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Collembola, the biological species concept, and the underestimation of global species richness.

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1	COLLEMBOLA AND THE BIOLOGICAL SPECIES CONCEPT
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20	Abstract Despite its ancient origin, global distribution, and abundance in nearly all habitats, the
21	class Collembola is comprised of only 8,000 described species, and is estimated to number no more
22	than 50,000. Many morphologically defined species have broad geographic ranges that span
23	continents, and recent molecular work has revealed high genetic diversity within species. However,
24	the evolutionary significance of this genetic diversity is unknown. In this study we sample five
25	morphological species of the globally distributed genus Lepidocyrtus from 14 Panamanian sites to
26	characterise genetic diversity and test morphospecies against the biological species concept.
27	Mitochondrial and nuclear DNA sequence data was analysed and a total of 58 molecular lineages
28	revealed. Very deep lineage diversification was recovered, with 30 evolutionary lineages estimated
29	to have established more than 10 Mya, and almost all contemporary lineages preceding the
30	Pleistocene (~2 Mya). Thirty-four lineages were sampled in sympatry revealing unambiguous co-
31	segregation of mitochondrial and nuclear DNA sequence variation, consistent with biological
32	species. Biological species richness within the class Collembola, and the geographic structure of
33	this diversity, are substantially misrepresented components of terrestrial animal biodiversity. We
34	speculate that global species richness could be at least an order of magnitude greater than a previous
35	estimate of 50,000 species.
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37	Keywords: Lepidocyrtus, molecular lineage, biodiversity, cryptic species, Panama
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41 Soil and canopy rainforest communities comprise representatives of almost all higher order 42 terrestrial taxonomic groups. They are tremendously diverse, both at local and global scales 43 (Ødegaard 2000; Lavelle et al. 2006), and it has been estimated that as much as 23% and 40% of 44 extant species reside in soil and canopy habitats respectively (Ozanne et al. 2003; Decaëns 2010). 45 The quantification and spatial characterization of this biodiversity is a key goal for biologists, 46 however, much of this diversity comprises animal species of very small body size, and as in any 47 ecosystem the void of knowledge is negatively related to the size class of organisms. Within both 48 soil and canopy communities the mesofauna (from $100 \,\mu\text{m}$ to 2 mm in size) of both habitats suffers 49 from a conspicuous deficit of interest from the scientific community (Decaëns 2010). Collembola 50 (springtails) are a class of wingless hexapods, and are often the most abundant mesofaunal 51 arthropods collected in rainforest canopies (Rodgers & Kitching 2010) and soil ecosystems (Hopkin 52 1997). Despite their worldwide distribution they are only moderately diverse in terms of species 53 number (approximately 8,000 described species) if compared with other hexapod groups (e.g. 54 360,000-400,000 species within the order Coleoptera alone (Scheffers et al. 2012)). Although 55 significant progress that has been made regarding Collembola taxonomy over recent decades, with 56 the identification of important new morphological characters and a significant growth of newly 57 described species (Deharveng 2004), global species richness remains low. Estimates of global 58 species richness within the hexapod class Insecta are in the order of 5,000,000 (Gaston 1991), while 59 for Collembola, the other major hexapod class, global species richness is estimated to be only 60 approximately 50,000 species when one takes into account the under-sampling of species with 61 restricted distributions (Hopkin 1997). However, in addition to geographic under-sampling of local 62 endemisms, recent molecular genetic studies present evidence that springtail biodiversity may be 63 vastly underestimated because of pervasive cryptic species diversity within morphologically defined 64 species of Collembola (e.g. Emerson et al. 2011; Porco et al. 2012a). Many morphologically defined 65 species investigated to date have revealed remarkable levels of deeply divergent DNA sequence 66 diversity at both broad and fine geographical scales (e.g. Timmermans et al. 2005; Stevens et al.

67 2006; Garrick et al. 2007; Cicconardi et al. 2010; Torricelli et al. 2010; Porco et al. 2012b; Ramirez-68 Gonzalez et al. 2013). More recently Emerson et al. (2011) have analysed geographically referenced 69 barcode sequences from the mitochondrial DNA (mtDNA) COI gene belonging to 105 70 morphologically defined species. Their analyses revealed that highly divergent mtDNA sequence 71 diversity within morphospecies is a taxonomically and geographically pervasive feature within 72 Collembola, and most likely a signature of cryptic species diversity. 73 A hypothesis of pervasive cryptic species diversity is also consistent with the Collembola 74 fossil record. From the geochronological point of view springtails have the most extensive record 75 among all hexapod orders. They stretch from the Lower Devonian ~ 410 million years ago (Mya) 76 (Hirst & Maulik 1926) to the Pleistocene ~ 1 Mya (Yosii 1974). There are two species reported 77 from the Paleozoic: Permobrya mirabilis from the Upper Permian of South Africa (Riek 1976), and 78 Rhyniella praecursor (397-391 Mya), originally described from the Rhynie Chert of England (Hirst 79 & Maulik 1926). The latter fossil was redescribed several times and finally placed in the modern 80 family Isotomidae because of the remarkably modern morphological characters (Whalley & 81 Jarzembowski 1981; Greenslade & Whalley 1986). Baltic amber fossils from the Early Eocene (50-82 45 Mya) are dominated by species belonging to contemporary genera, and in some cases these have 83 been assigned to extant species (Rapoport 1971). Morphological identity between fossil and modern 84 assemblages is also reported in Early Miocene amber from Chiapas (23-16 Mya), Mexico, and from 85 the Dominican Republic (23-20 Mya) where seven specimens could be placed into extant species 86 (Christiansen 1971; Mari-Mutt 1983a). This propensity for morphological stasis over long periods 87 of time fits well with the hypothesis that classically defined morphospecies are an underestimation 88 of the true collembolan biodiversity.

Many Collembola genera are distributed globally, with the distributions of many species
spanning continents. While extensive dispersal can be invoked to explain such distributions, it
remains equally plausible that the broad distributions of many morphospecies could be
representative of cryptic species diversity. The genus *Lepidocyrtus* Bourlet, 1839 (Entomobryidae:

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93	Lepidocyrtinae) is one of the largest genera of Collembola (Hopkin 1997). It has a worldwide
94	distribution and includes more than 250 described species, many of which have distributions
95	spanning more than one continent. A recent molecular analysis of the seven Lepidocyrtus
96	morphospecies inhabiting the North-Western Mediterranean basin, using both mitochondrial and
97	nuclear DNA sequence data, has revealed lineage diversity within the seven species that is
98	remarkable, both in terms of lineage number and age of origin (Cicconardi et al. 2010). Samples
99	from 24 distinct geographic locations revealed 52 molecular lineages predating the onset of the
100	Pleistocene 1.8 Mya, and 35 lineages originating more than 5.8 Mya. While the results of
101	Cicconardi et al. (2010) are remarkable, the implications for cryptic diversity within the Collembola
102	on a global scale are profound when one considers the broader geographical ranges of the seven
103	morphospecies investigated. Their ranges extend far beyond the North-Western Mediterranean
104	basin, with five of them spanning the Palaearctic and Nearctic regions. While evidence is mounting
105	that morphologically defined species of Collembola are frequently comprised of divergent genetic
106	lineages, the significance of these in term of species is unclear. Cicconardi et al. (2010) obtained
107	some evidence that the genetic lineages they identified conform to species under Mayr's (1942)
108	biological species concept (BSC), however their evidence was limited to an assessment of only two
109	sympatric lineages.

