Colonization and diversification of aquatic insects on three Macaronesian archipelagos using 59 nuclear loci derived from a draft genome

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Abstract

The study of processes driving diversification requires a fully sampled and well resolved phylogeny. Multilocus approaches to the study of recent diversification provide a powerful means to study the evolutionary process, but their application remains restricted because multiple unlinked loci with suitable variation for phylogenetic or coalescent analysis are not available for most non-model taxa. Here we identify novel, putative single-copy nuclear DNA (nDNA) phylogenetic markers to study the colonization and diversification of an aquatic insect species complex, *Cloeon dipterum* L. 1761 (Ephemeroptera: Baetidae), in Macaronesia. Whole-genome sequencing data from one member of the species complex were used to identify 59 nDNA loci (32,213 base pairs), followed by Sanger sequencing of 29 individuals sampled from 13 islands of three Macaronesian archipelagos. Multispecies coalescent analyses established six putative species. Three island species formed a monophyletic clade, with one species occurring on the Azores, Europe and North America. Ancestral state reconstruction indicated at least two colonization events from the mainland (Canaries, Azores) and one within the archipelago (between Madeira and the Canaries). Random subsets of the 59 loci showed a positive linear relationship between number of loci and node support. In contrast, node support in the multispecies coalescent tree was negatively correlated with mean number of phylogenetically informative sites per locus, suggesting a complex relationship between tree resolution and marker variability. Our approach highlights the value of combining coalescent-based phylogeography, species delimitation, and phylogenetic reconstruction to resolve recent diversification events in an archipelago species complex.

Keywords: *Baetidae, island radiation, multispecies coalescent, phylogeny, phylogeography*

1. Introduction

Any inference about the ecological and evolutionary processes driving diversification requires a well sampled and fully resolved phylogeny upon which traits can be mapped. Molecular phylogenetic studies historically have been limited to a small number of loci. The majority of studies are based largely on mitochondrial DNA (mtDNA) loci (Avise et al., 2000; Garrick et al., 2015) which have the benefit of small population size and high levels of polymorphism but suffer from several characteristics that can limit their suitability to reconstruct the evolutionary process. These include an inability to detect processes that confound gene trees and species trees such as hybridization and introgression, the inference of oversimplified or unresolved evolutionary relationships based on their matrilineal history, underestimated genetic diversity (Zhang and Hewitt 2003), and overestimation of divergence times (Zheng et al., 2011). Another major drawback is the presence of mtDNA genes that have been transposed to the nuclear genome, forming nuclear mitochondrial DNA (Numt; Lopez et al., 1994) which may appear homologous but give very different evolutionary signals from those of the real mtDNA. Phylogenetics has begun to benefit from more widespread use of single-copy nuclear DNA (nDNA) loci, and several recent studies have applied greater numbers of nDNA loci with success at the species (e.g. *Ambystoma tigrinum* (O'Neill et al., 2013); *Triturus cristatus* (Wielstra et al., 2014)), genus (e.g. *Takydromus* (Tseng et al., 2014); *Heliconius* (Kozak et al., 2015)), and higher taxonomic levels (e.g. Plethodontidae (Shen et al., 2016)).

The phylogenetic resolution of closely related taxa enables crucial insights in studies of evolution. In particular, the investigation of recent or ongoing species radiations helps to explain how components such as adaptation and hybridization are involved in the diversification process (e.g. Monaghan et al., 2006; Morvan et al., 2013; Giarla and Esselstyn 2015; Toussaint et al., 2015). A number of model systems in evolutionary biology come from

closely related species groups that have diversified in island archipelagos (Schluter 2000; Gillespie and Roderick 2002, and references therein). Examples include Darwin's finches (Grant and Grant 2008), *Anolis* lizards (Losos and Ricklefs 2009), or Hawaiian spiders (Gillespie et al., 1994). While a robust phylogeny is needed to study diversification and adaptation in such groups, phylogenetic analysis of close relatives can be problematic. Discordance between gene trees and species trees is more likely when speciation is recent and the effective population size of the ancestral population is large relative to the age of the species (Kubatko and Degnan 2007; Degnan et al., 2012). This discordance can arise through hybridization, gene duplication and loss, and incomplete lineage sorting (Maddison 1997; Degnan and Rosenberg 2009; Knowles and Kubatko 2010; Nakhleh 2013). Increasing arrays of methods exist for examining multilocus data that account for these processes (Rannala and Yang 2003; Edwards 2009; Heled and Drummond 2010; Knowles and Kubatko 2010). Unfortunately, the appropriate data for these analyses can be lacking because it is difficult to generate sequence data for a sufficient number of suitable nDNA loci from non-model systems. Most nDNA loci exhibit low levels of polymorphism and therefore many loci are needed, whereas identification of novel nDNA loci that are suitable as phylogenetic markers is generally not straightforward. Here we use a whole-genome draft of a non-model species to develop nDNA markers suitable for phylogenetic reconstruction.

Macaronesia consists of four archipelagos (Azores, Madeira, Canary Islands, and Cape Verde) whose flora and fauna have been used in several studies as model systems for evolutionary research. Their distances to the adjacent continental mainland vary from 110 km (Fuerteventura in Canary Islands to Morocco) to more than 2000 km (Flores in the Azores to Portugal). Several colonization pathways have been identified (Juan et al., 2000; Emerson 2002; Emerson and Kolm 2005), including a single colonization event followed by stepping-stone dispersal (Juan et al., 1997; Emerson and Oromi 2005; Illera et al., 2007; Arnedo et al.,

101 2008; Dimitrov et al., 2008), or multiple independent colonization events within the Canary
102 Islands (Nogales et al., 1998; Ribera et al., 2003a; Díaz-Pérez et al., 2012; Rutschmann et al.,
103 2014; Gohli et al., 2015; Stervander et al., 2015; Faria et al., 2016). While much research has
104 been carried out on island evolution and endemism of terrestrial organisms, comparatively
105 limited information exists for aquatic invertebrates (e.g. Stauder 1995; Drotz 2003; Ribera et
106 al., 2003b, 2003c; Jordal and Hewitt 2004; Hughes and Malmqvist 2005). This is a large
107 discrepancy considering that aquatic insects contribute a disproportionally large amount of
108 global biodiversity despite the relatively small extent of their habitat (Dijkstra et al., 2014).

109 Mayflies are well suited for phylogeographic studies considering their ancient origins (300
110 million years ago (Ma)), global distribution, and limited dispersal ability due to the strict
111 water habitat fidelity of larvae and very short life of the winged adults (Monaghan et al.,
112 2005; Barber-James et al., 2008). Several studies have pointed out their unusual potential for
113 dispersion, reporting mayfly species on remote islands such as the Azores (Brinck and
114 Scherer 1961; Raposeiro et al., 2012), trans-oceanic dispersal between Madagascar and
115 continental Africa (Monaghan et al., 2005; Vuataz et al., 2013), and recent colonization
116 processes of several lineages on the Canary Islands and Madeira ≈ 14 Ma, including a close
117 link to the African mainland (Rutschmann et al., 2014).

