applied Biosystems), and fragments were scored and binned using GENEMAPPER v4.0 (Applied Biosystems). In order to compare levels of polymorphism, we genotyped the same samples using eleven previously published microsatellites (Vliet et al., 2009). For this purpose, three additional

multiplex reactions were designed: multiplex 1 (loci Ppl2, Ppl3, Ppl5), multiplex 2 (Ppl1, Ppl12, Ppl13, Ppl14) and multiplex 3 (Ppl4, Ppl6, Ppl7, Ppl10). The markers Ppl8, Ppl9 and Ppl11 were also tested, however they presented some problems, such as dropout, non-specific amplification, and no amplification, respectively. PCR reactions were performed using the same conditions described above. Differences in PCR protocols across studies may account for the lower amplification success, since Vliet et al., (2009) used touch-down profiles for all but one loci.

MICROCHECKER v2.2.3 (Van Oosterhout et al., 2004) was used to test for evidence of stuttering, large allele dropout and presence of null alleles in each population, using a 99% confidence interval and 1,000 randomizations. We calculated the number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosity for each locus and population with GENALEX v6.5b5 (Peakall and Smouse, 2006). Deviations from Hardy-Weinberg equilibrium (HWE) and evidence of linkage disequilibrium (LD) were tested with the Markov chain method (Guo and Thompson, 1992) implemented in GENEPOP v4.2 (Rousset, 2008). The Markov chain was run with 10,000 dememorization steps, 1,000 batches and 10,000 iterations per batch. A sequential Bonferroni correction (Rice, 1989) was applied to adjust for multiple comparisons.

Among the 26 new loci tested, only 12 were polymorphic in the samples of *P. waltl*. Therefore, multiplex reactions were redesigned, and reduced to four reactions (Table 1). Potential null alleles were detected at locus Pleu2.21 (estimated frequency: 33.7%) in the population of Chiclana, which was the only instance of significant deviation from HWE. There was no evidence of significant LD in any population. This is in contrast to Vliet et al. (2009), who found significant LD between Ppl12/Ppl13, and significant deviation from HW in Ppl10. The number of alleles per locus ranged from two (Pleu2.31, Pleu2.34 and Pleu4.1) to eight (Pleu3.2). The average observed heterozygosity was 0.31 (range: 0.13–0.57) and the average expected heterozygosity was 0.35 (range: 0.21–0.64). The average number of alleles per locus and population ranged from two (Valdemanco) to 3.5 (Chiclana). Observed and expected

heterozygosities were 0.31/0.33 and 0.36/0.42 in the populations Valdemanco and Chiclana, respectively. In general, previously published markers were more polymorphic (number of alleles/observed/expected heterozygosities = 6.0/0.73/0.68 and 13.6/0.92/0.88 in Valdemanco and Chiclana, respectively). Reasons for this are unclear, although the use of different motifs in library construction may explain at least part of the differences.

Cross-species amplification with eight samples of *Pleurodeles nebulosus* was successful for eight of the thirteen new loci, although only six were polymorphic (number of alleles ranging from two to six, Table 2). With respect to previously published markers, seven out of the eleven loci tested also amplified in *P. nebulosus* samples (number of alleles: three to eleven) and showed higher levels of polymorphism (average number of alleles = 3.3 vs. 7.6, observed/expected heterozygosities: 0.42/0.47 vs. 0.71/0.76, in new vs. published loci, respectively). The overall similar values of polymorphism of the markers across species and the high cross-amplification success in *P. nebulosus* (despite at least 5.3 million years of independent evolution, see Carranza and Arnold, 2004; Veith et al., 2004) are in contrast to general expectations, given the overall low transferability of microsatellites in amphibians (Nair et al., 2012). The reasons for this are unclear, although biogeographic factors (for instance, lower diversity is expected in populations in recently-expanding parts of the range) are unlikely, since historical refugia for the species seem to have been located in the south (unpublished data), where one of the study populations (Chiclana) was sampled.

