DISCOMARK: Nuclear marker discovery from orthologous sequences using low coverage genome data

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Abstract

High-throughput sequencing has laid the foundation for fast and cost-effective development of phylogenetic markers. Here we present the program DISCOMARK, which streamlines the development of nuclear DNA (nDNA) markers from whole-genome (or whole-transcriptome) sequencing data, combining local alignment, alignment trimming, reference mapping and primer design based on multiple sequence alignments in order to design primer pairs from input orthologous sequences. In order to demonstrate the suitability of DISCOMARK we designed markers for two groups of species, one consisting of closely related species and one group of distantly related species. For the closely related members of the species complex of *Cloeon dipterum* s.l. (Insecta, Ephemeroptera), the program discovered a total of 77 markers. Among these, we randomly selected eight markers for amplification and Sanger sequencing. The exon sequence alignments (2,526 base pairs (bp)) were used to reconstruct a well supported phylogeny and to infer clearly structured haplotype networks. For the distantly related species we designed primers for several families in the insect order Ephemeroptera, using available genomic data from four sequenced species. We developed primer pairs for 23 markers that are designed to amplify across several families. The DISCOMARK program will enhance the development of new nDNA markers by providing a streamlined, automated approach to perform genome-scale scans for phylogenetic markers. The program is written in Python, released under a public license (GNU GPL v2), and together with a manual and example data set available at: https://github.com/hdetering/discomark.

Introduction

The inference of phylogenetic relationships has benefited profoundly from the availability of nuclear DNA (nDNA) sequences for an increasing number of organism groups. The development of new phylogenetic markers has provided unprecedented insight into the evolutionary relationships of non-model organisms in particular (Ellegren 2014). Large sets of nDNA markers (single copy genes) have recently been designed for taxonomic groups for which genomic resources were available, e.g. cichlid fish (Meyer *et al.* 2015), ray-finned fish (Near *et al.* 2012), reptiles (Ruane *et al.* 2014), birds (Kerr *et al.* 2014) and flowering plants (Zeng *et al.* 2014). However, for many taxonomic groups there are only a handful of nDNA markers available that are suitable for phylogenetic reconstruction. Other approaches, such as ultra-conserved element (UCE) sequencing (Faircloth *et al.* 2012), anchored hybrid enrichment (Lemmon and Lemmon 2012), restriction site-associated DNA (RAD) sequencing (Baird *et al.* 2008) or genotyping by sequencing (GBS, Elshire *et al.* 2011) have become popular for addressing specific questions in systematics or population genetics; however, these methods are still cost-intensive, require a comparatively high amount of starting DNA material and can depend on the availability of reference genomes (e.g. anchored hybrid enrichment). Consequently, standard Sanger sequencing approaches are still in high demand for various research questions.

Identification of novel phylogenetic markers has been a predominantly manual process, which impedes their large-scale development, and comprehensive primer design based on large sets of multiple sequence alignments remains challenging. Recently, tools have been developed for (1) specific primer design such as for automated primer design from transcriptome data (SCRIMER, Morkovsky *et al.* 2015), for individual degenerate primers (GEMI, Sobhy *et al.* 2012; PRIMER3, Untergasser *et al.* 2012; CEMASUITE, Lane *et al.* 2015),

70 for highly variable DNA targets (PRIMERDESIGN, Brodin *et al.* 2013; PRIMERDESIGN-M,
71 Yoon and Leitner 2015), viral genomes (PRISM, Yu *et al.* 2015), multiple primer design
72 (BATCHPRIMER3, You *et al.* 2008; PRIMERVIEW, O'Halloran 2015) and (2) the discovery of
73 specific markers, including single nucleotide polymorphism (SNP) markers (POLYMARKER,
74 Ramirez-Gonzalez *et al.* 2015), and putative single copy nuclear loci (MARKERMINER,
75 Chamala *et al.* 2015). In addition, the challenge of developing new markers lies both in the
76 discovery of conserved regions, the design of primer pairs and an estimation of their
77 suitability as phylogenetic markers.

78 Our aim was to develop a flexible, user-friendly program that works with FASTA-
79 formatted files of putative orthologous sequences from whole-genome or whole-transcriptome
80 data, identified conserved regions and and designs primers based on these multiple sequence
81 alignments. Here we present DISCOMARK (=Discovery of Markers), a program for the
82 discovery of phylogenetically suitable nDNA markers and design of primer pairs. The
83 program can be used to easily screen for nDNA markers and design primers that can be used
84 for Sanger sequencing as well as high-throughput sequencing. The program is structured into
85 several steps that can be individually optimized by the user and run independently. In terms of
86 input the program can be applied on large and small sets of taxa, including both closely and
87 distantly related species. Ideally, orthologous sequences in combination with a whole-genome
88 reference sequence are used. Thus, exon/intron boundaries can be inferred using the reference
89 for each marker. Under the default settings, the program will design several primer pairs that
90 anneal in conserved regions. The visualization of the alignments with potential primers allows
91 the user to choose between primers targeting exons or introns (e.g. exon-primed intron-
92 crossing (EPIC) markers). Additionally, information about the suitability as phylogenetic
93 markers is provided by an estimate of the number of SNPs per marker and the applicability

94 across species. Finally, we demonstrate the utility of DISCOMARK for (1) closely related
95 species (i.e. *Cloeon dipterum* s.l. species complex) using whole-genome data, and (2)
96 distantly related species (i.e. insect order Ephemeroptera) using whole-genome data derived
97 from genome sequencing projects.