110 The worldwide distribution of the genus *Lepidocyrtus*, provides ample opportunity for 111 extending the sampling of Cicconardi et al. (2010) to investigate the temporal and spatial properties, 112 and biological significance of cryptic lineage diversity within morphologically defined species of 113 Collembola. The Isthmus of Panama is a narrow strip of land which links Central America with 114 South America and it is among of the five most important global biodiversity hotspots (Myers et al. 115 2000; Briggs 2007; Joppa *et al.* 2011). For the purpose of our study the Isthmus represents a very 116 distinct biogeographic region from that sampled by Cicconardi et al. (2010) within which to 117 investigate morphospecies within the genus Lepidocyrtus. Contrasting with temperate species, that 118 typically exhibit a more habitual association with soil and leaf litter, tropical collembolan species

119 are in fact ecologically distinct from their temperate relatives, exhibit different seasonality and a 120 closer association with the sub-canopy. The combined processes of plate disassembly and 121 redistribution, phases of global warming and cooling (Molnar 2008), together with well documented 122 dating of volcanic arcs and geological formations (Coates et al. 2003; Wegner et al. 2010; Montes 123 et al. 2012) also renders the Isthmus of Panama an informative region for biogeographic inference. 124 In this study we sample five morphospecies within the genus *Lepidocyrtus* from 14 sites spanning 125 the Isthmus of Panama for the quantification of molecular lineage diversity, its temporal and spatial 126 structure, and the consistency of lineages with the BSC. To achieve this we analyse DNA sequences 127 from the mtDNA cytochrome c oxidase subumit II (COII) gene and the nuclear elongation factor 1α 128 $(EF1\alpha)$ gene. We reconstruct lineage origin in a temporal and spatial framework using molecular 129 phylogenetic tools, a molecular clock for the *Lepidocyrtus* and geological data. We then evaluate 130 divergent molecular lineages within morphospecies against the BSC, testing Hardy-Weinberg 131 equilibrium (HWE) and linkage disequilibrium (LD) among lineages in sympatry.

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134 **Methods**

135 Data Collection

136 Samples were collected from 14 sites across the Isthmus of Panama (Fig. 1; Table 1). Specimens 137 were collected from leaf litter and from plants to a height of two metres using Berlese/Tullgren 138 funnel and an automatic aspirator and stored at -80 °C in 95% alcohol. Specimens belonging to the 139 genus Lepidocyrtus Bourlet, 1839 were identified under a stereo microscope using characters as 140 described in Gisin (1964a; b, 1965) and classified to morphospecies according to the literature 141 available for Neotropical and Panama / Costa Rica springtails (Denis 1933; Mari-Mutt 1983b, 1986; 142 Mari-Mutt & Bellinger 1990, 1996). Total genomic DNA was extracted using the DNeasy Tissue 143 Kit (Oiagen Inc., Valencia, CA, USA) with the following variations. For all samples the entire 144 specimen were soaked in lysis buffer at 50 °C for 12-24h, depending on the specimen size, in order

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145	to allow for DNA extraction and the preservation of the thin cuticle. The cuticles were subsequently
146	mounted on slides for further morphological analysis. The mtDNA gene COII was amplified via the
147	polymerase chain reaction (PCR) using a combination of primers described in the literature
148	(Cicconardi et al. 2010). The nuclear gene $EF1\alpha$ amplification was accomplished using a
149	combination of primers designed specifically for Lepidocyrtus (EF1a_LepF: 5'-CAT GAT YAC
150	KGG TAC CTC TCA-3'; EF1α_LepR1: 5'-GCA TCM CCM GAT TTG ATA GC-3'; EF1α_LepR2:
151	5'-GCC TCA ACG CAC ATG GGC TTA-3'). PCR products were gel purified (Wizard SV Gel and
152	PCR Clean-Up kit, Promega) and sequenced in both directions at the John Innes Centre (Norwich,
153	UK). Sequences were manually checked and corrected using FINCHTV v. 1.4.0 (Geospiza Inc.,
154	Seattle, WA, USA). Double peaks in the nuclear sequences, corresponding to allelic differences in
155	heterozygous individuals, were scored and PHASE v. 2.1.1 (Stephens et al. 2001) was used to
156	resolve the two alleles for heterozygous individuals, as in Cicconardi et al. (2010). All alleles that
157	could not be phased were left unspecified and coded in accordance with the IUPAC code table.
158	Uncorrected pairwise distances (π) (Nei 1987) were calculated with PAUP* v. 4.0b10 (Swofford
159	2003). The partitioning of genetic variance within both the COII and EF1 α datasets among
160	morphological species, among sampling sites, and within sampling sites was measured using an
161	AMOVA strategy as implemented in ARLEQUIN v. 3.5.1.2 (Excoffier & Lischer 2010), and its
162	statistical significance tested based on 10,000 permutations.
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164 Phylogenetic analyses and divergence time estimation

165 Sequences from both markers were manually aligned with SEAVIEW v. 4.1 (Gouy et al. 2010) taking 166 into account the translated amino acid sequence. For COII sequences all gaps were retained in the 167 alignment. For *EF1a*, forward and reverse sequences were first assembled as contigs and the intron 168 regions removed due to the inability to align them unambiguously. For maximum likelihood (ML) 169 and Bayesian inference (BI) analyses the most appropriate substitution model was selected 170 excluding invariant characters (I) to avoid over-fitting. For each of the two loci the best nucleotide

171	substitution model was inferred among 44 (for GARLI and BEAST) and 12 models (for MRBAYES
172	for its more restricted model selection list) according to the Akaike Information Criterion as
173	implemented in JMODELTEST v. 0.1.1 (Posada 2008). Three individuals from undescribed Seira spp.
174	(Collembola: Entomobryidae: Seirinae), sampled in the same collection area, were used as
175	outgroups in all analyses. For each dataset the ML searches, as implemented in GARLI v. 2 (Zwickl
176	2006), were performed, retaining the best tree out of 20+5 runs from random starting trees, using
177	the best-fit model, allowing the algorithm to estimate parameters. Runs were continued until no
178	improvement in log-likelihood > 0.01 was observed for 50,000 generations. After the best tree was
179	found 1,000 ML non-parametric bootstrap replicates were performed and the result summarized
180	using the SUMTREES v. 3.3.1 tool (Sukumaran & Holder 2010). The BI search was performed with
181	the algorithm implemented in the MPI version of MRBAYES v. 3.2.1 (Huelsenbeck et al. 2001;
182	Altekar et al. 2004; Ronquist 2004) using the best-fit model, estimating parameters. Fine-tuning of
183	MCMC parameters such as number of runs, chains and temperature were conducted as in
184	Cicconardi et al. (2010).
185	To build a "species" tree, where "species" should represent any group of individuals that,
186	after some divergence time, are reproductively isolated respectively to individuals outside that
187	group, a coalescence tree model for species or divergent populations within species needs to be
188	identified (Heled & Drummond 2010). Because it has been demonstrated that mtDNA markers
189	provide a better indication of true species diversity (Tang et al. 2012), we used the COII gene tree to
190	define such coalescence units. A preliminary chronogram was built with BEAST v1.7.2
191	(Drummond <i>et al.</i> 2012) by performing two independent analyses (five independent runs of $5*10^6$
192	generations, a burn-in of 1*10 ⁶ generations, sampling every 100 th generation), using two different
193	tree priors (coalescence: constant size; speciation: Yule process). The two parallel analyses were