118 The species complex of *Cloeon dipterum* L. 1761 is one of the most common and abundant
119 species of freshwater insects in European standing water. The taxonomic classification and
120 phylogenetic relationships within the *C. dipterum* s.l. species complex, including its
121 complicated synonymy, remain largely unknown. The species complex belongs to the
122 subgenus *Cloeon* Leach, 1815. In Europe, *Cloeon* consists of *C. dipterum*, two other currently
123 recognized species (*C. peregrinator* Gattolliat and Sartori, 2008, and *C. saharense* Soldán and
124 Thomas, 1983), and three species with unclear status (*species inquirenda; C. cognatum*
125 Stephens, 1836, *C. inscriptum* Bengtsson, 1914, and *C. rabaudi* Verrier, 1949) that are often

126 considered to be synonyms of *C. dipterum*. Its distribution ranges from North America, across
127 Europe to Northern Asia (excluding China), making it one of the largest known distributions
128 among mayflies (Bauernfeind and Soldán 2012, and references therein). Larvae are found in a
129 variety of aquatic habitats, including natural standing or slow-flowing waters, brackish water,
130 intermittent watercourses, and artificial biotopes across a wide range of climatic zones
131 (Bauernfeind and Soldán 2012, and references therein).

132 For this study we used a draft genome sequence of *Cloeon* to develop 59 nDNA loci
133 suitable for phylogenetic reconstruction of closely related members of the *C. dipterum* s.l.
134 species complex of mayflies. We identified target genes and designed primer pairs for them.
135 Standard PCR and Sanger sequencing were used to generate sequences. We then applied
136 Bayesian phylogenetic inference using concatenated sequence alignments and multispecies-
137 coalescent approaches to delineate species, examine their colonization from the mainland, and
138 understand their diversification throughout Atlantic oceanic islands (Fig. 1, Azores, Madeira,
139 and Canary Islands). Additionally, we quantitatively examined the effect of increasing
140 numbers of nDNA loci on tree resolution. Our analyses show how marker development can
141 proceed efficiently from draft whole genomes and that large numbers of nDNA loci can
142 produce fully resolved trees in closely related taxa, revealing the evolution and diversification
143 of the geographically widespread *C. dipterum* s.l. species complex. The disentangled
144 colonization routes of the three species occurring on the Macaronesian Islands highlight trans-
145 oceanic dispersal abilities of aquatic insects as an important driver of allopatric speciation,
146 including sympatric occurring sister-species on the islands and the mainland.

147 2. Material and methods

148 *2.1 Development of nuclear DNA loci*

149 To develop a set of nuclear loci we sequenced a newly created whole-genome library of *C.*
150 *dipterum* (described in Rutschmann et al., 2016). Libraries were generated from laboratory-
151 reared subimagos of *C. dipterum* specimens (full siblings). DNA was extracted from pooled
152 specimens (five to 20) after removing eyes and wings using the Invisorb® Spin Tissue Mini
153 kit (STRATEC, Berlin, Germany). Extracted DNA was precipitated using Isopropanol and
154 pooled to obtain higher DNA yield. We prepared one 454 shotgun and one 454 paired-end
155 library according to the manufacturer´s guidelines (Rapid Library Preparation Method
156 Manual, GS FLX+ Series - XL+, May 2011; Paired End Library Preparation Method Manual
157 – 20 kb and 8 kb Span, GS FLX Titanium Series, October 2009). Fragments were amplified
158 with an emulsion PCR (emPCR Method Manual - Lib-L SV, GS FLX Titanum Series,
159 October 2009; Rev. Jan 2010). Four lanes per library were sequenced on a Roche (454) GS
160 FLX machine. The sequence reads were trimmed and *de novo* assembled using NEWBLER v.
161 2.5.3 (454 Life Sciences Corporation) with default settings for large datasets. We generated
162 two different assemblies, one using the reads from the shotgun library and one using the reads
163 from both shotgun and paired-end libraries. For ortholog prediction, the newly assembled
164 draft whole genomes were combined with 4,197 expressed sequence tag (EST) sequences
165 from *Baetis* sp. (FN198828–FN203024; Simon et al., 2009). *Cloeon* and *Baetis* belong to the
166 Baetidae subfamilies Cloeoninae and Baetinae. Primer pairs were designed in the conserved
167 regions of orthologous sequences from included taxa. The above analysis procedures have
168 been incorporated into the DISCOMARK pipeline for marker discovery and primer design
169 (Rutschmann at al., 2016; see Supplementary File 2).

170 ***2.2 Taxon sampling and DNA extraction***

171 We sampled individuals of the *C. dipterum* s.l. species complex from larval aquatic
172 habitats at 38 sampling sites on 13 islands including the Azorean archipelago, the Canary
173 Islands, Madeira (Fig. 1), and 32 sampling sites on the European and North American

174 mainland (Supplementary Tables 1 and 2). All samples were preserved in 99% ethanol in the
175 field and stored at 4°C until analysis. DNA was extracted from 107 individuals using
176 NucleoSpin® 96 tissue kits (Macherey-Nagel, Düren, Germany). Our analysis included
177 multiple populations of all currently recognized taxa (based on both morphological and
178 molecular data) on the islands (Brinck and Scherer 1961; Gattolliat et al., 2008; Rutschmann
179 et al., 2014).

180 ***2.3 PCR amplification, sequence alignment, and sequence heterogeneity***

181 We sequenced 60 loci for the study: the mtDNA barcoding gene (*cox1*) and 59 newly
182 developed nDNA markers from protein-coding gene regions. The *cox1* locus was amplified
183 and sequenced using the procedure described by Rutschmann et al., 2014. Based on a general
184 mixed Yule-coalescent (gmyc) model analysis (Fujisawa and Barraclough 2013) of *cox1*, we
185 selected a representative set of 29 individuals, including a set of one to nine individuals for
186 each gmyc species, depending on the number of locations at which a given gmyc species was
187 found (see 2.4 Species assignment and population structure analysis). For these individuals,
188 we obtained nDNA sequences using the 59 newly designed primer pairs (Supplementary
189 Table 3). Nuclear loci were amplified using standard polymerase chain reaction (PCR)
190 protocols with an annealing temperature of 55°C. The PCR products were custom purified
191 and sequenced at Beckman Coulter Genomics (Essex, UK) or Macrogen (Amsterdam, The
192 Netherlands). Forward and reverse sequences were assembled and edited using GENEIOUS R7
193 v.7.1.3 (Biomatters Ltd.). Length variation (i.e. heterozygous indels) was decoded using
194 CODONCODE ALIGNER v.3.5.6 (CodonCode Corporation, Centerville MA, USA).
195 Additionally, we included published sequences of six nDNA loci from four individuals
196 (KU971838-KU971840, KU971851, KU971919-KU971921, KU971933, KU972490-
197 KU972492, KU972503, KU972568-KU972570, KU972583, KU972653-KU972654,
198 KU972666, KU973060-KU973062, KU973074; Rutschmann et al., 2016).

199 Multiple sequence alignments were made for each locus using MAFFT v.7.050b (L-INS-I
200 algorithm with default settings; Katoh and Standley 2013). The predicted orthologous
201 sequences of *Baetis* sp. were used to check the correct exon-intron splicing boundaries
202 (canonical and non-canonical splice site pairs) of each alignment. Exon-intron boundaries of
203 locus 411912 could not be fully reconstructed and thus we used the exon sequence predicted
204 from tblastx searches for subsequent analyses. Locus alignments were split into coding and
205 non-coding parts using a custom script
206 (https://github.com/srutschmann/python_scripts/blob/master/extract_introns.py). All coding
207 alignments were checked for indels and stop codons using MESQUITE v.2.75 (Maddison and
208 Maddison 2011). Genotypes of the coding alignments were phased using the probabilistic
209 Bayesian algorithm implemented in PHASE v.2.1.1 (Stephens et al., 2001; Stephens and
210 Donnelly 2003) with a cutoff value of 0.6 (Harrigan et al., 2008; Garrick et al., 2010).
211 Multiple runs were performed for each alignment and phase calls checked for consistency.
212 Input and output files were formatted using the scripts from SEQPHASE (Flot 2010).
213 Heterozygous sites that could not be resolved were coded using ambiguity codes and
214 remained in the data set for subsequent sequence analyses. All alignments were re-aligned
215 after phasing with MAFFT. As an outgroup we used *Baetis* sp.. The number of variable sites,
216 informative sites, and Tajima´s D for each locus were determined using a custon script and
217 the package DENDROPY (Sukumaran and Holder 2010;
218 https://github.com/srutschmann/python_scripts/blob/master/alignment_stats.py) and a custom
219 script.

