Uncovering hidden specific diversity of Andean glassfrogs of the *Centrolene buckleyi* species complex (Anura: Centrolenidae)

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The glassfrog *Centrolene buckleyi* has been recognized as a species complex. Herein, using coalescence-based species delimitation methods, we evaluate the specific diversity within this taxon. Four coalescence approaches (GMYCs, bGMYC, PTP, and bPTP) were consistent with the delimitation results, identifying four lineages within what is currently recognized as *C. buckleyi*. We propose three new candidate species that should be tested with nuclear markers, morphological and behavioural data. In the meantime, for conservation purposes, candidate species should be considered evolutionary significant units, in light of observed population crashes in the *C. buckleyi* species complex. Finally, our results support the validity of *C. venezuelense*, formerly considered as a subspecies of *C. buckleyi*.

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2 **species complex (Anura: Centrolenidae)**

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17 Short title: Species delimitation of Andean glassfrogs

Abstract

The glassfrog *Centrolene buckleyi* has been recognized as a species complex. Herein, using

coalescence-based species delimitation methods, we evaluate the specific diversity within this

taxon. Four coalescence approaches (GMYCs, bGMYC, PTP, and bPTP) were consistent with

the delimitation results, identifying four lineages within what is currently recognized as *C.*

buckleyi. We propose three new candidate species that should be tested with nuclear markers,

morphological and behavioural data. In the meantime, for conservation purposes, candidate

species should be considered evolutionary significant units, in light of observed population

crashes in the *C. buckleyi* species complex. Finally, our results support the validity of *C.*

venezuelense, formerly considered as a subspecies of *C. buckleyi*.

Introduction

Species delimitation —the process by which species boundaries are determined— is important

and a challenge for characterizing the biota of biodiversity hotspots (Myers et al., 2000).

Achieving a taxonomic scheme that reflects the evolutionary history of organisms is critical for

both theoretical (characterizing biodiversity) and practical (designing conservation strategies)

reasons (e.g. Esselstyn et al., 2012). Although species delimitation ideally uses multiple lines of

evidence (Padial et al., 2010), DNA sequences play an important role in species-level lineage

identification (Chambers & Hebert, 2016; Pentinsaari, Vos & Mutanen, 2017). Sequences have

been recently, and more frequently, analyzed under coalescent-based methods (e.g. Pons et al.,

2006; Yang & Rannala, 2010; Camargo et al., 2012; Reid & Carstens, 2012; Fujisawa &

Barraclough, 2013; Zhang et al., 2013). The main goal of coalescent-based species delimitation

is to identify evolutionarily independent lineages, where each lineage represents a single species

40   (Fujita et al., 2012; for the conceptualization of the species category, in the so called Generalized

41   Lineage Concept, see de Queiroz, 1998, 1999, 2007). Coalescent-based methods, which allow

42   testing alternative hypotheses on the divergence of a lineage, are expected to reduce the

43   subjective bias introduced by researchers, avoiding using *ad hoc* thresholds (i.e., degree of

44   morphological, ecological, and/or percentage of sequence divergence) as criteria to establish

45   species limits. As such, these methods have become a common tool for delimiting species, both

46   to propose candidate species as well as to describe new species (e.g., Leaché & Fujita, 2010;

47   Páez-Moscoso & Guayasamin, 2012; Crivellaro et al., 2018). However, these sequences-based

48   methods have several assumptions that must be met (see Carstens et al., 2013; Talavera, Dincă &

49   Vila, 2013; White et al., 2014), as well as limitations and drawbacks according to the

50   characteristics of the analyzed data, including erroneous results with species of recent

51   diversification (Wei et al., 2016; Jacobs et al., 2018), and species diversity overestimation

52   (Sukumaran & Knowles, 2017). Therefore, conclusive species delimitation studies must have an

53   integrative approach (Dayrat, 2005; Padial et al., 2010).

54   Proposing candidate species, which currently is mostly done on the basis of molecular evidence

55   (e.g., Correa et al., 2017; Hurtado & D'Elía, 2018; Lin, Stur & Ekrem, 2018) can guide future

56   taxonomic research, allowing one to direct the costlier efforts (e.g., field collections,

57   morphological assessment of large specimen series) to specific taxonomic and geographic areas

58   of interest. In turn, these efforts result in an acceleration of the discovery and validation of new

59   species (Dellicour & Flot, 2015; Vitecek et al., 2017), which is relevant in the current era of

60   biodiversity crisis. In addition, candidate species can be considered evolutionary significant

61   units, which in turn can be subject to conservation actions (Moritz, 1994).

62   The taxon *Centrolene buckleyi* (Boulenger, 1882) has a large distribution inhabiting montane

63   primary and secondary forests in high tropical Andean zones (1,900–3,300 msnm), as well as

64   inter-Andean scrubland and Páramo environments of Colombia, Ecuador, and northern Peru

65   (Duellman & Wild, 1993; Guayasamin et al., 2006; Rada & Guayasamin, 2008; Guayasamin &

66   Funk, 2009) (Fig. 1). The glassfrog *C. buckleyi* has a relatively simple taxonomic history; only

67   two taxonomic forms, *venezuelense* (Rivero, 1968) and *johnelsi* (Cochran & Goin, 1970), are

68   associated to it. The later was synonymized under *C. buckleyi* by Ruiz-Carranza & Lynch

69   (1991). Meanwhile, the form *venezuelense* was considered as a subspecies of *C. buckleyi* until it

70   was elevated to species level as *C. venezuelense* by Myers & Donnelly (1997), based on the

71   argument that it is highly unlikely that the distribution of *C. buckleyi*, with its type locality in

72   Ecuador, reaches Venezuela. Señaris & Ayarzaguena (2005) agree in considering *venezuelense*

73   at the species level, given that it presents morphological and acoustic differences with respect to

74   typical *buckleyi*. Cisneros-Heredia & McDiarmid (2007), based on morphological characters,

75   stated that further research is needed to evaluate the distinction of *venezuelense* from *buckleyi*.