194 carried out to evaluate possible tree shape prior bias. All runs within each of the two analyses were

- 195 combined to produce a maximum clade credibility trees to define "intra-species" branching pattern
- under the general mixed yule coalescent (GMYC) model (Pons et al. 2006) as implemented in the

197	R-script GMYC (Powell 2012) (http://www.r-project.org), using default settings for single and
198	multiple threshold analysis. Coalescence units identified by the GMYC analysis were used as traits
199	and both genetic markers were used to define phylogenetic relationships among coalescent units
200	using the species tree method implemented in *BEAST (Heled & Drummond 2010), within BEAST
201	v1.7.2. The multispecies coalescent model estimates a species tree by assuming gene trees are
202	embedded inside it, and following the stochastic coalescent process back in time. The uncorrelated
203	relaxed clock (Drummond et al. 2006) was used for both markers, setting a conservative
204	Lepidocyrtus COII substitution rate of 2.45*10 ⁻² substitutions/site/myr (described by Cicconardi et
205	al. (2010)), and estimating the <i>EF1</i> α rate relative to mtDNA. The three specimens of <i>Seira spp</i> .
206	were used as outgroups and the Yule process tree prior used. Five independent runs of 5*10 ⁸ MCMC
207	generations were executed, sampling every 10,000 th generation and samples combined using
208	LOGCOMBINER v. 1.7.2 (Drummond et al. 2012). TRACER v. 1.5 (Rambaut & Drummond 2007) was
209	used to evaluate convergence among the independent runs and to define the appropriate burn-in.
210	Posterior distributions and parameters were also evaluated by examining their effective sample size
211	(ESS). TREEANNOTATOR v. 1.7.2 (Drummond et al. 2012) was subsequently used to summarize the
212	obtained trees as a single consensus tree representing the posterior distribution. All phylogenetic
213	analyses were performed independently and nodal support values extracted from the combined log
214	file and presented on the tree topology extracted from the *BEAST analysis. We used the Wilcoxon
215	rank-sum test (Wilcoxon-Mann-Whitney test), as implemented in R project (<u>http://www.r-</u>
216	project.org), to evaluate hypotheses concerning the ages of lineages and their geographic
217	distribution.

218

219 Molecular lineages and the biological species concept

220 We define a molecular lineage as a terminal monophyletic clade composed of the same set of

individuals in both *COII* and *EF1* α gene trees. To graphically illustrate molecular lineages we

222 constructed a visualization plot of terminal monophyletic clades for both the *COII* and *EF1* α gene

223	trees using the software package Circos (<u>http://www.circos.ca/</u>). Molecular lineages within
224	morphospecies were formally evaluated for consistency with the biological species concept by
225	testing for Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) within sampling
226	sites where two or more molecular lineages occur in sympatry. HWE and LD were calculated using
227	ARLEQUIN v. 3.5.1.2 (Excoffier & Lischer 2010) and statistical significance tested using 100,000
228	dememorisation steps and 100,000 steps in Markov chain. After Bonferroni adjustments (Benjamini
229	& Hochberg 1995), <i>p</i> -values were set to 0.05. We used the Wilcoxon rank-sum test to evaluate, for
230	both markers, differences in the distributions of pairwise genetic distance of intra-sympatric lineage
231	against intra-allopatric. We consider as null hypothesis that the distributions are equal (two-sided).
232	Using coalescence units and morphospecies as unit of analysis, we performed individual-based
233	rarefaction analyses, as implemented R package VEGAN v. 2.0-4 (Oksanen et al. 2007), to estimate
234	expected species richness based on the two different measures, in order to evaluate the implications
235	for biodiversity of the two different estimators of species richness.

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238 **RESULTS**

239 *Morphospecies delineation*

240 Given the very limited number of studies on the taxonomy of Central American springtails, 241 collected specimens (Table 1, Fig. 1) were named according to the closest literature description 242 available. Morphological characters such as labial, thoracic and abdominal chaetotaxy (especially 243 number and position of macrochaete and chaetotaxy of trichobothrial complex of abdomen II-IV), 244 body pigmentation, and shape of the foot complex were taken into consideration for a broad 245 assessment of morphological differences and characterisation of morphospecies groups. Five 246 different morphospecies were identified. Two of them appear closely related to the species 247 Lepidocyrtus balteatus Mari Mutt 1983. They differ from each other only by their body 248 pigmentation pattern and are named as L. cf. balteatus with one and two pigmented bands

249 respectively. A third species, well differentiated from the previous two, may be attributed to the 250 species L. vexans Denis, 1933. Compared with the previous species this latter shows higher 251 variability, specifically in the pigmentation pattern and macrochaetae distribution; nonetheless in 252 line with degree of morphological variability commonly considered among Entomobryidae (e.g. 253 Dallai 1967, 1969; Jordana & Baquero 2005; Zhang et al. 2009). These three morphospecies were 254 the most abundant and more geographically widespread. A further two species, one also referable to 255 the species L. balteatus Mutt, 1983 - with no pigmentation; and another completely undescribed (L. 256 sp.), whose habitus differs from the others by a clear and distinct distribution and abundance of 257 thoracic macrochaetotaxy, were less abundant and were recovered in only a few sampling sites 258 (Table 1).

259

260 DNA sequence variation

261 Sequences from the mitochondrial gene COII were obtained from 96 samples from across the 14 262 sampling sites, yielding 77 unique haplotypes from across 30 morphospecies populations. The 263 number of sequences per morphospecies ranges from 19 to 51 for the three more abundant species 264 and three sequences each for the remaining two species. MtDNA COII sequences range in length 265 from 665 to 677 nucleotides (nt). Length variation is explained by an indel of 12 nucleotides 266 corresponding to four contiguous amino acids, and another indel of a single triplet. The Hasegawa-267 Kishino-Yano model plus Gamma (HKY+ Γ) was selected as the most appropriate model for GARLI 268 (ML) and BEAST (BI), while the general time-reversible model plus gamma (GTR+ Γ) was selected 269 for MRBAYES (BI). Individuals with identical haplotypes belong to the same morphological species 270 from the same site, with the exception of a single haplotype sampled from two specimens of the 271 same morphospecies in two different sites. Genetic variation within the COII locus is remarkably 272 high. Considering COII pairwise p-distances (π), the distribution has a median of 0.23 π , a mean 273 0.21π and the highest value of 0.31π . Furthermore, almost all pairwise comparisons (97%) have 274 values above 0.10 π (Fig. S1). The ANOVA analysis reveals that 28.21% of the genetic variation

within the *COII* gene is explained by differences among morphospecies, 27.53% occurs among

populations within morphospecies, while the highest proportion, 44.26%, occurs within populations(Table 2, Fig. S2).