98 **Materials and Methods**

99 *DISCOMARK implementation*

100 The program DISCOMARK is written in Python and is developed to design primer pairs in
101 conserved regions of predicted orthologous genes. Orthologs are most suited for phylogenetic
102 studies. The ortholog identification step is not part of the DISCOMARK workflow but
103 DISCOMARK is designed to directly work with the output of several ortholog prediction
104 programs, e.g. HAMSTR (Ebersberger *et al.* 2009), or Orthograph
105 (https://github.com/mptrsen/Orthograph, last accessed March 25, 2016). Orthologous groups
106 may be derived from genomic or transcriptomic sequencing data. In addition to the
107 orthologous genes, genomic data such as whole-genome sequencing data can be provided to
108 DISCOMARK as a guide to detect exon/intron boundaries. DISCOMARK performs seven steps,
109 combining Python scripts with widely used bioinformatics programs (Fig. 1). The steps: (1)
110 combine orthologous groups of sequences, (2) align sequences of each orthologous group
111 using MAFFT v.7.205 (Katoh and Standley 2013), (3) trim sequence alignments with TRIMAL
112 v.1.4 (Capella-Gutierrez *et al.* 2009), (4) align sequences against a reference (e.g. whole-
113 genome dataset from the same or closely related taxa) with BLASTN v.2.2.29 (Altschul *et al.*
114 1997; Camacho *et al.* 2009) and re-alignment using MAFFT, (5) design primer pairs on
115 single-gene alignments using a modified version of PRIFI (Fredslund *et al.* 2005), adapted by
116 us into a Python package that uses BioPython v.1.65 and Python v.3.4.3, (6) check primer

117 specificity with BLASTN, and (7) generate output in several formats (visual HTML report,
118 tabular data and FASTA files of the primers). The results of each step can be inspected in the
119 respective output folders.

120 *1 Combine sequences*. In the first step, the putative orthologous sequences of different taxa
121 are combined according to the orthologous groups. The input files are expected to be
122 nucleotide sequences in FASTA format. We recommend using putative orthologous exon
123 sequences (e.g. CDS) in combination with whole-genome data (e.g. a draft genome
124 assembly). Each input file is expected to contain the sequences of one orthologous group;
125 orthologs of each input taxon are to be organized into a taxon folder. Importantly, file names
126 represent the ortholog identifiers used to combine orthologous sequences of the various input
127 taxa; by default, ortholog prediction tools follow that convention.

128 *2 Align sequences*. Orthologous sequences combined according to the orthologous groups are
129 separately aligned with the multiple sequence alignment (MSA) program MAFFT. Alignment
130 parameters can be specified by the user via a configuration file (discomark.conf, located in the
131 program folder). Default parameters are the following: '--localpair --maxiterate 16 --
132 inputorder --preservecase --quiet' (L-INS-i alignment method). We chose MAFFT as multiple
133 alignment tool because it combines accuracy and efficiency and has been adopted widely in
134 the scientific community (Pais *et al.* 2014; Szitenberg *et al.* 2015).

135 *3 Trim alignments*. In order to remove poorly aligned regions, sequence alignments are
136 trimmed using TRIMAL. The program TRIMAL analyzes the distribution of gaps and
137 mismatches in the alignment and discard alignment positions and sequences of low quality.
138 By default, DISCOMARK calls TRIMAL with the '-strictplus' method. The preset is used by
139 TRIMAL to derive the specific thresholds for alignment trimming (minimum gap score,

140 minimum residue similarity score, conserved block size). Since alignment trimming largely
141 depends on the input data and influences the downstream results, TRIMAL can also be run with
142 different settings (e.g. '-gappyout', '-strict', '-automated1'; but see Capella-Gutierrez *et al.*
143 (2009). Alternatively, there is also the option to deactivate the alignment trimming with the
144 DISCOMARK option '--no-trim' or use alternative trimming programs such as GBLOCKS
145 (Castresana 2000; Talavera and Castresana 2007) or GUIDANCE2 (Landan and Graur 2008;
146 Sela *et al.* 2015).