76   Even after the removal of *venezuelense*, distinct lines of evidence suggest that as currently

77   understood, *C. buckleyi* may encompass more than one species. For instance, Colombian

78   populations are regarded as *C.* aff. *buckleyi* as a way to denote uncertainties in their identity

79   (Guayasamin et al., 2008). Moreover, Guayasamin et al. (2006) identified significant call

80   variation among populations of *C. buckleyi*; similarly, several studies have shown the lack of

81   monophyly for this taxon (Guayasamin et al., 2006, 2008; Guayasamin & Funk, 2009; Pyron &

82   Wiens, 2011; Castroviejo-Fisher et al., 2014; Twomey, Delia & Castroviejo-Fisher, 2014). As

83   such, in current catalogues (e.g., Guayasamin et al., 2017) *C. buckleyi* is regarded as a species

84   complex.

85 Given these antecedents, herein, using an expanded molecular dataset, and phylogenetic and

86 coalescent-based species delimitation analyses, we evaluate the species limits of populations

87 currently allocated to the taxon *C. buckleyi*. Our study identifies four distinctive lineages, three

88 of which should be considered as candidate species.

89 **Materials & Methods**

90 A total of 34 sequences of the 12S and 16S mitochondrial genes of the genus *Centrolene* were

91 analysed; of these, ten belong to the *Centrolene buckleyi* species complex. The outgroup was

92 formed with sequences of *Nymphargus*, a genus closely related to *Centrolene* (Guayasamin et al.,

93 2008; Castroviejo-Fisher et al., 2014; Twomey, Delia & Castroviejo-Fisher, 2014). As such, the

94 analysed matrix totaled 47 sequences (see Table S1). Sequences were downloaded from

95 Genbank or gathered by us (Tables 1, S1). The matrix includes sequences of the *C. buckleyi*

96 species complex from four localities throughout its distributions in the eastern Andean foothills

97 of Ecuador (Table 1; Fig. 1). Specimen collection was done under research permit (MAE-DNB-

98 CM-2015-0017) issued by the Ministerio del Ambiente of Ecuador (MAE). Additionally,

99 morphological characters of all specimens were examined by one of the authors (JMG).

100 **DNA extraction and sequencing**

101 For newly generated sequences, genomic DNA was extracted from 96% ethanol-preserved

102 muscle tissue samples using a modified salt precipitation method based on the Puregene DNA

103 purification kit (Gentra Systems). We amplified two mitochondrial genes 12S and 16S using the

104 primers t-Phe-frog and t-Val-frog developed by Wiens et al. (2005). PCR reactions follow the

105 protocol described by Guayasamin et al. (2008). Cycle sequencing reactions were performed by

106   Macrogen Labs (Macrogen Inc., Korea). All fragments were sequenced in both forward and

107   reverse directions with amplification primers. Sequences were deposited in GenBank (Tables 1,

108   S1).

109   **Phylogenetic analyses**

110   Four matrices were created; a single matrix with 12S, a single matrix with 16S, concatenation of

111   both fragments (these three matrices included all sequences *Centrolene* + outgroup), and one

112   matrix formed only by sequences of *Centrolene* (34 terminals concatenated with 12S-16S

113   mtDNA genes). Sequences were aligned in MAFFT v7 under an automatic strategy (Katoh &

114   Standley, 2013). The aligned matrix was imported into Aliview (Larsson, 2014), where segments

115   that presented ambiguous alignments were excluded. All positions containing only gaps were

116   deleted. The best nucleotide substitution model was selected with ModelFinder

117   (Kalyaanamoorthy et al., 2017) using the Bayesian information criterion (BIC) and was the same

118   for the first three data sets with all sequences of *Centrolene* + *Nymphargus* (TIM2 + I + G). For

119   the matrix with only sequences belonging to *Centrolene* the selected best model was TIM2 + R3.

120   This last data set was used to infer the input genealogy in species delimitation analysis. The first

121   three were used to conduct phylogenetic analyses.

122   Phylogenetic trees were obtained using Maximum Likelihood (ML) and Bayesian inference (BI).

123   ML trees were inferred in IQ-TREE (Nguyen et al., 2015); nodal support was assessed with

124   1,000 ultrafast bootstrap replicates (Minh, Nguyen & Haeseler, 2013). Bayesian inference

125   analyses were conducted with MrBayes 3.2 (Ronquist et al., 2012) using two parallel runs of four

126   Markov chains that were allowed to run for ten million generations and that were sampled every

127   1,000 generations. The first 25% of the sampled trees were discarded as a burnin, prior to

128   constructing a consensus tree with the remaining sample. Phylogenetic trees were visualized

129   using FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Clades with ML values (BV)

130   equal or above 75% and posterior probabilities values (PP) equal or greater than 0.95 were

131   considered as strongly supported. MEGA 7.0 (Kumar, Stecher & Tamura, 2016) was used to

132   estimate genetic distances between sequences of the 16S gene with a bootstrap procedure of

133   1,000 replicates.

134   **Methods for delimiting species**

135   We used two coalescence based methods, and their Bayesian implementation, to delimit species

136   on the basis of variation of 12S and 16S sequences.

137   *Generalized Mixed Yule Coalescent (GMYC) and Bayesian General Mixed Yule-Coalescent*

138   *(bGMYC)*. -- The GMYC method uses maximum likelihood statistics and takes an estimated

139   ultrametric and bifurcating genealogy from a single-locus as input (Pons et al., 2006; Fujisawa &

140   Barraclough, 2013). The time calibrated-ultrametric tree was obtained with BEAST 2 (Bouckaert

141   et al., 2014) using the temporal calibration scheme outlined by Castroviejo-Fisher et al. (2014)

142   for the most recent common ancestor (MRCA) of *Centrolene* and *Nymphargus*. We conducted

143   two independent analyses to check for consistency in the results under a relaxed clock model and

144   a birth-death model of speciation. Each analysis was run for 20 million generations logging

145   every 1,000 generations. BEAST log files were checked for convergence and for ESS values

146   above 200 using Tracer v1.6 (Rambaut, Suchard & Drummond, 2014). Maximum clade

147   credibility tree was estimated with TreeAnnotator v2 (distributed as part of BEAST) with the

148   sampled trees after discarding the first 25% as burn-in. Outgroups were removed with

149   *drop.tip.simmap* function of R v. 3.3.2 (R Core Team, 2016) package phytools (Revell, 2012).

150   The GMYC method attempts to model the transition point between cladogenesis (Yule process)

151   and the population level process of allelic coalescence, using the assumption that cladogenesis

152   will occur at a much lower rate than coalescence (Carstens et al., 2013; Tang et al., 2014).