278 Sequences from the nuclear gene $EF1\alpha$ were obtained from 80 specimens from 14 sampling 279 sites, representing a total of 29 morphospecies populations. Sixteen samples could not be amplified 280 due to limited DNA template. The number of sequenced individuals per morphospecies ranges from 281 15 to 45 in the three more common species, and two and three in the remaining two species. The 282 Lepidocyrtus $EF1\alpha$ has three exons of differing length (exon I: 396bp; exon II: 219bp; exon III: 283 236bp), and four intronic sequences with high nucleotide polymorphism and length variation (intron 284 I: 46-102bp; intron II: 84-108bp; intron III: 108-130bp; intron IV: 52-131bp). High variation within the $EF1\alpha$ locus is also exemplified by the 3rd intron that is found in only three individuals, 285 286 representing three different morphospecies from three different sites. The model of molecular 287 evolution that best fits the data for both GARLI (ML) and BEAST (BI) is the equal-frequency 288 Tamura-Nei model plus Gamma ($TrNef+\Gamma$) model while the general time-reversible model plus 289 gamma (GTR+F) model was selected for MRBAYES (BI). A total of 77 unique alleles are recovered 290 after identical sequences are collapsed. Considering only the coding region of the gene, the locus 291 shows an observed heterozygosity (H_{o}) of 0.28, which increases to 0.53 when introns are also taken 292 into account. Fourteen alleles were sampled from more than one specimen, of which 13 were 293 sampled from different specimens from the same site, with the remaining allele sampled from two 294 geographically very close populations (SF and CM, Fig. 1). As expected the nuclear locus exhibits 295 less genetic divergence with respect to mtDNA loci, but divergences within and among 296 morphospecies are still substantial for a nuclear locus. Considering the distribution of nucleotide 297 diversity, the median value is 0.11π , with a mean value of 0.08π and a maximum value of 0.14π 298 (Fig. S1). An AMOVA analysis reveals that 70.82% of the observed variation is explained by 299 differences among morphospecies, while 16.04% and 13.14% of variation is explained by 300 differences among populations within morphospecies and within populations respectively (Table 2,

301 Fig. S2).

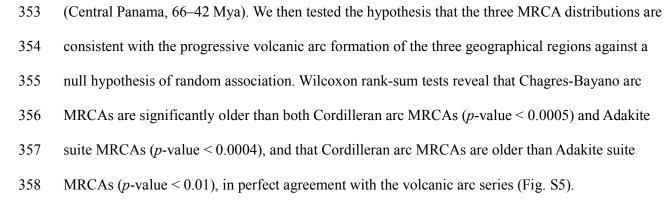
302

303 *Phylogenetic analysis and divergent time estimation*

304 Phylogenetic analyses using both ML (Garli) and BI (MRBAYES) with the same molecular marker 305 resulted in the same topology, with the exception of only a few nodes characterized by very low 306 support (Fig. S3-S4). For both markers and methods the topologies are composed of two very well 307 characterized clades: one composed by the morphospecies L. cf. vexans, and another with the 308 remaining samples, where morphospecies are highly polyphyletic. The sample compositions of 309 terminal clades are highly consistent between the two gene trees, with very few exceptions. In order 310 to estimate a species tree with *BEAST, coalescence units were first defined by two BEAST 311 analyses for the mtDNA partition, the first with a Yule tree prior, and the second with a coalescent 312 tree prior (see Methods). Both analyses yielded high effective sampling size values (ESS > 400) after sampling $2*10^7$ states. GMYC analyses (threshold probability of 0.90) gave very consistent 313 314 values for input trees derived from both BEAST analyses, yielding 65 coalescent units for the 315 coalescence tree prior and 62 units for the Yule prior. The more conservative estimate of 62 units 316 was subsequently used for the *BEAST analysis. Five independent runs for the species tree analysis converged and reached stability after 25×10^7 generations. After applying a burn-in of 5×10^7 317 generations log files were merged, yielding a total of $1*10^9$ states giving high ESS values, higher 318 319 than 5,000 for 85% of the priors.

Using a conservative *Lepidocyrtus COII* evolutionary rate of $2.45*10^{-2}$ *substitutions/site/myr* (see Cicconardi et al. 2010), BEAST estimated the *EF1a* rate to be $1.38*10^{-3}$ *substitutions/site/myr*, approximately 18 times slower than *COII*. The use of a conservative (fast) substitution rate means that our estimations of lineage ages are more likely to be underestimates than overestimates (Cicconardi et al. 2010). Adopting this conservative approach to date the species tree (Fig. 2) reveals very deep lineage diversification, with the origin of almost all lineages estimated to precede the Pleistocene (~2 Mya), and basal divergence within the ingroup to have initiated at least 40 Mya

327 (upper HPD: 51.2; lower HPD: 33.3). The restricted geographic ranges of Collembola lineages, 328 combined with the geologically old branching pattern among lineages, reflect long-term persistence 329 of evolutionary lineages within restricted geographic limits. Very similar findings have been 330 observed in other studies of Collembola, where geologically old and geographically discrete 331 lineages have also been recovered (e.g. Garrick et al. 2007; Cicconardi et al. 2010; Torricelli et al. 332 2010; Ramirez-Gonzalez et al. 2013). 333 The number of lineages through time plot (Fig. 2) indicates two inflation points, one 334 between 20 and 15 Mya (T_1) and another more recent, approximately 2.5 Mya (T_2) . Although we 335 consider our rate calibration to potentially underestimate the timing of divergence events, these 336 inflation points exhibit good agreement with major paleogeographic and paleoclimatic phenomena. 337 The Panama region went through a general and rapid shallowing (19-12 Mya), from a deep-marine 338 (lower-bathyal) environment to a coastal/fluvial environment, with continual emergence and erosion 339 of volcanic arcs in the early-late Miocene (Coates et al. 2003; Wegner et al. 2010; Montes et al. 340 2012). At the beginning of the Pleistocene paleogeographic and paleoclimatic events influenced the 341 area, with a global climate change towards cooler temperatures (~ 2.8 Mya), and the final closure of 342 the Isthmus of Panama (2.7-2.6 Mya) triggering a significant biogeographical event, the great biotic 343 American interchange (see Molnar 2008 for a review). Given the apparent fit of phylogenetic events 344 with paleogeographic and paleoclimatic events, we further evaluated the hypothesis that the 345 geographical persistence of lineages and their diversification may correlate with the regional 346 geological history of land formation. To do this we used available dates for the magmatic evolution 347 of Isthmus of Panama to test whether sequentially formed terrains were randomly colonized by 348 lineages (H_0) , or if the temporal emergence of lineages correlates with the temporal sequence of 349 magmatic evolution and the formation of new colonisable territories (H_i) . Based on their 350 geographic distributions we assigned the most recent common ancestor (MRCA) of each lineage to 351 one of the three main volcanic arcs designated by Wegner et al. (2010): Adakite suite (Western 352 Panama, < 2 Mya), Cordilleran arc (Midwestern Panama, 22–7 Mya) and Chagres-Bayano arc