220 ***2.4 Species assignment and population structure analysis***

221 Most analyses that use phylogenetic or multilocus species tree approaches require *a priori*
222 species assignment. Because of the partly unknown and largely incomplete taxonomy of the
223 group, we used two approaches to first assign the 29 *C. dipterum* individuals to putative

224 species. The first was a gmyc analysis (Fujisawa and Barraclough 2013) of mitochondrial
225 *coxI* and the second was a Bayesian clustering algorithm using all nuclear loci to assign
226 individuals to 'populations' (STRUCTURE, Pritchard et al., 2000; Falush et al., 2003). The
227 gmyc approach was carried out using *cox1* from 147 specimens that included all newly
228 sequenced *Cloeon* individuals, published sequences that were available as of February 2016
229 (Supplementary Table 2), six newly sequenced individuals of *C. simile* Eaton, 1870, and
230 *Baetis rhodani* (KF438126) as an outgroup. The analysis followed that of Rutschmann et al.,
231 (2014) except that we used BEAST v.2.3.2 (Bouckaert et al., 2014) and the first two codon
232 positions were modeled with HKY + I while the third codon position was modeled with HKY
233 + Γ. For the Bayesian clustering we used the exon_all_data matrix (see 2.5 Phylogenetic and
234 species tree reconstructions). We assumed 1-10 genotypic clusters (K) and ran nine replicate
235 analyses for each K, using 10^6 MCMC generations with a burn-in of 10%. All individuals
236 were assigned probabilistically without *a priori* knowledge to genetic clusters. We applied an
237 admixture model with default settings (Supplementary File 3).

238 ***2.5 Phylogenetic and species tree reconstructions***

239 We prepared three data matrices (Table 1), contianing all nDNA sequences (all_data), all
240 coding genotypes (exon_all_data), and all coding haplotypes (exonhap_all_data). Because
241 data matrices were not 100% complete (see 3.1 Development of nuclear DNA loci) we
242 compiled a second set of matrices that were 100% complete using only the 17 loci that were
243 successfully sequenced in all 29 individuals (complete_matrix, exon_complete_matrix,
244 exonhap_complete_matrix). All individual locus alignments were concatenated using a
245 Python script (https://github.com/srutschmann/python_scripts/blob/master/fasta_concat.py).
246 We used two partitioning schemes for the phylogenetic analysis. One used the most
247 appropriate substitution model for each locus ("partition_locus") according to a Bayesian
248 Information Criterion using the program JMODELTEST v.2.1.5 (Guindon and Gascuel 2003;

249 Darriba et al., 2012) (Supplementary Table 3). The other used the best-fit partitioning scheme
250 identified by PARTITIONFINDER v.2 (https://github.com/brettc/partitionfinder)
251 ("partition_whole"). The latter was determined using the greedy algorithm (Lanfear et al.,
252 2012), whereby PHYML (Guindon et al., 2010) was used for model evaluation prior to the
253 Bayesian analysis, and RAXML v.8.2.8 (Stamatakis 2014) was used for model evaluation
254 prior to maximum likelihood analysis (see 2.7 Relationship between node support and number
255 of loci).

256 Bayesian phylogenetic analysis was carried out on each partitioning scheme using
257 MRBAYES v.3.2.3 (Ronquist et al., 2012) using exons, one analysis using all data and one
258 using only the complete matrix (Table 1; exon_all_data, exon_complete_matrix). We
259 unlinked the nucleotide frequencies, gamma distributions, substitution rates and the
260 proportion of invariant sites across partitions. Each run consisted of two independent analyses
261 of four MCMC chains, each with 10^7 generations and 25% burn-in.

262 Species tree reconstructions were carried out under a multispecies coalescent framework
263 (Drummond and Rambaut 2007; Heled and Drummond 2010) as implemented in the program
264 *BEAST v.2.1.3 (Bouckaert et al., 2014). All analyses were performed using exons, one
265 analysis using all data and one using only the complete matrix (Table 1; exonhap_all_data,
266 exonhap_complete_matrix). All individuals were *a priori* assigned to species based on the
267 gmyc and Bayesian clustering analyses (see 2.4 Species assignment and population structure
268 analysis). In the Bayesian clustering analysis, one individual sampled from Russia was
269 considered to be admixed based on Bayesian posterior probability (PP) assignment values >
270 0.05 for more than one cluster (Supplementary File 3, Supplementary Fig. 1, Supplementary
271 Table 5). This individual was therefore excluded from further analysis. We used a relaxed
272 uncorrelated lognormal clock for gene tree estimation at each locus and a Yule speciation-
273 process prior. Six independent runs of 8×10^8 million generations each were conducted. Runs

274 were combined in LogCombiner v.2.1.3 (Bouckaert et al., 2014), whereby all parameters
275 reached effective sample sizes (ESS) > 600. Maximum clade credibility trees for each species
276 tree were obtained using TreeAnnotator v.2.1.3 (Bouckaert et al., 2014).

277 ***2.6 Ancestral state reconstruction***

278 Ancestral state reconstruction was used to test the geographical direction of the radiation
279 (i.e. Continental to Island or Island to Continental). Ancestral range patterns of each
280 individual were defined into four geographic areas: (1) a broadly defined Continental
281 referring to the European and North American mainland, (2) Canary Islands, (3) Madeira, and
282 (4) Azores. As input tree, we used the concatenated tree based on the exon_all_data inferred
283 with MrBayes. A chronogram was fit to the tree using the chronos function in the ape v.3.4
284 (Paradis et al., 2004) package for R. Ancestral states were estimated under an equal-rates
285 (ER) model using the function ace, and the scaled likelihoods of each ancestral state were
286 calculated using the function lik.anc. An MCMC approach was used to sample character
287 histories from their PP distribution generating 1,000 stochastic character maps with the
288 function make.simmap of the phytools v.0.4.98 (Revell 2012) package in R (R Core Team,
289 2016; Supplementary File 4).

290 ***2.7 Relationship between node support and number of loci***

291 To investigate how the number of analyzed loci affected node support values, we
292 performed phylogenetic reconstructions based on multiple subsets of randomly selected loci
293 (Supplementary Table 4). This included twelve subsets for the phylogenetic analysis and four
294 subsets for the multispecies coalescent analysis. For the Bayesian phylogenetic analysis of
295 subsets of loci we used MrBayes (see 2.5 Phylogenetic and species tree reconstructions),
296 applying both partition schemes. As an alternative to Bayesian PP, we calculated Shimodaira-
297 Hasegawa approximate likelihood ratio test (SH-aLRT; Guindon et al., 2010) supports of

298 each node using maximum likelihood phylogenies. For this, we first estimated the best tree
299 with RAxML v.8.2.9 applying the "partition_whole" partitioning scheme (Supplementary
300 Table 4) and rapid bootstrap analysis with 1,000 replicates. Linear regressions were used to
301 model the number of supported nodes for PP ≥ 0.95 and PP = 1, and the SH-aLRT supports ≥
302 0.95 and SH-aLRT supports = 1 as a function of the number of loci used in the analysis. The
303 Pearson correlation between the number of loci and number of supported nodes was
304 calculated using the stats package for R.