153   GMYC was fitted to the ultrametric gene tree to delimit the species boundaries using single

154   (GMYCs, Pons et al., 2006) and multiple threshold models (GMYCm, Monaghan et al., 2009).

155   We compared the likelihood of single and multiple transition model with likelihood of null

156   model via a likelihood ratio test. These analyses were performed with the package Splits (Ezard,

157   Fujisawa & Barraclough, 2009) in R, after removing zero-length branches and making the tree

158   fully dichotomous. We also performed a Bayesian General Mixed Yule-Coalescent (bGMYC)

159   analysis (Reid & Carstens, 2012), which takes into account the uncertainties in the estimation of

160   the genealogy. The analysis was done with the R package bGMYC (Reid & Carstens, 2012) in

161   R, which calculates the posterior marginal probabilities of species boundaries. This was

162   performed with a post-burn-in sample of 100 trees sampled from the posterior distribution of

163   trees. For the bGMYC analysis, the priors of parameters t1 and t2 were set at 4 and 100,

164   respectively. The bGMYC analysis was performed with 50,000 generations, with a burnin of

165   10%, and a thinning interval of 1,000 samples.

166   *Poisson Tree Processes (PTP) and Bayesian Poisson Tree Processes (bPTP) methods.* --This

167   method models speciation and coalescence events in terms of numbers of substitutions (Zhang et

168   al., 2013). PTP provides hypothesis of species delimitation based on a gene tree (not necessarily

169   ultrametric), using heuristic algorithms to identify the most likely classification of branches in

170   processes at the level of populations and species (Tang et al., 2014). We also used bPTP, which

171   is the Bayesian implementation and updated version of the PTP method. Moreover, the result of

172   the search for the maximum probability in PTP is part of the results of bPTP. This

173   implementation produces Bayesian posterior probability values (PPV) of delimited species using

174   as input the phylogenetic tree (the same as in PTP). A higher Bayesian value (PPV > 0.90) at one

175   node indicates that all descendants of that node are more likely to belong to the same species

176   (Zhang et al., 2013). The PTP and bPTP analyses were performed on the web server

177   http://species.h-its.org/ptp/.

178   To discern among incongruent results of the species delimitation analyses, we followed the

179   reasoning of Carstens et al. (2013), relying on those delimitations schemes that are recovered in

180   the majority of the analyses.

181   **Results**

182   **Phylogenetic relationships**

183   The alignment of 47 nucleotide sequences, including the outgroups, resulted in a total of 1,641

184   positions in the final dataset. All new sequences were deposited in GenBank. The methods of

185   phylogenetic reconstruction (ML and BI) inferred identical evolutionary relationships, in

186   particular regarding the lineages of the *Centrolene buckleyi* species complex (Fig. 2).

187   The *Centrolene buckleyi* species complex is not recovered as monophyletic; sequences recovered

188   from specimens of *C. buckleyi* form four main lineages, namely *C. buckleyi* sensu stricto, *C.*

189   *buckleyi* [Ca1], *C. buckleyi* [Ca2] and *C.* aff. *buckleyi* [Ca3] (Fig. 2). Specimens of the first

190   lineage come from the proximities of the type locality of *Centrolene buckleyi* (Intag, Imbabura

191   province, Ecuador), as such, hereafter we refer to the first lineage as *C. buckleyi* sensu stricto.

192   This form is sister to *C. ballux* in a strongly supported clade (BV=99%, PP=1); *C. buckleyi* [Ca1]

193   is sister to *C. sabini* (BV=84%, PP=0.98), while *C. buckleyi* [Ca2] is sister to a clade formed by

194 *C. venezuelense* and *C.* aff. *buckleyi* [Ca3] from Colombia in a highly supported clade

195 (BV=96%, PP=1) (Fig. 2).

Molecular species delimitation

197 The results obtained with the GMYCs approach, delimited 26 putative species of the matrix of

198 34 sequences of *Centrolene*; recognizing *C. buckleyi* sensu stricto, *C. venezuelense*, *C.* aff.

199 *buckleyi* [Ca3], *C. buckleyi* [Ca1] and *C. buckleyi* [Ca2] as different species (Fig. 3, see also Fig.

200 S1). The two specimens of *C. buckleyi* sensu stricto (KU17803, Cuicocha Lake, Imbabura

201 province, and MZUTI763, Oyacachi-El Chaco road, Napo province; distance between locations:

202 about 60 km) were clustered in a single lineage. Similarly, the two specimens of *C. buckleyi*

203 [Ca1] (MRy547 and MRy548, Shucos, Zamora Chinchipe province) and the three individuals of

204 *C. buckleyi* [Ca2] (MZUTI83 – MZUTI85, Yanayacu, Napo province) were clustered in a single

205 lineage, respectively. Furthermore, the two specimens of *C. venezuelense* were grouped in a

206 single lineage as well as *C.* aff. *buckleyi* [Ca3] was recovered as a different candidate species.

207 Meanwhile the GMYCm approach found 21 species, GMYCm was the only one of the species

208 delimitation methods that yielded distinct delimitation results, greatly departing from the results

209 of the other analyses (Fig. S2). For instance, GMYCm separated the three specimens of *C.*

210 *buckleyi* [Ca2] into two different species, while consolidating *C. buckleyi* sensu stricto and *C.*

211 *ballux* as a single species; similarly, *C. venezuelense* and *C.* aff *buckleyi* [Ca3] that were

212 recovered as a single entity. For both methods (GMYCs and GMYCm), the result of Likelihood

213 ratio test was not significant ($LRtest_{SINGLE} = 0.063$, $LRtest_{MULTIPLE} = 0.058$) and the likelihood

214 value of the GMYC model was always higher at both methods, single and multiple ($ML_{SINGLE} =$

215 202.026, $ML_{MULTIPLE} = 202.117$) than the value of the likelihood of null model ($L = 199.268$).

216   The Bayesian implementation of the method (bGMYC) also delimited 26 putative species, the

217   same that were recovered with GMYCs (Fig. 3, see also the Klee diagram in Fig. S3).

218   The ML implementation of PTP and the Bayesian implementation of the method (bPTP),

219   considered the topology recovered with MrBayes as a guide tree. These methods delimited 26

220   putative species with good support, recovering the same delimitation results obtained with

221   GMYC and bGMYC (Figs. 3 and S4). Further details of the results of the analysis of species

222   delimitation are in Supplementary Information.