359

360 Molecular lineages, species boundaries and species richness

361 An assessment of terminal monophyletic clades, comprised of the same individuals in both the COII 362 and $EF1\alpha$ trees (Fig. S3-S4), results in a total of 58 molecular lineages (Fig. 3). Across the 14 363 sampling sites 34 molecular lineages are found in sympatry with at least one other molecular 364 lineage belonging to the same morphospecies. Perhaps not surprisingly, sympatric lineages (s.l.) are 365 found only within the more densely sampled morphospecies (L.ve: 22 sympatric lineages; L.b1b: 7; 366 L.b2b: 5). To evaluate the significance of apparent mitochondrial and nuclear DNA sequence co-367 segregation patterns among sympatric molecular lineages, we tested for linkage disequilibrium (LD) 368 and Hardy-Weinberg equilibrium (HWE), considering as a null hypothesis that individuals of the 369 same morphospecies within a sampling site belong to a panmictic population. Tests were performed 370 for morphospecies populations in which sympatric molecular lineages are represented by four or 371 more individuals. This resulted in the analysis of six morphospecies populations comprising a total 372 of 19 molecular lineages (L.b1b-SanFelix 3 sympatric lineages; L.b2b-ElValle 3; L.ve-Darien 4; 373 L.ve-Fortuna 3; L.ve-P.I.L.A. 3; L.ve-SanFelix 3). Within all the six morphospecies populations both 374 null hypotheses of linkage equilibrium and HWE were rejected (*p*-adj < 0.04). As a more rigorous 375 test, we performed pairwise analyses between sympatric molecular lineages sampled for two or 376 more specimens (L.b1b-SanFelix-2 vs -3; L.ve-Fortuna-2 vs -3; L.ve-P.I.L.A.-1 vs -2; L.ve-377 SanFelix-1 vs -2). All pairwise comparisons resulted in the rejection of both random association of 378 alleles between the two loci and HWE (*p*-adj < 0.03). The two results are therefore consistent with

reproductive isolation among cryptic species. We applied the Wilcoxon rank-sum test to evaluate whether the distribution of pairwise genetic *p*-distances (π) within allopatric molecular lineages differs from that of sympatric molecular lineages. We found no significant difference between the two distributions (*COII p*-value = 0.06; *EF1* α *p*-value = 0.95) indicating that allopatric molecular lineages exhibit genetic divergences consistent with reproductive isolation from other such lineages (Fig. S6).

A rarefaction analysis was performed for both morphospecies and molecular lineages (Fig. 4). While the rarefaction analysis suggests it is unlikely that there are additional morphospecies that we have not sampled, the implications for molecular lineages are quite different. The steepness of the molecular lineage curve suggests that regional diversity is much greater than the 58 molecular lineages sampled. A rarefaction analysis of data generated by Cicconardi et al. (2010) yields a similar result for morphospecies and molecular lineages within the North-Western Mediterranean basin (Fig. 4).

392

393

DISCUSSION

395 *Temporal and geographical lineage diversification*

396 The five morphologically defined species of Lepidocyrtus sampled within the region of Panama 397 reveal a striking level of molecular genetic differentiation, with both mitochondrial and nuclear 398 markers exhibiting divergent DNA sequences with restricted geographic ranges. The few identical 399 sequences recovered in our analyses were always sampled from the same morphospecies within the 400 same sampling site, with only two exceptions: a single mtDNA haplotype and a single nuclear allele 401 are shared between two geographically proximate sampling sites. Diversification within the focal 402 group is estimated to have initiated at least 40 Mya, resulting in 62 coalescent units defined from a 403 joint Bayesian analysis of mitochondrial and nuclear DNA sequence data. Thirty molecular lineages 404 were already distinct more than 10 Mya, while the Pleistocene is characterised by 46 molecular

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405	lineages prior to its onset that survived through this 1.8 Mya period. An estimated increase in the
406	rate of diversification approximately 16 Mya coincides with the emergence of the first colonisable
407	lands within the Isthmus, and our analyses indicate that the geographic distribution of molecular
408	lineages within Collembola has been shaped by paleogeographic events within the region. The
409	temporal pattern of establishment of molecular lineages follows a westward progression, with more
410	ancient eastern populations subsequently and progressively colonising newly emergent terrains to
411	the west. This pattern is in general accordance with that observed within freshwater fishes of the
412	region, where two dispersal events occurred in the area, both with a westward direction, before (\sim 7
413	Mya) and after (~3 Mya) the closure of the Isthmus of Panama (Bermingham & Martin 1998).
414	The pattern of deeply divergent but geographically localised molecular lineages observed
415	within Panamanian morphospecies of <i>Lepidocyrtus</i> is consistent with results obtained by Cicconardi
416	et al. (2010), who examined seven traditionally described morphospecies from the genus
417	Lepidocyrtus in the North-Western Mediterranean basin. Their combined analysis of highly
418	concordant mtDNA and nuclear $EF1\alpha$ gene sequences revealed an Oligocenic or pre-Oligocenic
419	origin of more than 23 Mya for lineage diversity within and among morphospecies. Cicconardi et
420	al. (2010) identified 35 evolutionary lineages in the North-Western Mediterranean basin that were
421	already distinct more than 5.8 Mya, indicating their survival and persistence through the dramatic
422	environmental perturbations of the Messinian Salinity Crisis. The Pleistocene is characterized by 52
423	evolutionary lineages prior to its onset that survived through the climatic oscillations during this 1.8
424	Mya period. Deeply divergent DNA sequence lineages are frequently recovered within
425	morphologically defined species of Collembola, with lineages often exhibiting narrow geographic
426	ranges (e.g. Timmermans et al. 2005; Stevens et al. 2006; Garrick et al. 2007; Cicconardi et al.
427	2010; Torricelli et al. 2010; Ramirez-Gonzalez et al. 2013). While this diversity has been suggested
428	to be indicative of cryptic species diversity (Cicconardi et al. 2010; Torricelli et al. 2010; Emerson
429	et al. 2011), until now only limited evidence has been available to evaluate this hypothesis
430	(Cicconardi et al. 2010). The fortuitous sampling of divergent molecular lineages in sympatry

431 within the Panama has allowed us to formally evaluate sequence diversity within morphospecies

432 agains the BSC.