305 The multispecies coalescent tree reconstructions were performed and summarized as
306 described in section 2.5. We calculated the correlation between the mean number of
307 parsimony-informative sites per locus and the mean node support values of the randomly
308 selected loci and the resulting tree reconstruction using the cor.test function of the stats
309 package for R.

310 3. Results

311 *3.1 Development of nuclear DNA loci*

312 Whole-genome sequencing resulted in 1,109,684 raw reads, including 651,306 reads for
313 the shotgun library and 458,378 reads for the paired-end library, with an average large contig
314 length of 1,187 and 736 base pairs (bp), respectively (BioSample SAMN03202660,
315 BioProject PRJNA268073, Sequence Read Archive SRP050093). All reads were assembled
316 into 68,473 contigs with an N50 of 1,116 bp. The reads of the shotgun library were assembled
317 into 31,827 contigs with an N50 of 1,260 bp. We detected 918 putative orthologous gene
318 sequences for *C. dipterum* from the contigs derived from the shotgun library, 1,298 putative
319 orthologous gene sequences from the contigs of the combined assembly, and 416 for *Baetis*
320 sp. (Supplementary Table 6). From these, we haphazardly selected 65 markers for primer
321 design, approximately 80% of which included orthologous sequences from both taxa. These

322 were chosen based on the presence of conserved regions and short introns suitable for primer
323 design. Based on preliminary laboratory testing, 59 markers were selected that amplified
324 consistently and had similar annealing temperatures in order to simplify the large number of
325 PCR reactions (Supplementary Table 3).

326 Total fragment length per sequenced locus ranged from 210 - 1,007 bp with a mean of 545
327 bp. Exon sequence length ranged from 210 - 710 bp with a mean of 410 bp (Supplementary
328 Table 3) (KF438124-KF438125, KU757080-KU757184, and KU971616-KU973191). The
329 full data matrix of all 29 individuals and all 59 loci including exons and introns (all_data) was
330 32,213 bp in length when concatenated and when introns were removed (exon_all_data) it
331 was 24,168 bp (Table 1). All individuals were successfully sequenced for at least 44 loci, and
332 the above matrices were >75% complete. The 100% complete matrix included 17 loci that
333 were sequenced successfully for all 29 individuals. All heterozygous indels were located in
334 the intron sequences. However, 100 heterozygous sites could not been resolved and remained
335 in the exonhap alignments.

336 The number of variable sites per locus ranged from six to 65 (mean: 18.95). In the
337 exon_all_data matrix, there was one SNP per every 21.62 nucleotides sequenced (i.e. total
338 length per total number of variable sites). The loci included between six and 54 informative
339 sites (mean: 16) and one to 26 ambiguous sites (mean: 8.4). Nucleotide diversity ranged from
340 0.007 to 0.04 (mean: 0.017), and Tajima´s D varied between -0.85 and 1.97 (mean: 0.29)
341 (Supplementary Table 7).

342 ***3.2 Species assignment and population structure***

343 There were 62 unique *cox1* haplotypes of *Cloeon* and the gmyc model was a significantly
344 better fit to the data than the null model ($\chi^2 = 31.00$, $p < 0.001$). Seven putative species were
345 delineated within *C. dipterum* s.l. (Fig. 2a): One occurred only in Asia (South Korea) while

346 the remaining six comprised three species with distributions that included the Macaronesian
347 Islands (IS1, IS2, IS3) and three species only occurring on the European and North American
348 continents (CT1, CT2, CT3). The population assignments from the Bayesian clustering
349 analyses of nDNA agreed with the results from the gmyc analysis for the six species of
350 interest (Supplementary File 3, Supplementary Fig. 1, and Supplementary Table 5). No
351 nuclear data were available for the seventh gmyc species from Asia so it was not included in
352 the clustering analysis. Among the six, one widespread species (IS1) was found on all
353 Azorean islands, in Greece and Italy, and in North America; one was found on the Canary
354 Islands and Madeira (IS2), and one was found only on four Canarian islands (IS3). The gmyc
355 model delineated all seven *C. dipterum* species even when using the most conservative
356 estimate (95% confidence interval based on two log likelihood units: 16-19 gmyc species).
357 The two *C. cognatum* specimens from the North American DNA barcoding project (Webb et
358 al., 2012) had *cox1* haplotypes identical to our gmyc species IS1.

359 ***3.3 Phylogenetic reconstruction***

360 Analyses based on both exon matrices (exon_all_data; exon_complete_matrix) and both
361 partition schemes recovered the same tree topology with strong node support, resolving each
362 of the three species occurring on Macaronesia (IS1-IS3) as monophyletic and members of a
363 monophyletic 'Island clade' (Fig. 3). The geographically widespread species IS1 was sister
364 taxon to the two others. Species CT2 and CT3 were both monophyletic and sister group to the
365 Island clade (Fig. 3). All individuals in CT1 were monophyletic except for a single individual
366 that was sister taxon to the entire *C. dipterum* s.l. lineage. There were 27 resolved (PP $\geq$ 0.95)
367 nodes in the tree resulting from the large, >75% complete matrix with all available data
368 (exon_all_data, 59 loci). The only unresolved node was between the two Azorean individuals
369 (Fig. 3). In contrast, the tree resulting from the smaller matrix with no missing data

370 (exon_complete_matrix, 17 loci) contained only 19 resolved nodes, with lack of resolution
371 most pronounced in IS2 (Supplementary Fig. 2).

372 All species tree phylogenies had identical topologies and these matched the Bayesian
373 phylogenies (Figs. 2b, 3a) in that Island and Continental clades were both monophyletic, with
374 IS1 sister taxon to IS2 + IS3, and with CT1 sister taxon to CT2 + CT3. Using the
375 exon_complete_matrix, all nodes were highly supported (PP ≥ 0.99; Table 2). All individuals
376 clustered into six species in the same way in both the multilocus nDNA tree and the single-
377 locus (*cox1*) mtDNA tree (PP = 1), but the relationships among the species were different.
378 The mtDNA tree did not support the sister relationship of IS2 + IS3 or the monophyly of the
379 Continental clade (Fig. 2a; Table 2).

380 The analysis of subsets of loci showed a positive relationship between the number of loci
381 employed and the number of nodes resolved for phylogenetic analyses (both Bayesian and
382 maximum likelihood) (Fig. 4). The results for the multispecies coalescent were not as clear.
383 Support of key nodes varied widely with the number of loci employed (Fig. 2b, Table 2). The
384 highest overall support came from analysis of 17 and 40 loci, although only the analysis using
385 20 loci failed to recover either node in the Macaronesian clade and resulted in no resolution
386 other than continental monophyly (Fig. 2b, Table 2). There was a strong negative correlation
387 (Pearson R = -0.95, $p < 0.05$) between the mean number of informative sites per locus and
388 mean node support (Supplementary Tables 4, 7).

389 ***3.4 Ancestral state reconstruction***

390 The ancestral state reconstruction identified four nodes having marginal states with less
391 than 0.9 PP for one character, including the sister relationship between the individual
392 CH010_SR21B07 and the remaining species, the ancestral node of IS2 + IS3 (Canary Islands
393 and Madeira), and the nodes separating Madeiran from Canarian individuals within IS2 (Fig.
394 5). The Island clade had a continental origin, further a Canarian origin was estimated for IS2

395 + IS3. The clade IS2 was estimated to have an ancestral state of 0.59 for Madeira and 0.4 for
396 the Canary Islands (Supplementary File 4).