223   Average genetic distances of the 16S matrix, within and between candidate species pairs of the

224   *C. buckleyi* species complex are presented in Table S2. The maximum values between candidate

225   species were observed for the comparison between *C. buckleyi* [Ca1] with *C. buckleyi* sensu

226   stricto and *C. buckleyi* [Ca2] (1.6% and 1.8% respectively); while the lower values correspond to

227   the comparisons between *C. venezuelense* with *C. buckleyi* [Ca2] and *C.* aff. *buckleyi* [Ca3]

228   (0.7% and 0.4% respectively). It is also worth highlighting the low values recovered between *C.*

229   *buckleyi* sensu stricto with its sister taxa *C. ballux* (0.6%) and *C. buckleyi* [Ca1] with its sister

230   taxa *C. sabini* (0.4%).

231   **Discussion**

232   *Centrolene buckleyi* has been suggested to represent a species complex, requiring therefore, a

233   taxonomic revision (Guayasamin et al., 2006; Cisneros-Heredia & McDiarmid, 2007; Hutter,

234   Guayasamin & Wiens, 2013; Castroviejo-Fisher et al., 2014), which in turn would have direct

235   consequence on conservation status and strategies (the *C. buckleyi* species complex is currently

236   listed as Vulnerable by IUCN; Guayasamin, 2010).

237   In this study, we find four distinct lineages within the *buckleyi* species complex and corroborate

238   the distinction of *C. venezuelense* (but see the results of the GMYCm analysis, Fig. 3). Within

239   the current taxonomic definition of *C. buckleyi*, we propose three candidate species (namely *C.*

240   *buckleyi* [Ca1], *C. buckleyi* [Ca2] and *C.* aff. *buckleyi* [Ca3]) in addition to the typical form (*C.*

241   *buckleyi* sensu stricto). This scenario is supported by phylogenetic (ML, BI) and coalescence-

242   based species delimitation analyses (GMYC, PTP). The ML and BI analyses resulted in a tree

243   with topology similar to those of previous studies on glassfrogs (e.g. Hutter, Guayasamin &

244   Wiens, 2013; Castroviejo-Fisher et al., 2014; Delia, Bravo-Valencia & Warkentin, 2017), but

245   with an increased sampling within the *C. buckleyi* species complex. In all phylogenetic analyses

246   performed in this study, *C. buckleyi* is not recovered as monophyletic; specimens allocated to

247   this taxon fall into four main lineages: *C. buckleyi* sensu stricto, *C. buckleyi* [Ca1], *C. buckleyi*

248   [Ca2] and *C.* aff. *buckleyi* [Ca3], which are not closely related (except for *C. buckleyi* [Ca2] and

249   *C.* aff. *buckleyi* [Ca3], which are recovered in the same clade with *C. venezuelense* in ML and BI

250   analysis). These lineages, instead, are recovered as sister to distinct glassfrog species (*C.*

251   *buckleyi* sensu stricto - *C. ballux*, *C. buckleyi* [Ca1] - *C. sabini*, and *C.* aff. *buckleyi* [Ca3] - *C.*

252   *venezuelense*, respectively). Therefore, the topologies obtained here suggests that there are at

253   least four species in the *C. buckleyi* species complex. We acknowledge, however, that the

254   observed lack of monophyly of the *C. buckleyi* species complex at the mitochondrial genome

255   may be a case of differences between gene and species trees, which may be caused by distinct

256   biological processes, such as mitochondrial DNA introgression. This process has been suggested

257   for cases of other amphibians such as the Nearctic treefrogs of the genus *Dryophytes* (Bryson et

258   al., 2010, 2014) and the toads of the genus *Rhinella* (Sequeira et al., 2011). However, without

259   nuclear DNA data we cannot test if the mitochondrial-based tree inferred for the *C. buckleyi*

260   species complex departs from the species tree; as such, we have no reasons to reject these data

261   and assume they represent the true evolutionary history of the group.

262   Coalescent-based species delimitation methods provide hypotheses for the delimitation of

263   species based on gene trees inferred from DNA sequences (Fujita et al. 2012). Previous studies

264   have reported the congruence in the results of methods such as GMYC and PTP when defining

265   putative species (Lang, Bocksberger & Stech, 2015; Thormann et al., 2016; Conte-Grand et al.,

266   2017). Although GMYC and PTP differ in assumptions (e.g., GMYC uses an ultrametric tree and

267   in PTP it is not required), we obtained similar results with both methods. In fact, most of the

268   used species delimitation methods agree in considering *Centrolene buckleyi* as a species complex

269   composed of at least four independently evolving lineages. GMYC provided consistent diversity

270   estimates for BEAST trees (Talavera, Dincă & Vila, 2013; Tang et al., 2014). This method gives

271   better results when the guide tree is well supported; otherwise it may tend to overestimate (or

272   underestimate) the number of candidate species (Fujisawa & Barraclough, 2013; Leavitt, Moreau

273   & Lumbsch, 2015). In addition, GMYC is generally stable in the presence of a certain number of

274   singletons, as is our case (Monaghan et al., 2009; Pons et al., 2006). The results of GMYC with

275   multiple threshold (which searches for more than one probable scenario of speciation) gave a

276   smaller number of candidate species that the other methods; when the GMYC method was used

277   with a single threshold (GMYCs), results were in line with those of bGMYC, PTP and bPTP. It

278   should be noted that bGMYC has been successfully applied in other studies that examined

279   species delimitation in amphibians (Lawson et al., 2015; De Andrade et al., 2016), however, this

280   method could also fail due to errors associated with unilocus data (e.g., selection, error in gene

281   tree estimation, incomplete lineage sorting; Satler, Carstens & Hedin, 2013). Both PTP

282   approaches, ML-PTP and bPTP (adding Bayesian PPV) gave exactly the same result; these

283   methods simultaneously inferring speciation events based on change in the number of

284   substitutions in the internal nodes (Zhang et al., 2013). When visualizing the likelihood plot of

285   each delimitation method, we observed that the MCMC chains converged, which suggests that

286   the PTP results are reliable (Fig. S5). In bPTP, high values of Bayesian support were obtained,

287   which were calculated as the number of occurrences of all the descendants under a particular

288   node, and are the posterior probabilities that these taxa form a single species under the PTP

289   model (Zhang et al., 2013).