433

434 *Genetic diversity and its biological significance*

435 The class Collembola has a worldwide distribution, but it is only moderately diverse in terms of 436 species number, with only approximately 8,000 species described on the basis of morphological 437 variation. While few in number, morphologically defined species of Collembola frequently exhibit 438 unusually broad geographic ranges for species incapable of flight. While 8,000 species is 439 recognised to be an underestimation of true species diversity, a projected estimate of approximately 440 50,000 species after taking into account the probable under sampling of locally endemic species 441 (Hopkin 1997) remains a small number of species when compared to estimates of up to 5,000,000 442 species within the class Insecta (Gaston, 1991). Recent molecular studies raise the possibility that 443 the under-sampling hypothesis is not the only plausible explanation to interpret low species richness 444 within the Collembola, and Emerson et al. (2011) presented evidence supporting a hypothesis of 445 taxonomically and geographically pervasive cryptic species diversity within morphologically 446 defined species. While Emerson et al. (2011) were able to document the widespread occurrence of 447 divergent genetic lineages within morphologically defined species of Collembola, the significance 448 of this diversity in terms of biological species can only be assumed. Cicconardi et al. (2010) were 449 able to directly assess two molecular lineages within one of their seven morphospecies of 450 Lepidocyrtus against the BSC, finding support for their status as biological species, but the 451 remainder of the lineages they described could not be assessed as they were only sampled in 452 allopatry. Our sampling of five morphologically defined species of *Lepidocvrtus* in the Isthmus of 453 Panama recovered 58 well-defined molecular lineages (Fig. 2-3) in only 14 sampling sites. Among 454 these 58 lineages, 34 were sampled in sympatry, among which 19 were sufficiently sampled to 455 allow us to formally test expectations from the BSC concerning LD and HWE. Our analyses 456 revealed unambiguous co-segregation between COII and EF1 α markers in sympatry and the

disruption of HWE. Four sympatric pairs of molecular lineages were sufficiently sampled to
perform pairwise analyses that were in each case consistent with reproductive isolation among
molecular lineages. The pairwise genetic distances among molecular lineages in sympatry were not
significantly different from pairwise distances among the remaining allopatric lineages, suggesting
all 58 molecular lineages are likely to exhibit reproductive isolation.

462

463 Morphospecies diversity vs biological species diversity

464 Molecular sampling of *Lepidocyrtus* within only 39 sites across Panama and the North-Western 465 Mediterranean yields a species richness estimate of more than half that of a morphologically based 466 global species richness estimate for the genus. The genus *Lepidocyrtus* is globally distributed with 467 266 described morphospeices (www.collembola.org). We have ascertained that molecular lineages 468 within morphospecies conform to biological species, and we have characterised 58 such lineages 469 across 14 sites in Panama. A previous investigation of *Lepidocyrtus* in the North-Western 470 Mediterranean (Cicconardi et al. 2010) recovered 87 molecular lineages across 25 sampling sites, 471 yielding an estimated 145 biological species. Rarefaction analysis (Fig 4) reveals that 472 morphospecies diversity has been exhaustively sampled while, in contrast, it is clear that biological 473 species diversity, as estimated by molecular lineages, has only been partially sampled within each 474 region. These results have significant implications for global biological species diversity, both 475 within the genus Lepidocyrtus and within the many other genera of Collembola with molecular 476 signals of cryptic species diversity (Emerson et al. 2011).

477

478 **CONCLUSIONS**

479 Collembola are one of the more ancient groups of arthropods to have colonized the terrestrial

480 environment, with broad environmental tolerances among species appearing to have facilitated their

481 ubiquitous occurrence across a wide range of ecosystems, from dry arctic environments through

482 alpine tundra and deserts to humid tropical rain forests. Their low species diversity and typically

483 broad geographic ranges have been an enigma that has recently been challenged, with the 484 suggestion that morphology may be an inappropriate tool for the delineation of species (Emerson et 485 al. 2011). This is supported by recent molecular studies reporting high levels of DNA sequence 486 divergence within morphologically defined species, but without a demonstrated relationship 487 between this variation and biological species boundaries. We have assessed 58 intra-morphospecies 488 molecular lineages, sampled from within five morphologically defined species of Collembola 489 sampled in the Isthmus of Panama, against the biological species concept, finding that molecular 490 lineages are consistent with biological species. Biological species richness within the class 491 Collembola is clearly underestimated by morphological approaches, with local species richness 492 estimates from molecular lineage sampling indicating the need for intensive sampling to estimate 493 species richness, even at a small local scale. Biological species richness within the class 494 Collembola, and the geographic structure of this diversity, are substantially misrepresented 495 components of terrestrial animal biodiversity. We speculate that global species richness could be at 496 least an order of magnitude greater than a previous estimate of 50,000 species (Hopkin 1997). 497

498

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513	

514 **References**

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F.C. and B.C.E designed the study and wrote the manuscript, F.C. performed sampling, sequencing and analysis.

Data Accessibility:

- DNA sequences: Genbank accessions XXXXXXXXXX

- Final DNA sequence assembly uploaded as online supplemental material

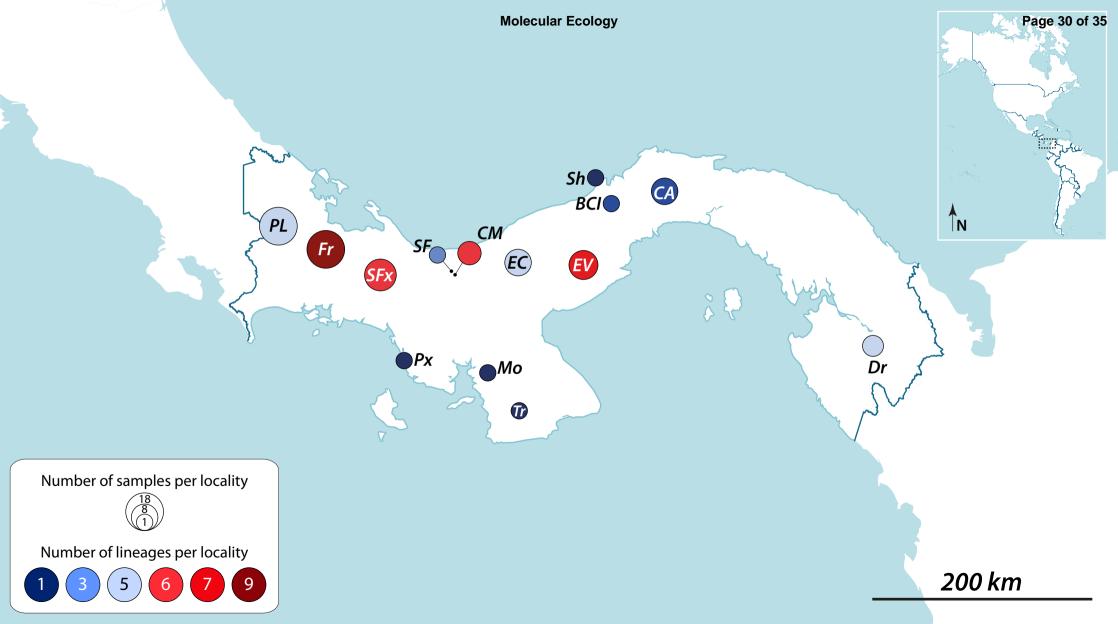
Figure legends

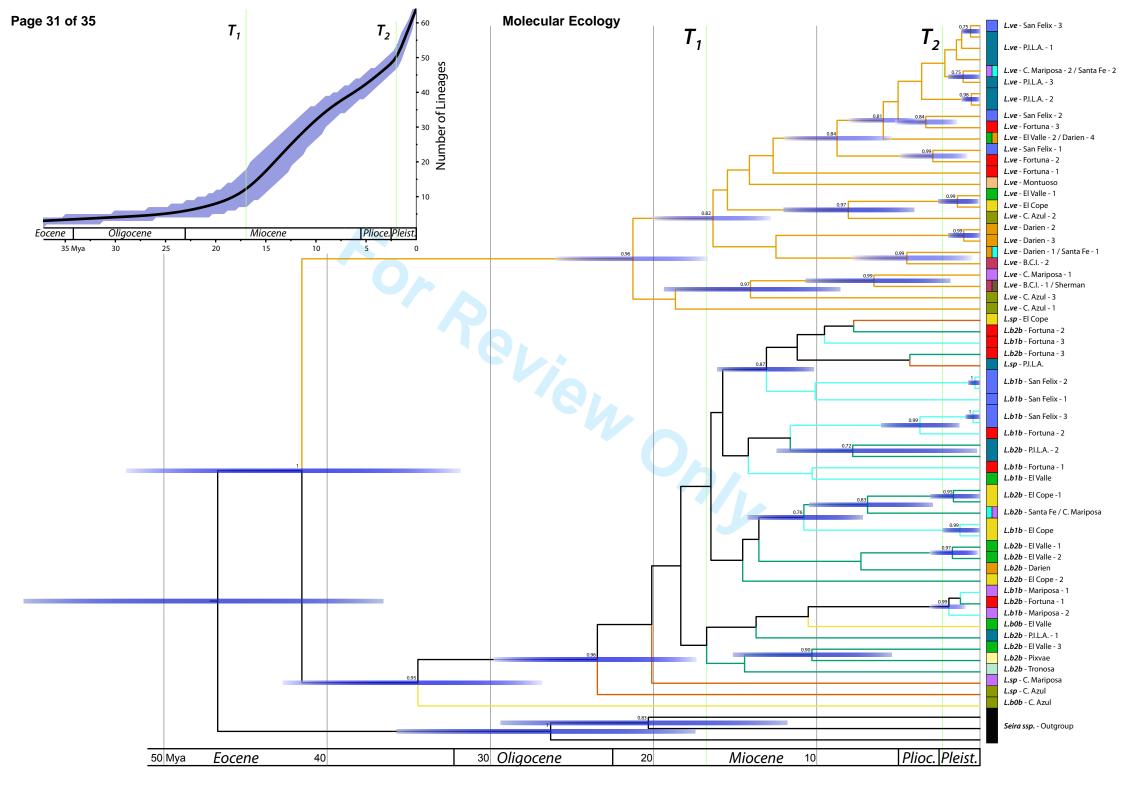
FIGURE 1. Map of sampling sites within the Isthmus of Panama for morphospecies within the genus *Lepidocyrtus*, as coded as in Table 1. Circle size represents the number of sampled specimens, colours indicate the number of mtDNA lineages within each site.

FIGURE 2. Species tree derived from the Bayesian analysis of *COII* and *EF1* α DNA sequence data from morphospecies within the genus *Lepidocyrtus* using *BEAST. Posterior probabilities are indicated above nodes only if higher than 0.70. Branch colors represent morphospecies, while colored boxes represent molecular lineages within each sampling site. The plot in the insert represents lineages through time (LTT). The black line indicates the mean, while the blue area represents the 95% HPD.

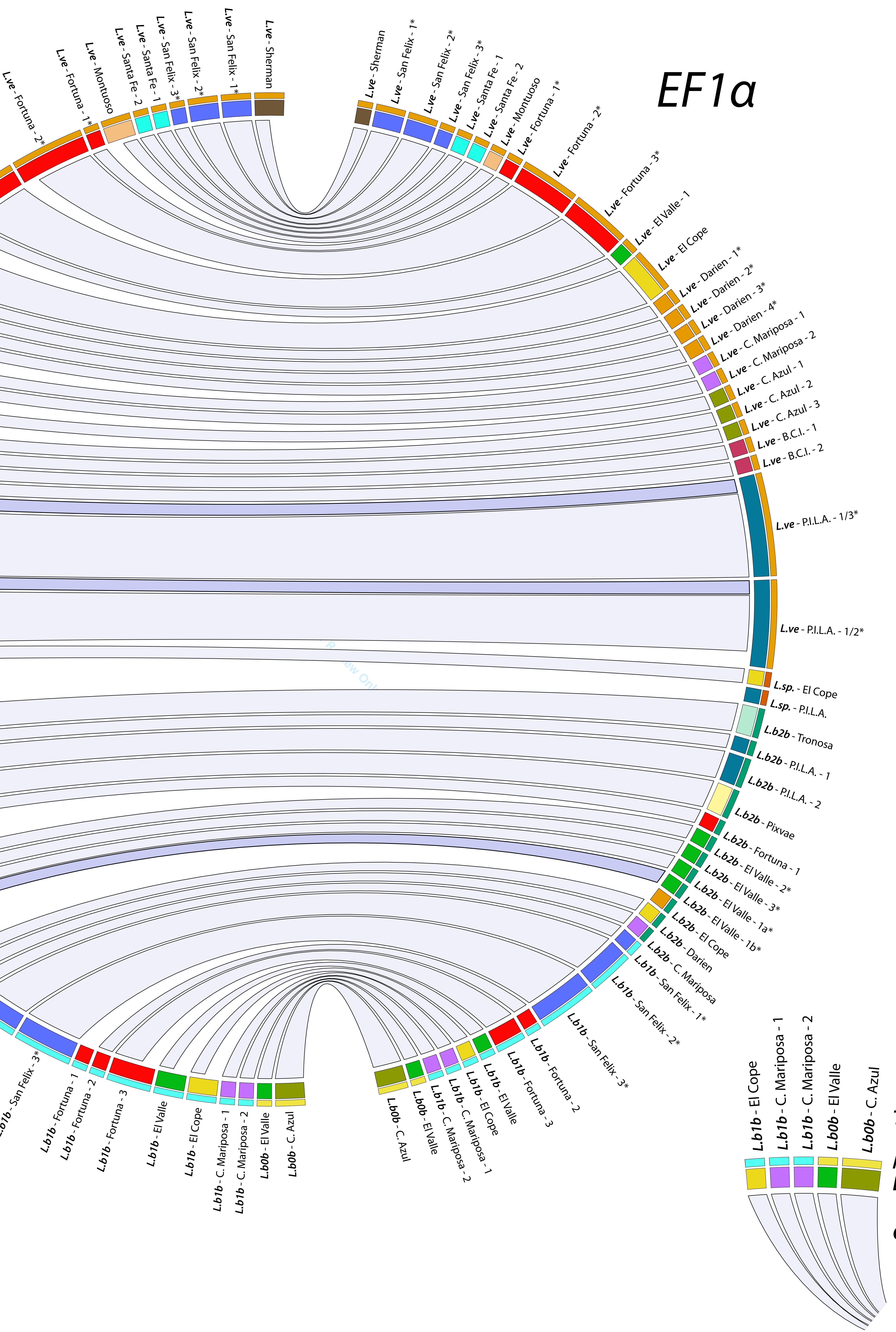
FIGURE 3. Segregation of molecular loci within morphospecies of the genus *Lepidocyrtus*. The correspondence between mitochondrial (left) and nuclear (right) molecular lineages is plotted with Circos. Differently coloured outer rectangles represent different morphospecies, while differently coloured inner rectangles represent different sampling sites. Rectangle width reflects sample size. Internally, light-gray ribbons represent co-segregation of markers, while darker ribbons represent mixed segregation. Tags show species name and sampling site, with numbers differentiating sympatric lineage of the same morphospecies.