397 # 4. Discussion

398 ### *4.1 Species delineation*

399 The agreement of the multispecies coalescent and mitochondrial gmyc approaches for *a*
400 *priori* species delineation support the use of *cox1* as barcoding gene for the taxa studied (e.g.
401 Lucentini et al., 2011; Pereira-da-Conceicoa et al., 2012; Webb et al., 2012; Rutschmann et
402 al., 2014; Gattolliat et al., 2015). Nonetheless, the gene tree based on *cox1* did not resolve the
403 relationships among the six closely related *C. dipterum* s.l., with no support for the sister
404 relationship of IS2 + IS3 or the monophyly of the Continental clade. The distant clustering of
405 one individual (CH010_SR21B07) in the concatenated tree analyses might be explained by
406 incomplete lineage sorting since the species tree inferences using *BEAST did result in a
407 clear clustering of CT1 with low frequency of different topology (Fig. 3). Moreover, when
408 incomplete lineage sorting is present, standard methods for estimating species trees, such as
409 concatenation and consensus methods, can be statistically inconsistent (Degnan et al., 2009;
410 Roch and Steel 2014), and produce highly supported but incorrect trees (Kubatko and Degnan
411 2007). The majority of gene trees could support an incorrect species tree if the phylogeny is in
412 the anomaly zone (Degnan and Rosenberg 2006). Here this does not appear to be the case,
413 because one would expect the concatenation and coalescent approach to support different
414 topologies (Kubatko and Degnan 2007; Liu and Edwards 2009).

415 ### *4.2 Species diversity*

416 The use of nDNA and geographically extensive sampling uncovered a largely
417 underestimated species diversity for *C. dipterum* s.l. species complex, supporting the

418 existence of six geographically relevant species from our study (with a seventh in Asia).
419 Recent evidence from the study of other mayfly species found fine-scale ecological
420 differences among cryptic species detected with molecular methods (Leys et al., 2016;
421 Macher et al., 2016), lending support to the ecological and evolutionary significance of these
422 and other DNA-based findings. Another widespread species, *Baetis harrisoni*, was also found
423 to consist of several cryptic species (Pereira-da-Conceicoa et al., 2012). In light of the
424 unusually broad ecological tolerance (among mayflies) observed for *C. dipterum*, we also
425 conclude that the lineage clearly consists of multiple independent species, as has been
426 recognized by morphological taxonomy for some of the members (e.g. *C. peregrinator*). All
427 of our analyses grouped the two specimens of *C. peregrinator* from Madeira with individuals
428 from several Canary Islands into species IS2. Gattolliat et al., (2008) described *C.*
429 *peregrinator* as an endemic Madeiran species based on morphological characters and support
430 from mtDNA cytochrome-oxidase *b* sequences. At the time, there were no nDNA sequences
431 of Canarian *C. dipterum* s.l. specimens available. Rutschmann et al., (2014) assigned all
432 Madeiran *Cloeon* individuals to *C. peregrinator* for their mtDNA phylogeny, but the
433 specimens were not included in their gmyc analysis because there were no *cox1* sequences
434 available. Based on our findings here, there is no endemic *Cloeon* species on Madeira.

435 The focus of our study was Macaronesia and therefore nDNA results are only applicable to
436 these taxa, but the mtDNA gene tree provides evidence for broad cryptic diversity within the
437 subfamily Cloeoninae. *Cloeon simile* included two geographically widespread European
438 gmyc species, and *C. smaeleni* Lestage 1924 was two gmyc species, one with Saudi Arabian
439 and one with Afrotropical distribution. The species *C. praetexum* was clearly distinct from all
440 other examined European specimens, which was surprising because it is thought to belong to
441 *C. simile* s.l.. The two specimens of *C. cognatum*, which is thought to be a junior synonym of
442 *C. dipterum* by some authors, were nested within the IS1 clade. All of the above findings

443 must be considered preliminary because they are based on mtDNA, although we note that
444 mtDNA and nDNA markers agreed in all of the *Cloeon* species that were directly compared.
445 Further studies on these other *Cloeon* taxa with nuclear markers, using morphological
446 characteristics, and including comparisons with previously described species that are now
447 considered junior synonyms or *species inquirenda* would be a valuable complement to the
448 work presented here.

449 ***4.3 Evolution, colonization, and diversification***

450 For the species occurring in the Macaronesian region, one species appeared widely
451 distributed on all Canary Islands and Madeira (IS2), one species was found only on the
452 western group of the Canary Islands (IS3), and one species was found on five islands of the
453 Azores, as well as in Italy, Greece and North America (IS1). The short branches of
454 individuals from IS1 support very recent or perhaps ongoing gene flow. Other studies have
455 found evidence for recent or ongoing dispersal in *Cloeon* (e.g., Monaghan et al., 2005)
456 including a recent introduction of African *Cloeon* to South America (*C. smaeleni*, Salles et
457 al., 2014). This long-distance dispersal ability is probably at least partly related to their
458 reproductive flexibility including ovovivipary and their ability to survive in anthropogenic
459 habitats. Our ancestral state reconstruction indicated that IS2 may have first colonized
460 Madeira and then the Canaries from west to east. Colonization routes between these two
461 archipelagos have been suggested for several taxa (Emerson et al., 2000a; Emerson et al.,
462 2000b; Trusty et al., 2005; Illera et al., 2007; Dimitrov et al., 2008; Amorim et al., 2012). The
463 species IS3 seems not to have reached La Palma and the two most eastern Canarian islands of
464 Fuerteventura and Lanzarote. The dispersal of IS3 appears to have followed the progression
465 rule, in which older islands are inhabited by older clades, which is further supported by
466 stepping-stone dispersal along an east-western gradient.

467 Our data confirm at least three and possibly four independent colonization events of the
468 islands studied, with a European origin for the Macaronesian *C. dipterum* s.l.. However, long
469 branches between the Continental clades and the Island clade suggest there may be missing
470 intermediates. These may occur in the Iberian Peninsula or North Africa. Several studies have
471 proposed a North African origin for both the Canarian and Madeiran fauna (Brunton and
472 Hurst 1998; Kvist et al., 2005; Weingartner et al., 2006; Gohli et al., 2015; Stervander et al.,
473 2015). The Continental clades are also distantly related to one another and the long branches
474 within both clades, compared to the Island clades, suggest there may be additional European
475 species that are not included here.

476 Our results suggest a strong effect of different habitat preferences between the two
477 Canarian species, which might affect their colonization success. Although our dataset was not
478 quantitative, we observed that species IS3 generally occurred on islands with more potential
479 habitats in comparison to IS2, which seems to have better dispersal abilities and might
480 therefore be able to more successfully colonize islands with very little water occurrence. This
481 pattern may be linked with the occurrence of suitable water habitats on the Canarian Islands.
482 The four islands of Gran Canaria, Tenerife, La Gomera, and La Palma all have permanent
483 natural water sources, and the island of El Hierro has several artificial water habitats due to
484 the mostly temperate climatic conditions. In contrast, there are only a few habitats on
485 Fuerteventura and Lanzarote due to the arid climatic conditions. The effect of habitat use on
486 species richness has been shown for aquatic beetles (Ribera et al., 2003c), with running water
487 bodies generally containing more species than standing ones. This pattern also applies to the
488 Macaronesian mayflies. The genus *Baetis* occurs in running waters and is species-rich,
489 including eight island endemic species on five islands of Madeira and the Canary Islands
490 (Rutschmann et al., 2014). In contrast, the genus *Cloeon* comprises three species, none of
491 which are restricted to a single island. The impact of agriculture and tourism on natural

492 habitats (Malmqvist et al., 1995; Nilsson et al., 1998) has clearly threatened the occurrence of
493 species living in running water habitats (*Baetis canariensis* and *B. pseudorhodani*,
494 Rutschmann et al., 2014), but it may have had less of an effect on *C. dipterum*. The records of
495 mayflies from El Hierro indicate a recent anthropogenic import of the species, moreover
496 because it is the youngest island of the Canarian archipelago and its remote geographical
497 position.