290   The results obtained with the methods GMYC and PTP are consistent; however, this scheme

291   should be viewed with caution, mainly due to intrinsic factors of the mitochondrial genes (e.g.

292   smaller genome size, high mutation rates), to the sampling coverage of the taxa, and to the time

293   of divergence between taxa (Talavera, Dincă & Vila, 2013; Luo et al., 2018), which could regard

294   intraspecific structure as distinct species (Satler, Carstens & Hedin, 2013; Sukumaran &

295   Knowles, 2017). For instance, there are results (not within the *Centrolene buckleyi* species

296   complex) that are clearly erroneous, such as the union into a single lineage of two

297   morphologically distant species (*C. charapita* and *C. geckoideum*) with GMYCm method.

298   Therefore, it is also vital to evaluate these molecular species delimitation methods in light of

299   other sources of data (e.g., morphological distinctiveness).

300   One of the main limitations of our study is that our genetic sampling covers only a fraction of the

301   historical distribution range of the *Centrolene buckleyi* species complex. The limited sampling is

302   consequence of population crashes observed across distributional range of this species complex.

303   For instance, Bustamante et al. (2005) mentioned that *C. buckleyi* has disappeared or is rarely

304   found at localities where it used to be abundant. Similarly, Guayasamin et al. (2006) carried out

305   intensively fieldwork in Yanayacu during three years and only found three individuals of what

306   we recognize as *C. buckleyi* [Ca2]. Finally, historically *C. buckleyi* has been reported in several

307   localities and different vegetation formations in 10 different provinces of Ecuador; however,

308   only four populations had been recorded between the years 1997 and 2007 (Cisneros-Heredia &

309   McDiarmid, 2007). Two of these populations correspond to the candidate species that we

310   propose herein (*C. buckleyi* [Ca1] and *C. buckleyi* [Ca2]). Thus, in the scenario of widespread

311   population declines, our findings of distinct independently evolving lineages within the *C.*

312   *buckleyi* species complex make a strong case for increasing efforts aimed to avoid their

313   disappearance.

314   Here we show the existence of candidate species in the *Centrolene buckleyi* species complex.

315   Although the morphology of all candidate species matches the description of *C. buckleyi*, calls

316   from the so far studied candidate species are different (see Guayasamin et al., 2006). This

317   observation matches the fact that calls of populations from the Cordillera Occidental of

318   Colombia, approximately 180 kilometers west of the locality of *C.* aff. *buckleyi* [Ca3] (Bolívar,

319   Grant & Osorio, 1999) and those of *C. buckleyi* [Ca2] (Yanayacu Biological Station (YBS) in

320   northeast of Ecuador) are different. Guayasamin et al. (2006) found that the call of *C. buckleyi*

321   [Ca2] in YBS consisted of 1-5 notes and fundamental frequency = 4,139 Hz which is

322   considerably distinct from that of specimens from Colombia that consists of a single note and

323   fundamental frequency = 5,200 Hz.

324   It is worth noting that, in some cases, genetic differentiation between species pairs is not related

325   with geographic distance. For example, despite the large geographic gap separating the analyzed

326   populations of *C. buckleyi* [Ca2] with those of *C. venezuelense* and *C.* aff. *buckleyi* [Ca3]

327   (approximately 1300 km and 700 km, respectively; see Table 1), the genetic differences for the

328   16S matrix are low (0.7% and 0.9%). The same pattern is seen for *C. sabini* and *C. buckleyi*

329   [Ca1] that have a very low genetic distance (0.4%) and approximately 1300 km of geographic

330   distance. On the other hand, for the pair *C. buckleyi* sensu stricto and *C. buckleyi* [Ca2], whose

331   populations in the cloud forests of the Ecuadorian Napo province, are separated by only 45 km,

332   present a large genetic distance than the previous comparisons (1.4%). Moreover, in general,

333   sister species of *Centrolene* show low levels of genetic divergence. For example, between *C.*

334   *altitudinale* and *C. notostictum* there is a divergence of 0.7%, a similar low value (0.6%) is found

335   between *C. huilense* and *C. muelleri* (CORBIDI 14667) (see Table S2), these two last sister

336   species were recovered as a single species by the GMYCm method. It has been suggested that

337   most speciation events of *Centrolene* occurred during the last five million years, mostly mediated

338   by the Andes uplift (Lynch & Duellman, 1997; Hutter, Guayasamin & Wiens, 2013; Castroviejo-

339   Fisher et al., 2014). This rapid and recent speciation could explain both the low genetic

340   differences found among species, as well as the little morphological divergence observed in the

341   *C. buckleyi* species complex. A similar pattern has been observed in the plump toad

342   *Osornophryne bufoniformis*, another high-Andean anuran species distributed in the northern

343   Andes of Ecuador and Colombia, where highland species also exhibit shallow genetic

344   differentiation (Páez-Moscoso & Guayasamin, 2012).

345   Some of the candidate species that are suggested in our study (Fig. 3) seem to be separated by

346   well-characterized biogeographic barriers. For example, *C. sabini* (Kosñipata valley, Cusco

347   Department, Peru) and *C. buckleyi* [Ca1] (Zamora Chinchipe, southern Ecuador) are separated

348   by the Huancabamba Depression, an important geographic barrier delimiting distinct

349   communities of high-Andean amphibians (see Catenazzi et al., 2012; Hutter, Guayasamin &

350   Wiens, 2013; Castroviejo-Fisher et al., 2014; Hutter, Lambert & Wiens, 2017). Another well-

351   supported example of sister species of Andean frogs separated by geographical barriers are

352   rainfrogs of genus *Pristimantis*, such as *P. cedros* and *P. pahuma* separated by the Guayllabamba

353   River in northern Ecuador (Hutter & Guayasamin, 2015).

354   The inferences drawn from this study should be taken as conservative when evaluating species

355   boundaries of the *Centrolene buckleyi* species complex, mainly because our population sampling

356   is relatively low and to the use of single-locus based methods. The hypothesis posed here should

357   be used as a preliminary perspective of species boundaries and not as the only evidence

358   necessary to circumscribe species (Leavitt, Moreau & Lumbsch, 2015). However, the lack of

359   monophyly of *C. buckleyi* as currently delimited, constitutes strong evidence of the existence of

360   hidden specific diversity. Having said that, before formalizing any taxonomic change (i.e.,

361   describing and naming any new species), further studies integrating morphological variation, as

362   well more geographical samples, additional behavioural (calls) and genetic data (nuclear