These new markers complement those previously published and will help to address a variety of questions on the population dynamics and demography (mating system, low-scale dispersal, effective population size), genetic structure (identification

of potential barriers to gene flow), and evolutionary history (delineation of lineages and their contact zones) of *P. waltil*. In addition, the success of cross-amplification tests in the closely related species *P. nebulosus* is important from a conservation perspective. This species is listed as Vulnerable by the IUCN, because its distribution area in northern Algeria and Tunisia is less than 2000 km² and is very fragmented (Donaire-Barroso et al., 2013) and thus these markers will provide valuable information on the spatial genetic structure of its populations. Further tests should be conducted on the third extant species in the genus, *P. poireti*, which has the smallest range (less than 500 km², restricted to the Edough peninsula in northern Algeria) and is listed as Endangered by the IUCN (Geniez and Mateo, 2006).

Acknowledgements

We thank D. Buckley, E. Recuero, S. Nouira and J. Ben Hassine for help collecting samples and S. Bogdanowicz at Cornell University for help with the microsatellite library. All samples were collected with permits issued by legal authorities in Spain and Tunisia. The manuscript was improved with the comments of two anonymous reviewers and the Associate Editor. This research was funded by grants CGL2008-04271-C02-01/BOS and CGL2011-28300 (Ministerio de Ciencia e Innovación -MICINN-), Ministerio de Economía y Competitividad -MEC-, Spain, and FEDER) and PPII10-0097- 4200 (Junta de Comunidades de Castilla la Mancha) to IMS. JGR is supported by the Consejo Superior de Investigaciones Científicas of Spain (CSIC) and the European Social Fund (ESF) (JAE-pre PhD fellowship), EGG is a “Juan de la Cierva” postdoctoral fellow supported by the MEC, and IMS was supported by a ‘Ramón y Cajal’ postdoctoral fellowship (MICINN and Universidad de Castilla la Mancha).

References

195 Beja, P., Bosch, J., Tejedo, M., Edgar, P., Donaire-Barroso, D., Lizana, M., Martínez-
 196 Solano, I., Salvador, A., García-París, M., Recuero Gil, E., Slimani, T., El
 197 Mouden, E.H., Geniez, P. (2009): *Pleurodeles waltl*. In: IUCN 2012. IUCN Red
 198 List of Threatened Species. Version 2012.2. <http://www.iucnredlist.org>.
 199 Accessed 3 June 2013.

200 Beukema, W., De Pous, P., Donaire-Barroso, D., Bogaerts, S., García-Porta, J.,
 201 Escoriza, D., Arribas, O.J., El Mouden, E.H., Carranza, S. (2013): Review of the
 202 systematics, distribution, biogeography and natural history of Moroccan
 203 amphibians. *Zootaxa* **3661**: 1-60.

204 Busack, S.D. (1986): Biogeographic analysis of the herpetofauna separated by the
 205 formation of the Strait of Gibraltar. *National Geographic Research* **2**: 17-36.

206 Carranza, S., Arnold, E.N. (2004): History of West Mediterranean newts, *Pleurodeles*
 207 (Amphibia: Salamandridae), inferred from old and recent DNA sequences.
 208 *Systematics and Biodiversity* **1**: 327-337.

209 Donaire-Barroso, D., Salvador, A., Slimani, T., El Mouden, E.H., Geniez, P., Mateo,
 210 J.A. (2006): *Pleurodeles nebulosus*. In: IUCN 2012. IUCN Red List of
 211 Threatened Species. Version 2012.2. <http://www.iucnredlist.org>. Accessed 19
 212 June 2013.

213 Ellegren, H. (2004): Microsatellites: simple sequences with complex evolution. *Nat.*
 214 *Rev. Genet.* **5**: 435-445.

215 Geniez, P., Mateo, J.A. (2006): *Pleurodeles poireti*. In: IUCN 2012. IUCN Red List of
 216 Threatened Species. Version 2012.2. <http://www.iucnredlist.org>. Accessed 19
 217 June 2013.

218 Guo, S.W., Thompson, E.A. (1992): A Monte Carlo method for combined segregation
219 and linkage analysis. *Am. J. Hum. Genet.* **51**: 1111-1126.

220 Hamilton, M.B., Pincus, E.L., Di Fiore, A., Fleischer, R.C. (1999): Universal linker and
221 ligation procedures for construction of genomic DNA libraries enriched for
222 microsatellites. *BioTechniques* **27**: 500-507.