498 Interestingly, there were eight sampling sites (out of 32 examined sites, i.e. 25%) in which
499 both species IS2 and IS3 occurred sympatrically. Four of these localities were natural
500 habitats. However, more work needs to be done to make quantitative assessments on species
501 occurrence and local abundance of the two distinct species occurring on the same habitats. A
502 wider geographic sampling, focusing on specimens from the European mainland and North
503 Africa will be needed to clarify the origin and distribution of the *C. dipterum* s.l. species
504 complex. We expect to find more individuals from distinct geographic localities belonging to
505 the species IS1, since this species appears to exhibit trans-oceanic dispersal abilities.

506 ***4.4 Number of loci for phylogenetics***

507 A recent study by O'Neill et al., (2013) examined how multilocus species tree inferences
508 varied with differing numbers of loci. Their analysis based on the 20 and 30 most informative
509 loci (using a parsimony criterion) in their data set resulted in high PPs, whereas node support
510 values were lower and likelihoods failed to converge when loci that were less informative
511 were added to the analysis. They concluded this was the result of the increasing number of
512 parameters while adding loci with decreasing levels of information. Our results are not
513 directly comparable to those of O'Neill et al. (2013) for the species tree reconstruction,
514 because we did not explicitly order loci by parsimony-informative sites in our tests.
515 Nonetheless, we found a strong negative correlation between the mean number of informative
516 sites per locus and mean node support in the coalescent species tree. This suggests that the

517 number of informative sites was not able to explain variation in support alone, and that
518 multiple characteristics of individual loci play an important role in whether or not analyses
519 achieve convergence and tree resolution. For the species phylogeny, we found a positive
520 linear correlation between number of loci and node support. This was despite the larger
521 number of parameters. In our study we observed that the reduction in node support when
522 using a reduced set of loci (exon_complete_matrix *vs.* exon_all_data, see 2.3 PCR
523 amplification, sequence alignment, and sequence heterogeneity) primarily affected the most
524 derived clade (IS2), which highlights the importance of large nDNA marker sets for the
525 reconstruction of shallow phylogenies.

526 **5. Conclusion**

527 Our aims were to delineate the species boundaries within the *C. dipterum* species complex
528 and place these lineages within a phylogenetic framework, in order to better understand their
529 evolution on the Macaronesian Islands. Robust phylogenetic reconstruction of such closely
530 related species can be challenging, but is a necessary step in the understanding of
531 evolutionary processes of diversification and adaptation. Most of the widely used nDNA loci
532 (e.g. rRNA) do not exhibit suitable polymorphism, resulting in a large dependence on mtDNA
533 for phylogenetics (Garrick et al., 2015). A distinct advantage of using multiple nDNA loci
534 comes from the advent of multilocus species tree reconstruction methods. These are important
535 tools in the reconstruction of relationships between close relatives, which is often intractable
536 based on single-locus (i.e. mtDNA) data. The difficulty in developing large numbers of
537 nDNA loci remains one of the primary reasons that there are few model systems available for
538 detailed studies of speciation and diversification processes. This is particularly true for
539 freshwater insects, despite their overwhelming contribution to global biodiversity (Dijkstra et
540 al., 2014). Here we developed a large set of nDNA loci using draft whole-genome
541 sequencing. All of the procedures we used have since been incorporated into a single analysis

542 pipeline (Rutschmann et al., 2016). Our results show that even for taxa with very limited
543 available genomic resources, it is possible to develop sets of nuclear loci that produce fully
544 resolved and supported coalescent-based species trees and species-level phylogenetic trees.
545 Using these results, we were able to infer species boundaries within the largely cryptic *C.*
546 *dipterum* s.l. species complex and reconstruct the diversification and island colonization
547 history of these species with confidence.

Author contributions

S.R., M.S. and M.T.M. conceived the study. S.R., H.D., D.H.F., J.-L.G., S.J.H., P.M.R., and M.S. collected and identified specimens. H.D., S.S., and R.D. contributed analytical tools. S.R. and M.T.M. performed and interpreted the analyses, and wrote the manuscript. All authors commented and approved the final manuscript.

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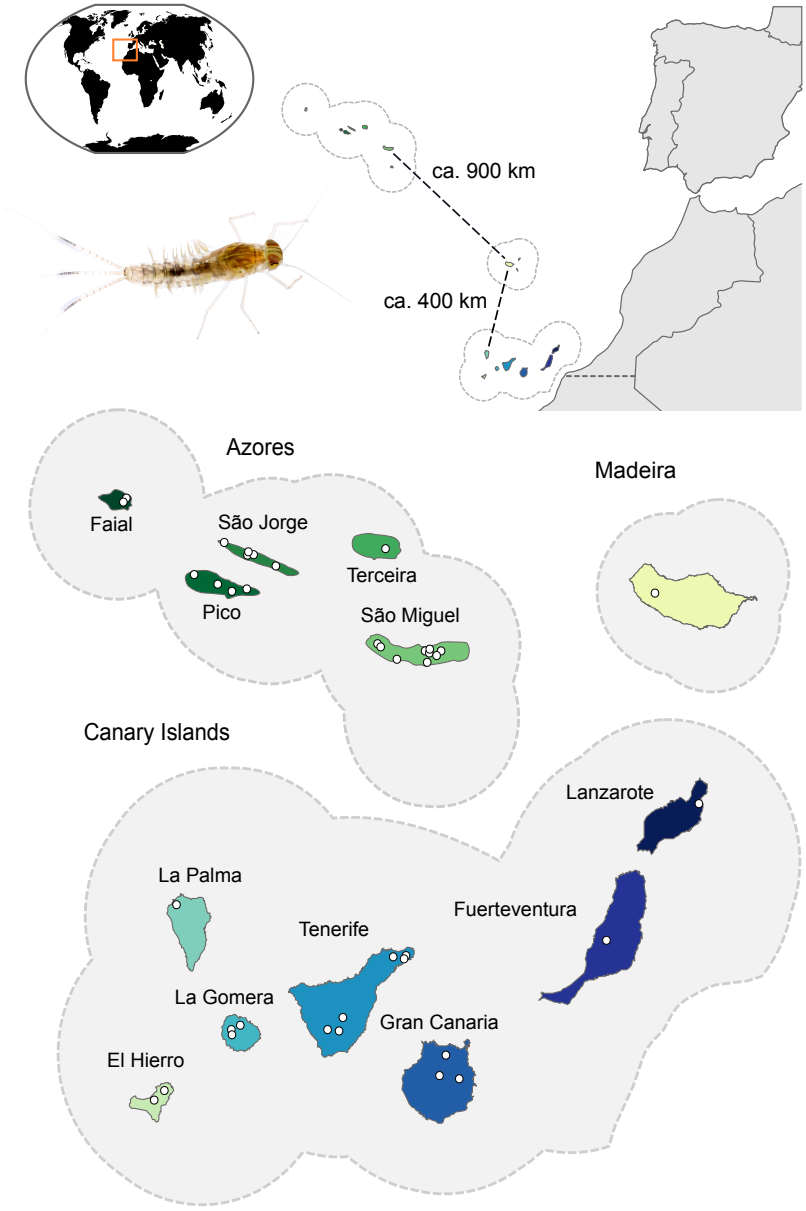
847 **Figure legends**



848

849 **Fig. 1.** Overview of the sampling localities in the Macaronesian region. The three
850 archipelagos of the Azores, Madeira, and the Canary Islands are shown in detail, whereby the
851 38 sampling sites are indicated by white dots. For the Azores and Madeira only islands with
852 sampling sites are shown in the detailed view. Photo of *Cloeon dipterum* s.l. larvae by
853 Amanda44/CC BY 3.0.