363   markers), are needed to test our taxonomic hypothesis (Olave, Solà & Knowles, 2014;

364   Sukumaran & Knowles, 2017).

365   **Conclusions**

366   We highlight that our study is in line with several others showing a pattern of high levels of

367   cryptic diversity in amphibians of tropical South America, including glassfrogs (e.g. Páez-Vacas,

368   Coloma & Santos, 2010; Funk, Caminer & Ron, 2012; Hutter & Guayasamin, 2012, 2015;

369   Gehara et al., 2014; Twomey, Delia & Castroviejo-Fisher, 2014; Ortega-Andrade et al., 2015;

370   Tarvin et al., 2017). As such, we note, that in this era of biodiversity crisis, it is urgent to

371   increase the rate in which the biodiversity is characterized, taking advantage of new and

372   traditional tools, but, mainly, by facilitating research, an issue that requires the collaboration of

373   both scientists and governmental authorities that regulate research activities.

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656 **Table legends**

657 **Table 1.** Names, museum codes, localities and GenBank accession numbers of sequences of

658 specimens of *Centrolene buckleyi* species complex analysed in this study. Sequences gathered

659 here are written in bold.

660 **Figure legends**

661 **Figure 1.** Distribution of *Centrolene buckleyi* species complex. *Centrolene buckleyi* sensu stricto

662 (KU 178031 Imbabura province, Ecuador and MZUTI 763 Napo province, Ecuador, blue

663 circles); *C. buckleyi* [Ca1] Shucos, Zamora province, Ecuador (orange circle); *C. buckleyi* [Ca2]

664 Yanayacu, Napo province, Ecuador (green circle); *C.* aff. *buckleyi* [Ca3] MAR 371 Chingaza

665 National Park, Cundinamarca, Colombia (purple circle); *C. venezuelense* EBRG 5244 Páramo de

666 Maraisa, Mérida, Venezuela (red circle). *C. ballux* in Ecuador (black triangle) and *C. sabini* in

667 Peru (black star).

668 **Figure 2.** ML tree depicting phylogenetic relationships of species of the genus *Centrolene* based

669 on the concatenated dataset of 12S + 16S sequences. Gray nodes represent Bayesian posterior

670 probabilities equal or greater than 0.95 and ML bootstrap values equal or greater than 75%.

671 Species names with different colours represent lineages within the *Centrolene buckleyi* species

672 complex.

673 **Figure 3.** Maximum clade credibility of DNA sequences of glassfrogs of the genus *Centrolene*

674 based on mitochondrial DNA (12S and 16S) showing a comparison of the results of distinct

675 species delimitation methods (see text for details). Each coloured bar represents a candidate

676 species delimited by each method employed. The calibrated gene tree is from a BEAST 2

677 analysis under a relaxed clock with a Birth-Death model tree prior. Node height was determined

678 using mean height across the posterior distribution. Asterisks above branches represent Bayesian

679 posterior probabilities equal or greater than 0.95. The outgroup (*Nymphargus*) was removed prior

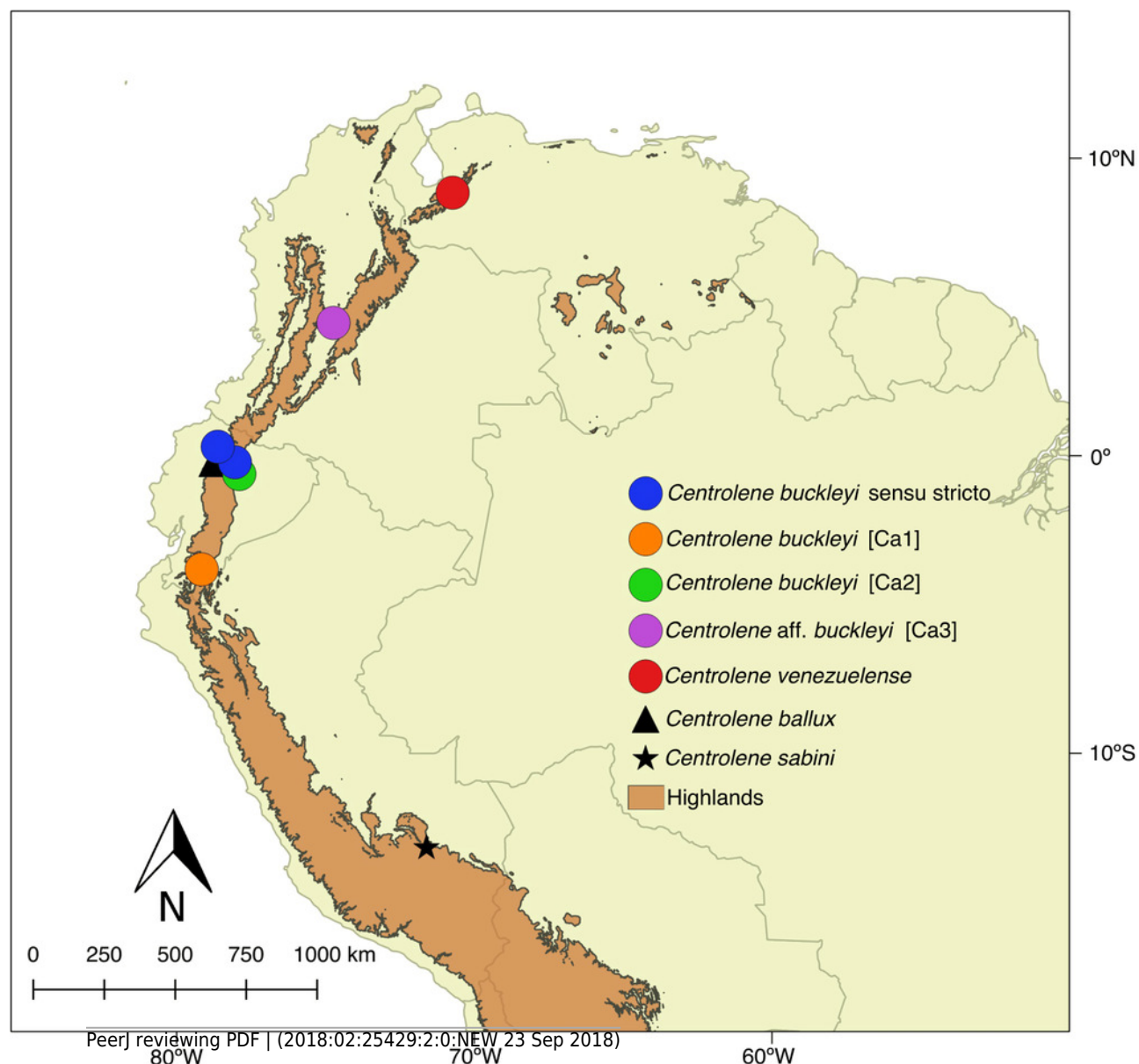
680 to the analyses. Figure inserted in the upper left end corresponds to log lineages through time