223 Holleley, C.E., Geerts, P.G. (2009): Multiplex Manager 1.0: a cross-platform computer
224 program that plans and optimizes multiplex PCR. *BioTechniques* **46**: 511-517.

225 Montori, A., Llorente, G.A., Santos, X., Carretero, M.A. (2002): *Pleurodeles waltl*
226 Michahelles, 1830. Gallipato. In: Atlas y Libro Rojo de los Anfibios y Reptiles
227 de España, pp. 51-53. Pleguezuelos, J.M., Márquez, R., Lizana, M., Eds.
228 Dirección General de Conservación de la Naturaleza-Asociación Herpetológica
229 Española, Madrid, Spain.

230 Peakall, R., Smouse, P.E. (2006): Genalex 6: genetic analysis in Excel. Population
231 genetic software for teaching and research. *Mol. Ecol. Notes* **6**: 288-295.

232 Nair, A., Gopalan, S.V., George, S., Kumar, K.S., & Merilä, J. (2012): Cross-species
233 testing and utility of microsatellite loci in Indirana frogs. *BMC Research Notes*
234 **5**: 389.

235 Rice, W.R. (1989): Analyzing Tables of Statistical Tests. *Evolution* **43**: 223-225.

236 Rousset, F. (2008): Genepop'007: a complete re-implementation of the genepop
237 software for Windows and Linux. *Mol. Ecol. Resources* **8**: 103-106.

238 Salvador, A. (2002): Gallipato - *Pleurodeles waltl*. In: Enciclopedia Virtual de los
239 Vertebrados Españoles. Carrascal, L.M., Salvador, A., Eds. Museo Nacional de
240 Ciencias Naturales, Madrid. <http://www.vertebradosibericos.org>. Accessed June
241 19 2013.

242 Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A.M.
 243 (2007): Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids*
 244 *Research* **35**: 71-74.

245 Van Oosterhout, C., Hutchinson, W.F., Wills, D.P.M., Shipley, P. (2004): Micro-
 246 checker: software for identifying and correcting genotyping errors in
 247 microsatellite data. *Mol. Ecol. Notes* **4**: 535-538.

248 Veith, M., Mayer, C., Samraoui, B., Barroso, D.D., Bogaerts, S. (2004): From Europe to
 249 Africa and vice versa: evidence for multiple intercontinental dispersal in ribbed
 250 salamanders (Genus *Pleurodeles*). *J. Biogeogr.* **31**: 159-171.

251 Vliet, M.S.V.d., Diekmann, O.E., Serrão, E.A., Beja, P. (2009): Isolation of highly
 252 polymorphic microsatellite loci for a species with a large genome size: sharp-
 253 ribbed salamander (*Pleurodeles waltl*). *Mol Ecol. Resources* **9**: 425-428.
 254
 255

Table 1. Description of 12 new polymorphic and one monomorphic microsatellite loci isolated for the Iberian Ribbed newt (*Pleurodeles waltl*). Locus name, primer sequences, fluorescent dye, repeat motif, multiplex reaction, annealing temperature, size of amplified product (bp), and GenBank accession numbers are listed for each locus. Also indicated are the number of individuals successfully genotyped (n), number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities for each locus and population (Valdemanco/Chiclana), as well as cross-species amplification of each locus in *P. nebulosus* (+: positive, -: negative).