854

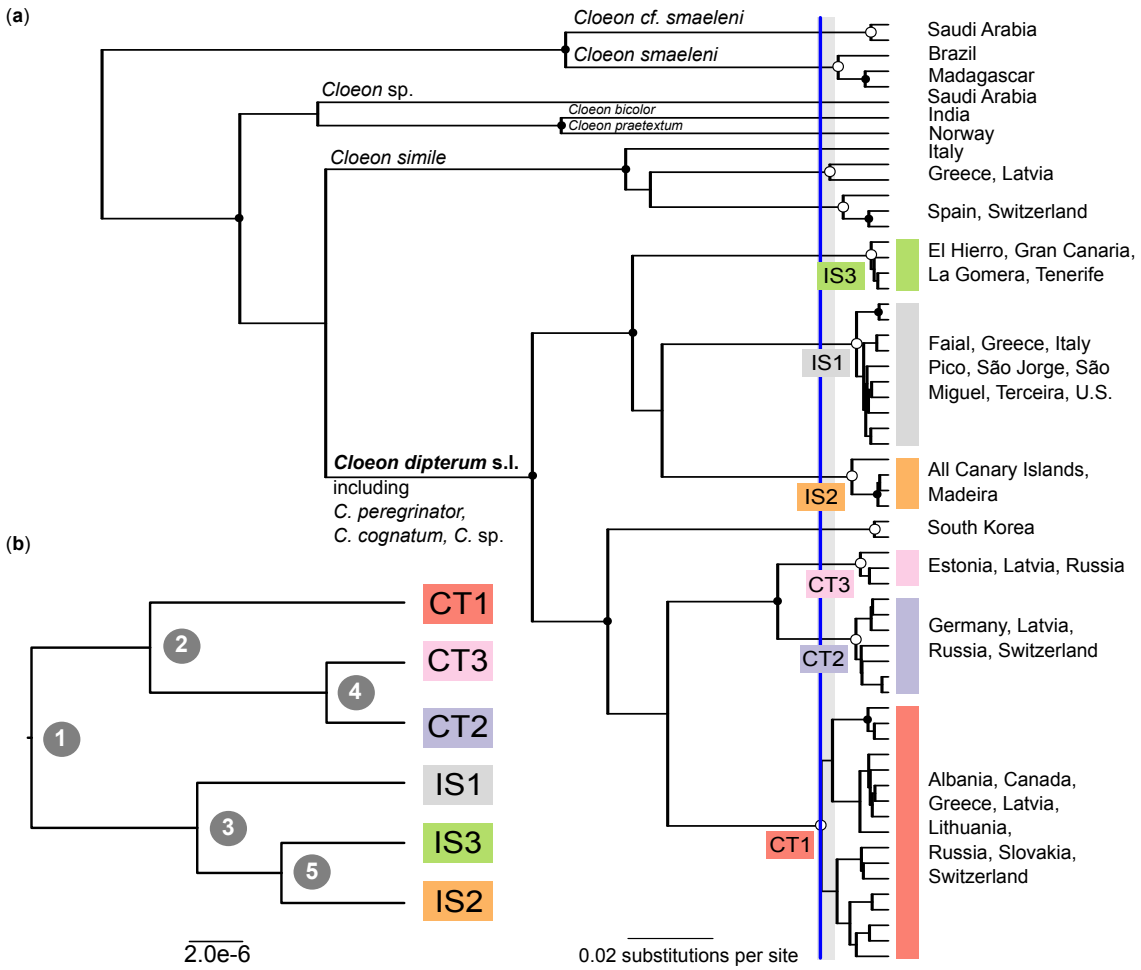


Fig. 2. Mitochondrial gene tree and nuclear species tree topology. Species delimitation based on (**a**) the general mixed Yule-coalescent (gmyc) approach using mitochondrial data (single-locus); and (**b**) the multispecies coalescent approach using nuclear data (59 loci). An ultrametric mitochondrial *cox1* gene tree was used as input for the gmyc analysis of *Cloeon* sp (**a**). All circles indicate well supported nodes (Bayesian posterior probability ≥ 0.95). Open circles at subtending nodes indicate sequence clusters corresponding to single gmyc species. Colored rectangles and alphanumeric codes indicate the six gmyc species within *C. dipterum* s.l. The outgroup *Baetis rhodani* is not shown. The vertical blue line indicates the point of maximum-likelihood fit of the single-threshold gmyc model and 95% confidence intervals are indicated by grey shading. Terminal labels indicate sampling regions (Supplementary Table 1). The species trees of *C. dipterum* s.l. inferred using a multispecies coalescent approach based on the exonhap_all_data matrix (**b**). Posterior probabilities of the five nodes varied with the number of loci analysed and these are indicated in Table 2.

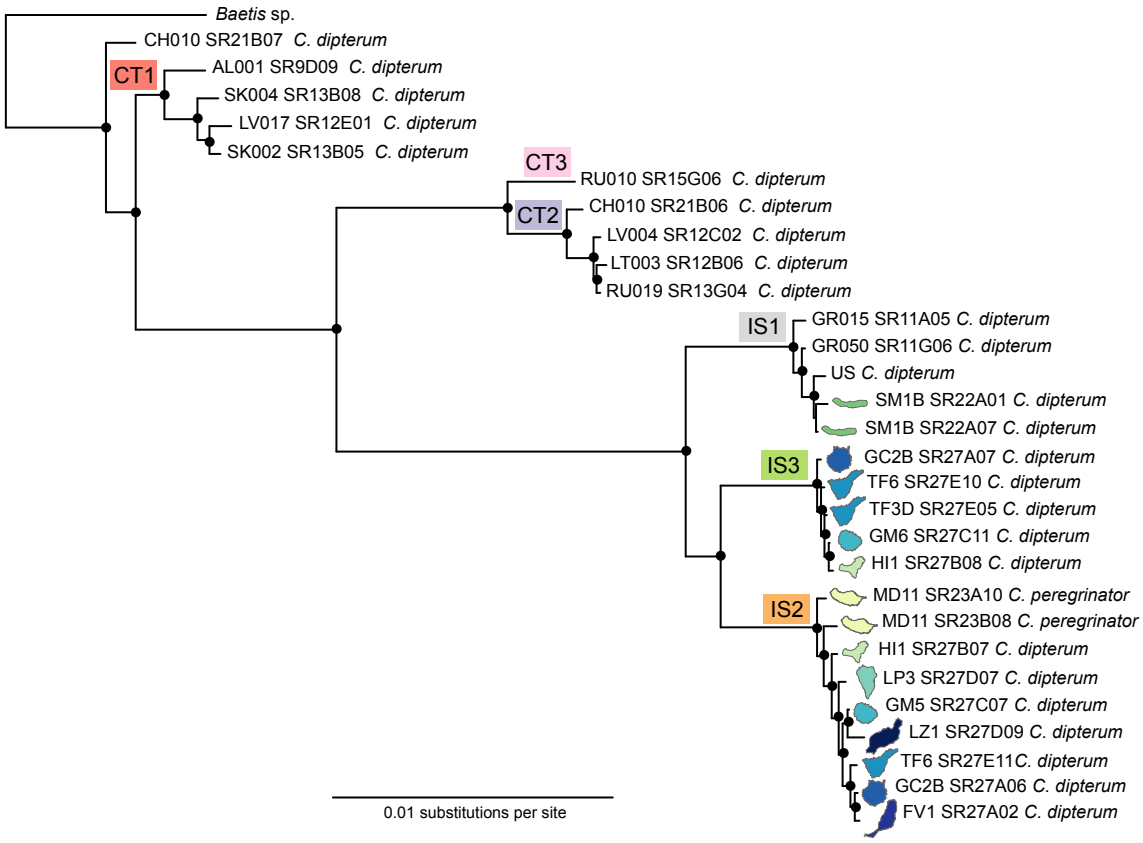


Fig. 3. Phylogenetic relationships among *Cloeon dipterum* s.l. (including *C. peregrinator*) based on the Bayesian reconstruction using the concatenated exon_all_data matrix of 59 nuclear and one mitochondrial marker. Filled circles indicate nodes with Bayesian posterior probability ≥ 0.95. Species inferred from multilocus coalescent and gmyc analyses are indicated with colored rectangles and alphanumeric codes as in Fig. 2.