FIGURE 4. Rarefaction curves from sampling at the regional scale within the Isthmus of Panama and the northwestern Mediterranean basin for species within the genus *Lepidocyrtus* defined upon the basis of morphology or molecular lineages.





Q/, C/ Ke Oarien Z* · ve Darien 3* * ·ve Darien A* L.ve. C. Mariposa. L.ve. C. Mariposa - 2 L.ve-C. Azul-7 L.ve-C. Azul-2 L.ve-C. Azul-3 L.ve-B.C.1. - 1 L.ve - B.C.I. - 2 L.ve - P.I.L.A. - 3* L.ve - P.I.L.A. - 1* *L.ve* - P.I.L.A. - 2* L.sp. - El Cope L.sp. - C. Mariposa - C. Maripose L.sp. - C. Azul L.b2b - Santa Fe L.b2b - Tronosa L.b2b - P.I.L.A. - 1 L.b2b-P.I.L.A.-2 L.b2b-Pixvae L.b2b-Fortuna-L.b2b-Fortuna-L L.b2b-Fortuna-3-2* r.p3p-ElAgue 1.620 Elvalle 1020 V.

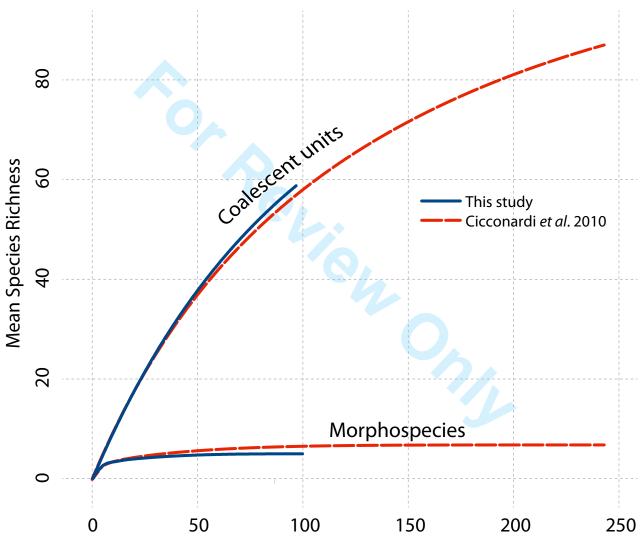


Tag

Morphospecies Locality

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Gene mix



Individuals in Subsample

Table 1 Sampling sites

Table 1. Sampling sites within the Isthmus of Panama for morphospecies within the genus Lepidocyrtus

Code	Location	Province	Latitude	Longitude	Altitude a.s.l.	Morphotype (# of specimen)	# Lineages	Vegetation
BCI	Barro Colorado Island	Panamà	9° 9'5.33"N	79°51'32.47"W	130m	L.ve (2)	2	1-2
CA	Cerro Azul	Panamà	9°14'16"N	79°24'38"W	890m	L.b0b (2); L.sp. (1); L.ve (5)	4	2-1
CM	Cerro Mariposa	Veraguas	8°29'59.80"N	81° 6'53.20"W	1200m	L.b1b (2); L.b2b (1); L.sp. (1); L.ve (3	6	3-1
Dr	Darién	Darién	8° 0'1.86"N	77°42'23.72"W	480m	L.b2b (1); L.ve (4)	5	1-1
EC	El Copé	Coclé	8°39'15"N	80°37'30"W	1180m	L.b1b (2); L.b2b (3); L.sp. (1); L.ve (3	5	2-1
EV	El Valle	Coclé	8°37'27"N	80° 7'6"W	1000	L.b0b (1); L.b1b (2); L.b2b (5); L.ve (2	7	2-1
Sh	Fort Sherman	Colón	9°21'11.98"N	79°57'45.17"W	40m	L.ve (2)	1	1-1
Fo	Fortuna station	Bocas del Toro	8°47'12.50"N	82°13'17.80"W	1230m	L.b1b (5); L.b2b (3); L.ve (10)	9	2-1
Tr	La Tronosa Forest Res.	Los Santos	7°23'52"N	80°38'15"W	560m	L.b2b (2)	1	1-2
Мо	Montuoso Forest Res.	Herrera	7°43'36"N	80°50'54"W	930m	L.ve (2)	1	1-2
PL	P.I.L.A.	Chiriqui	8°53'58.60"N	82°37'11.60"W	2430m	L.b2b (3); <i>L.sp.</i> (1); <i>L.ve</i> (12)	5	4-1
Px	Pixvae	Veraguas	7°52'7"N	81°31'55"W	430m	L.b2b (2)	1	1-2
Sfx	San Felix	Ngöbe-Buglé	8°29'57.80"N	81°46'21.10"W	1690m	L.b1b (8); L.ve (5)	6	3-1
SF	Santa Fe	Veraguas	8°33'36"N	81°10'29"W	570m	L.b2b (1); L.ve (2)	3	2-1
Code 1-1 1-2 2-1 3-1	Low-land tropical evergre Low-land tropical semide Sub-mountain tropical evergre Mountain tropical evergre	ciduous rainfores ergreen broadlea een broadleaf oml	t f ombrophilous i brophilous rainfo	rainforest prest				
4-1	High mountain tropical ev	vergreen broadlea	at ombrophilous	rainforest				

- 1-1 Low-land tropical evergreen broadleaf ombrophilous rainforest
- 1-2 Low-land tropical semideciduous rainforest
- 2-1 Sub-mountain tropical evergreen broadleaf ombrophilous rainforest
- 3-1 Mountain tropical evergreen broadleaf ombrophilous rainforest
- 4-1 High mountain tropical evergreen broadleaf ombrophilous rainforest

Source of variation COII	Variance components	Percentage of variation
Among morphospecies	23.83	28 %
Among population within morphospecies	23.25	28 %
Within morphospecies populations	37.38	44 %
total	84.46	100 %
Among T ₁ lineages	26.80	32 %
Among coalescent units within T_1 lineages	53.56	64 %
Within coalescent units	3.78	4 %
total	84.14	100 %
Among T ₂ lineages	14.37	44 %
Among coalescent units within T_2 lineages	17.46	54 %
Within coalescent units	0.73	2 %
total	32.55	100 %

Table 2. Amova analyses of mtDNA COII and nuclear EF1α sequence data from morphospecies within the genus *Lepidocyrtus*.

Source of variation EF1α	Variance components	Percentage of variation
Among morphospecies	31.14	71 %
Among population within morphospecies	7.05	16 %
Within morphospecies populations	5.78	13 %
total	43.97	100 %
Among T ₁ lineages	29.23	70 %
Among coalescent units within T ₁ lineages	12.08	29 %
Within coalescent units	0.73	2 %
total	42.04	100 %
Among T ₂ lineages	14.37	44 %
Among coalescent units within T ₂ lineages	17.46	54 %
Within coalescent units	0.73	2 %
total	32.55	100 %