Locus	Primer sequence	Labelling dye	Repeated motif	Multiplex reaction	T _a	Size ranges (bp)	n	N _A	H _O	H _E	<i>P. nebulosus</i>	GenBank accession no.
Pleu2.3	5' TTGGAACCACTGATGAA 3' 5' ATTTCTCTCTCCACACCA 3'	NED	(TC) ₁₀	Multiplex 1	60	226 - 240	20/20	1/4	0/0.5	0/0.42	+	KF647652
Pleu2.19	5' ATGAACCCCTGGAGACTG 3' 5' ACGCTCAGTCCGTTTCATTTT 3'	VIC	(TG) ₁₄	Multiplex 1	60	228 - 236	20/20	4/3	0.6/0.45	0.49/0.53	-	KF647653
Pleu2.34	5' GAGACATGACTTGCCCAACA 3' 5' CTTTGCCCCGGTAATACTCC 3'	6-FAM	(CA) ₁₂	Multiplex 1	60	138 - 148	20/16	2/1	0.35/0	0.47/0	-	KF647658
Pleu2.16	5' CTATTGTGGTGACCCGCAIT 3' 5' GCCTTATGAGGGTTTCATGC 3'	PET	(TG) ₁₆ (AG) ₁₉	Multiplex 2	57	219 - 251	20/20	2/7	0.5/0.6	0.46/0.68	+	KF647655
Pleu2.31	5' AGCCTCTTGGTTGGTCTCAG 3' 5' CATGTCCCGTTCTTTGTCCT 3'	VIC	(GA) ₁₀	Multiplex 2	57	210 - 216	20/20	2/1	0.4/0	0.5/0	-	KF647657
Pleu3.2	5' GACCGCTGCTGCAAGTATCT 3' 5' AGAGCGCTTTACACCAGAGC 3'	6-FAM	(TTC) ₂₁	Multiplex 2	57	184 - 223	20/20	2/7	0.4/0.75	0.5/0.78	+	KF647661
Pleu3.5	5' CAAGCAGGGTTTGCCTTTA 3' 5' CCTGCTTGCAACTCTCTCC 3'	6-FAM	(TGA) ₃ GGAAGCTGTGGGGC(TGA) ₉	Multiplex 3	60	178 - 199	20/20	1/4	0/0.55	0/0.58	+	KF647662
Pleu4.1	5' CTAGTGAGCAGCAGTCCAC 3' 5' ATGAAAACCTCCGTTGAAGG 3'	VIC	(ATTG) ₂ ACTG(ATTG) ₅	Multiplex 3	60	194 - 198	20/20	2/2	0.2/0.15	0.48/0.14	+	KF647660
Pleu2.35	5' GACCTATCCTAGTTTCTCATCCTCAC 3' 5' TTGTTATGCAAAATTCAGGAGA 3'	6-FAM	(TC) ₁₄	Multiplex 4	62	139 - 151	20/19	1/4	0/0.26	0/0.42	+	KF647659

Pleu3.14	5' CAGCTGGATAAACATGGCAAC 3' 5' TTTCGAATGCAAAATCAAAGC 3'	6-FAM	(ATT) ₅ (GTT) ₉	Multiplex 4	62	257 - 266	20/19	2/4	0.05/0.53	0.05/0.59	-	KF647664
Pleu3.11	5' GGGAAATCTCAGTCCGAACA 3' 5' ACCTCTTTCCCAGCTCCACT 3'	VIC	(CTT) ₉	Multiplex 4	62	225 - 237	20/20	2/3	0.55/0.4	0.49/0.48	+	KF647663
Pleu2.21	5' TTGGAATGCAGCTATGTTGG 3' 5' TTACCATCTTGAAAAGGCTCT 3'	NED	(TG) ₁₃	Multiplex 4	62	195 - 223	20/19	3/2	0.7/0.11	0.55/0.5	-	KF647654
Pleu2.29	5' GGACACCTGCTTGACCCTTA 3' 5' CTTCTGCGACCTTGGTAAA 3'	NED	(TC) ₁₀ TGTCTG(TC) ₄	-	57	165	20/20	1	-	-	+	KF647656

Table 2. Polymorphism in loci isolated from *P. waltl* in *P. nebulosus* (n: 8): locus name, number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities. * = loci from Vliet et al. (2009).

Locus	N_A	Size range (bp)	H_E	H_O
Pleu2.3	2	220 - 226	0.125	0.305
Pleu2.16	6	210 - 242	0.875	0.672
Pleu2.29	3	165 - 169	0.375	0.461
Pleu3.2	2	157 - 172	0.250	0.219
Pleu3.5	1	131	-	-
Pleu4.1	3	190 - 198	0.375	0.570
Pleu2.35	4	118 - 140	0.500	0.609
Pleu3.11	1	231	-	-
Ppl3*	10	139 - 185	1.000	0.883
Ppl5*	7	146 - 184	0.750	0.844
Ppl7*	9	167 - 195	0.875	0.789
Ppl10*	3	138 - 142	0.125	0.320
Ppl12*	7	110 - 168	0.625	0.813
Ppl13*	11	184 - 278	1.000	0.875
Ppl14*	5	306 - 322	0.625	0.773