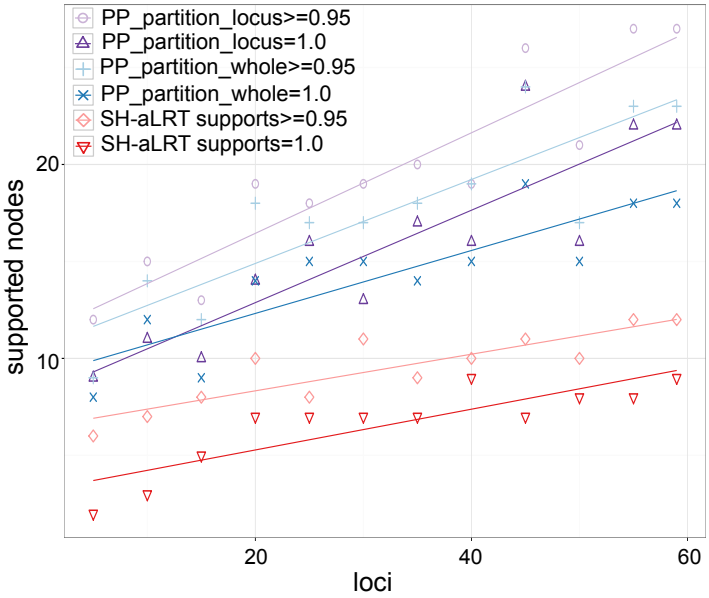


Fig. 4. Linear relationships between number of supported nodes and number of loci analyzed, including as node support values the Posterior probability (PP) and Shimodaira-Hasegawa approximate likelihood ratio (SH-aLRT) supports for the two partition schemes, using each locus as partition (partition_locus) and using the whole alignment as one partition (partition_whole). Purple circles for PP_partition_locus ≥ 0.95 ($R^2 = 0.83$, $p < 0.001$), purple triangles for PP_partition_locus = 1 ($R^2 = 0.75$, $p < 0.001$), blue pluses for PP_partition_whole ≥ 0.95 ($R^2 = 0.72$, $p < 0.001$), blue crosses for PP_partition_whole = 1 ($R^2 = 0.72$, $p < 0.001$), red squares for SH-aLRT supports ≥ 0.95 ($R^2 = 0.74$, $p < 0.001$), and red triangles for SH-aLRT supports =1 ($R^2 = 0.71$, $p < 0.001$). Sets of loci are reported in Supplementary Table 4.

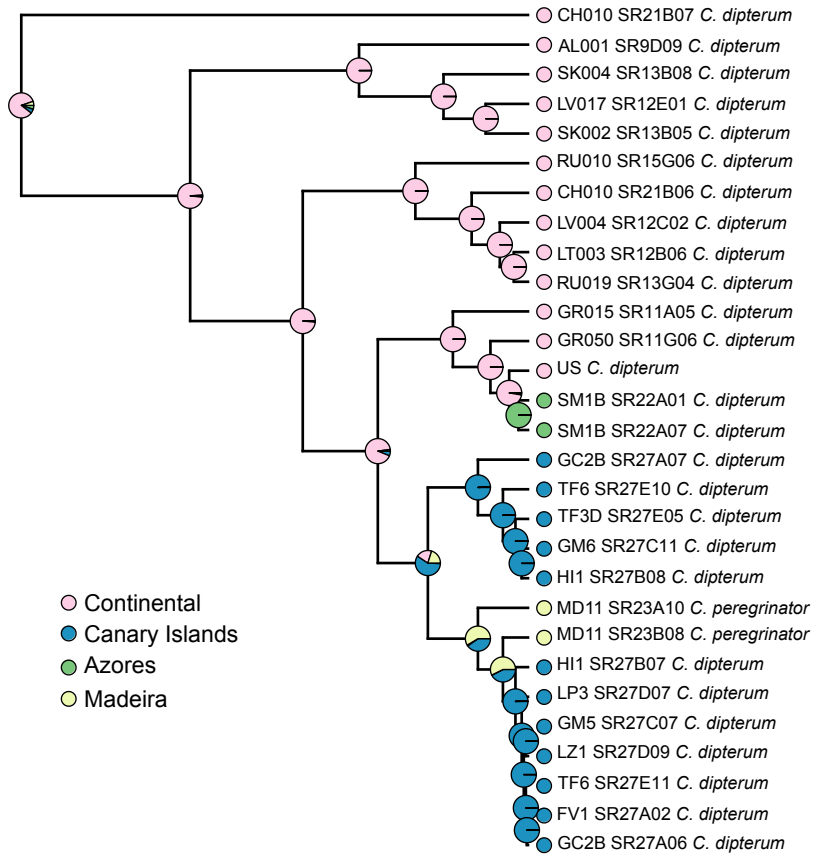


Fig. 5. Ancestral state reconstruction of the origin estimated under an equal-rates model based on the concatenated tree. Colors highlight the four defined origins : Continental, including European and North American mainland (pink), Azores (green), Madeira (yellow), and Canary Islands (blue).

896 **Tables**

897 **Table 1.** Overview of seven sequence alignments, including one based on mitochondrial sequences and six concatenated matrices based on
898 nuclear sequences. The mitochondrial sequence alignment including only the species group of *Cloeon dipterum* s.l. comprised 130 taxa with 148
899 variable sites. Matrices containing all taxa and all loci were >75% complete; the matrices containing only loci sequenced for all taxa were 100%
900 complete. Exon matrices refer to exon sequence alignments and the exonhap matrices refer to exon haplotype sequences.

901

Data Matrix	Concatenated Length [bp]	Number of Taxa	Number of Loci	Number of Variable Sites	Description
mitochondrial	658	148	1	240	All *cox1* sequences used for putative species assignment
all_data	32,213	29	59	2,481	All taxa and loci; introns and exons
exon_all_data	24,168	29	59	1,118	All taxa and loci; exons
complete_matrix	8,565	29	17	648	Only loci sequenced for all taxa; introns and exons
exon_complete_matrix	6,485	29	17	293	Only loci sequenced for all taxa; exons
exonhap_all_data		29	59	1,390	All taxa and loci; haplotypes of exons
exonhap_complete_matrix		29	17	361	Only loci sequenced for all taxa; haplotypes of exons

Table 2. Node support values of the species tree analysis (Fig. 2b) using six different sets of loci (Supplementary Table 4). Support values are given as Bayesian posterior probability (PP).

	Node Support					
Number of Loci	All (1)	Continental (2)	Island (3)	CT2+CT3 (4)	IS2+IS3 (5)	Mean
17	1.00	0.99	1.00	1.00	1.00	1.00
20	1.00	1.00	-	1.00	-	0.60
30	1.00	0.85	0.87	0.87	0.99	0.92
40	1.00	1.00	1.00	0.67	0.83	0.70
50	1.00	0.83	0.83	0.83	1.00	0.87
59	1.00	0.83	0.83	0.83	1.00	0.87