681 plot, which shows an increase in the rates of branching to the present, probably corresponds to

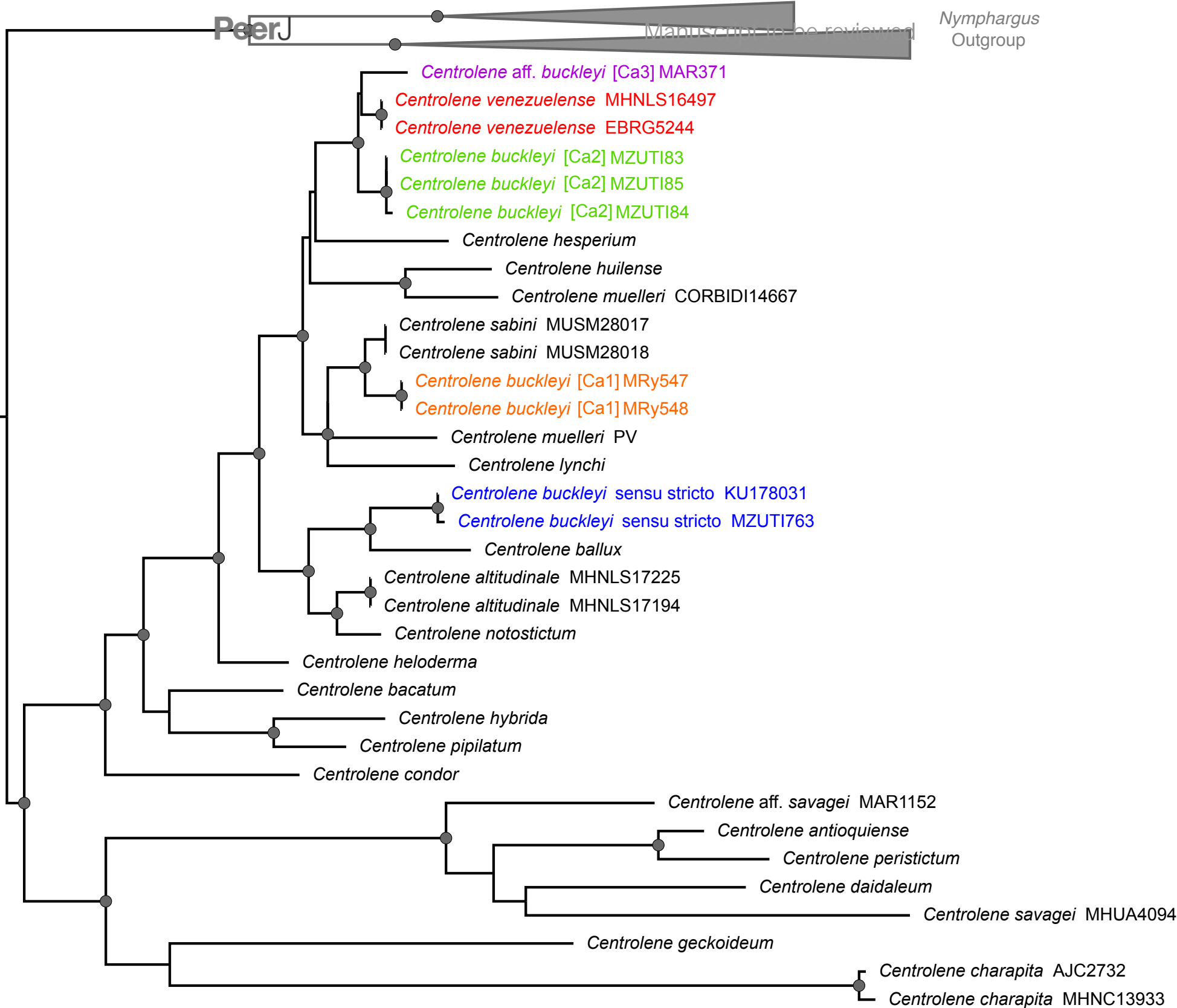
682 the change from interspecific to intraspecific branching events.

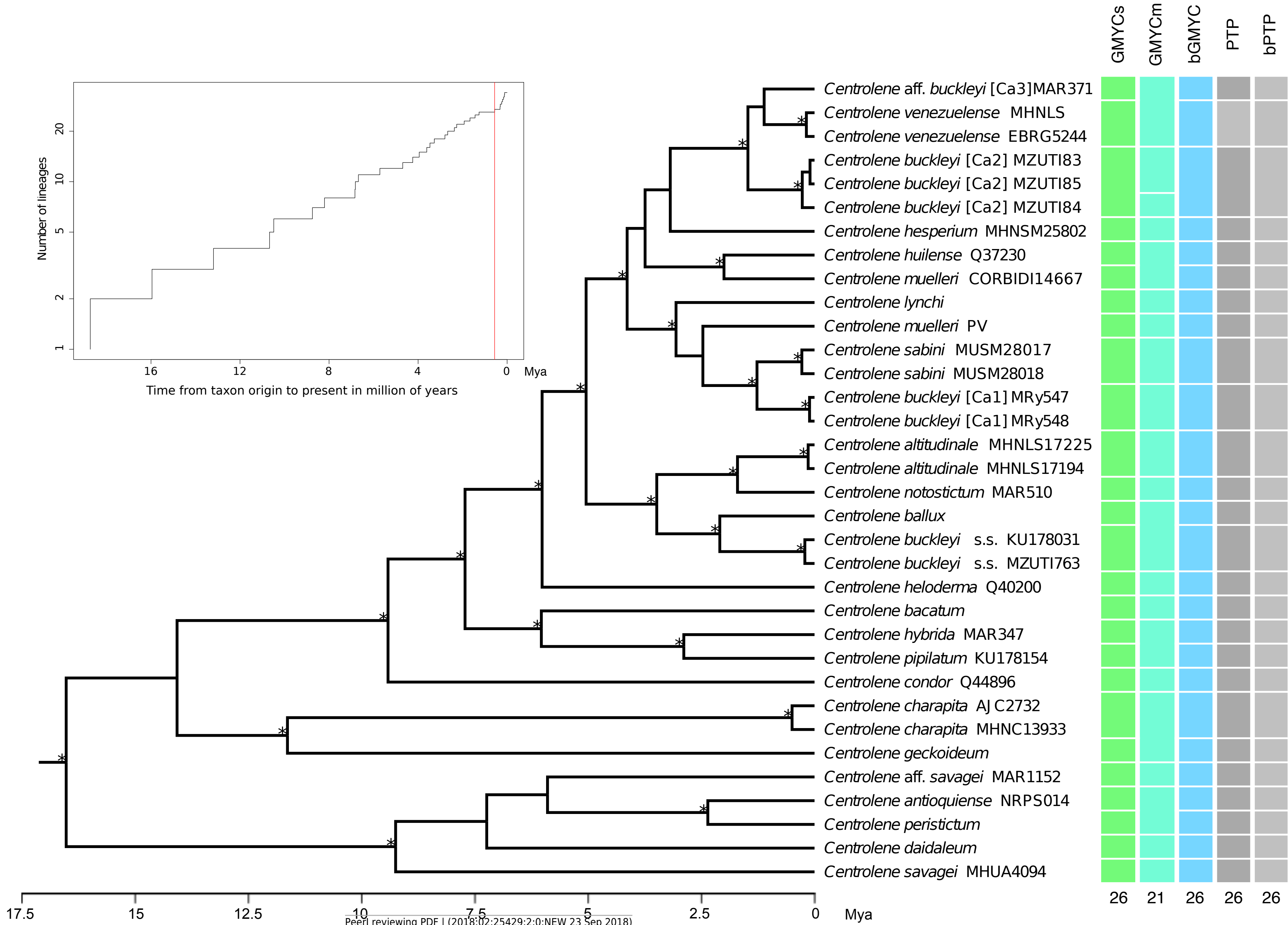
Figure 1

Distribution of *Centrolene buckleyi* species complex.

Centrolene buckleyi sensu stricto (KU 178031 Imbabura province, Ecuador and MZUTI 763 Napo province, Ecuador, blue circles); *C. buckleyi* [Ca1] Shucos, Zamora province, Ecuador (orange circle); *C. buckleyi* [Ca2] Yanayacu, Napo province, Ecuador (green circle); *C.*aff. *buckleyi* [Ca3] MAR 371 Chingaza National Park, Cundinamarca, Colombia (purple circle); *C. venezuelense* EBRG 5244 Páramo de Maraisa, Mérida, Venezuela (red circle). *C. ballux* in Ecuador (black triangle) and *C. sabini* in Peru (black star).

ML tree depicting phylogenetic relationships of species of the genus *Centrolene* based on the concatenated dataset of 12S + 16S sequences.

Gray nodes represent Bayesian posterior probabilities equal or greater than 0.95 and ML bootstrap values equal or greater than 75%. Species names with different colours represent lineages within the *Centrolene buckleyi* species complex.

0.01

Maximum clade credibility of DNA sequences of glassfrogs of the genus *Centrolene* based on mitochondrial DNA (12S and 16S) showing a comparison of the results of distinct species delimitation methods (see text for details).

Each coloured bar represents a candidate species delimited by each method employed. The calibrated gene tree is from a BEAST 2 analysis under a relaxed clock with a Birth-Death model tree prior. Node height was determined using mean height across the posterior distribution. Asterisks above branches represent Bayesian posterior probabilities equal or greater than 0.95. The outgroup (*Nymphargus*) was removed prior to the analyses. Figure inserted in the upper left end corresponds to log lineages through time plot, which shows an increase in the rates of branching to the present, probably corresponds to the change from interspecific to intraspecific branching events.

Names, museum codes, localities and GenBank accession numbers of sequences of specimens of *Centrolene buckleyi* species complex analysed in this study.

Sequences gathered here are written in bold.

1   **Table 1.** Names, museum codes, localities and GenBank accession numbers of sequences of specimens of *Centrolene buckleyi* species
2   complex analysed in this study. Sequences gathered here are written in bold.

Specie/Taxon	Museum code	GenBank number 12S	GenBank number 16S	Latitude	Longitude	Elevation msnm	Locality	Country/State
Centrolene buckleyi s.s	KU 178031	EU663338	EU662979	0.3025	-78.6186	3010	cerca a Lago Cuicocha	Ecuador. Imbabura
***Centrolene buckleyi* s.s**	MZUTI 763	MH844843	MH844849	-0.2189	-78.0444	3012	Zona de humedal en camino Oyacachi-El Chaco	Ecuador. Napo
***Centrolene buckleyi* [Ca1]**	MRy 547	MH844838	MH844844	-3.8193	-79.1592	2633-2923	Shucos	Ecuador. Zamora Chinchipe
***Centrolene buckleyi* [Ca1]**	Mry 548	MH844839	MH844845	-3.8193	-79.1592	2633-2923	Shucos	Ecuador. Zamora Chinchipe
***Centrolene buckleyi* [Ca2]**	MZUTI 83	MH844840	MH844846	-0.6133	-77.8974	2187-2190	Yanayacu	Ecuador. Napo
***Centrolene buckleyi* [Ca2]**	MZUTI 84	MH844841	MH844847	-0.6133	-77.8974	2187-2190	Yanayacu	Ecuador. Napo
***Centrolene buckleyi* [Ca2]**	MZUTI 85	MH844842	MH844848	-0.6133	-77.8974	2187-2190	Yanayacu	Ecuador. Napo
Centrolene aff. buckleyi [Ca3]	MAR 371	EU663339	EU662980	4.4660	-74.7333	3035	Sitio Monte Redondo.P.N. Chingaza	Colombia. Cundinamarca
Centrolene venezuelense	EBRG 5244	EU663359	EU663000	8.8419	-70.7311	2450	Páramo de Maraisa	Venezuela. Mérida
Centrolene venezuelense	MHNLS 16497	EU663360	EU663001	8.7092	-70.9822	2100–3050	Cordillera de Mérida	Venezuela. Mérida