147 *4 Blast and alignment to reference*. In this step a genomic reference sequence for each input
148 ortholog is identified and added to the trimmed alignment. This step is particularly important
149 when working with coding sequences which do not contain intron sequences; thus, a genomic
150 sequence is needed to infer intron/exon boundaries. Working with coding sequences is
151 advisable for more distantly related taxa which may include intron length polymorphisms, or
152 to target EPIC markers. Any whole-genome data set (from one of the included taxa or a
153 closely related taxa) can be used as reference for mapping the ortholog sequences. Here,
154 mapping means that the input sequences are compared to the reference sequences, which are
155 defined by the user using the local alignment program BLASTN. The best locally aligning
156 reference sequence (the one that yields the longest alignment among all input sequences) for
157 each orthologous group is added to the corresponding sequence alignment. Reference
158 sequences are cut to 100 base pairs (bp) upstream and downstream of the first, respectively
159 last, BLAST hit to avoid alignment length inflation. Then, the extended alignments are re-
160 aligned with MAFFT. The reference alignment step is optional; however, the inclusion of
161 whole-genome data is essential for estimating intron/exon boundaries. Given that information,
162 the focus of target sequences to be amplified can be on entire exon markers, EPIC markers, or
163 a combination.

164 *5 Design primers*. The single-gene alignments, after trimming, mapping and re-aligning to a
165 reference, are used as input to design primer pairs. We integrated the webtool PRIFI
166 (http://cgi-www.daimi.au.dk/cgi-chili/PriFi/main, last accessed December 20, 2015) as a
167 Python package that provides a comprehensive set of parameters. As default settings for
168 DISCOMARK we chose the following: estimated product length between 200-1,000 bp
169 ('OptimalProductLength = [400, 600, 800, 1000], MinProductLength = 200,
170 MaxProductLength = 1000'), maximum number of ambiguity positions within the primer
171 sequences ('MaxMismatches = 2'), primer length between 20-30 bp ('MinPrimerLength = 20,
172 MaxPrimerLength = 30, OptimalPrimerLength = [20, 25]'), melting temperature of the primer
173 pairs between 50-60°C ('MinTm = 50.0, MinTmWithMismatchesAllowed = 58.0,
174 SuggestedMaxTm = 60.0'), and we set the maximum number of primer pairs per alignment to
175 six (note: only settings different from the PRIFI default are mentioned above). The program
176 PRIFI was originally developed to design intron-spanning markers (but see Fredslund et al.
177 2005). Here we use it because it enables primer design based on MSA input. Parameters for
178 PRIFI can be specified in the DISCOMARK configuration file ('discomark.conf').

179 *6 Check marker specificity*. To ensure the specificity of the designed primer pairs, we
180 compare their sequences against the NCBI database ('refseq_mrna'). Primer sequences are
181 searched in the NCBI database ('refseq_mrna') using the online BLASTN interface. The
182 default search settings are restricted to human and bacterial targets using the Entrez query
183 'txid2[ORGN] OR txid9606[ORGN]' because these are most likely to be present as
184 contaminants in sequencing libraries. The result hits of the BLAST search are indicated to the
185 user in the HTML output.

186 *7 Visualize results*. As final step, the program produces a HTML report containing the list of

designed primers, an alignment viewer and plots visualizing the discovered set of markers. Besides the primer sequences the report lists several features such as the melting temperatures, predicted sequence length, and the number of taxa amplified by each primer set. Selected primer pairs and primer lists can be downloaded as FASTA or CSV files, respectively. In order to provide a measure of the suitability of the markers for phylogenetic reconstruction the program calculates the number of SNPs between a primer pair by comparing the aligned input sequences against each other. The number of SNPs between each primer pair is visualized in relation to the estimated product length (see Fig. 2 for an example) and reported in the tabular output. Furthermore, the report highlights the species coverage achieved by the discovered markers, i.e. how many species' sequences each primer set is expected to amplify, as an estimate of how universal each primer set can be applied. Additionally, functional annotations are reported, if available, to guide the user in the selection of markers of interest. Annotations can be supplied in form of a tab-delimited file with the '-a' option. In principle, any kind of annotations can be used depending on the desired research objective. In our usage scenarios, we used gene ontology (GO) terms which were retrieved by mapping the gene IDs contained in the HAMSTR core ortholog set via the UniProt website (http://www.uniprot.org/, last accessed December 20, 2015).

Usage cases

Closely related species - Cloeon dipterum s.l. species complex. To test the suitability of DISCOMARK for closely related species, we designed primer pairs for the species complex of *Cloeon dipterum* s.l. (Ephemeroptera: Baetidae). The species complex consists of several closely related species, including *Cloeon peregrinator* GATTOLLIAT & SARTORI, 2008 from Madeira (Gattolliat *et al.* 2008; Rutschmann *et al.* 2014; Table 1). As input to design the

210 primer pairs data we used whole-genome sequencing data of *Cloeon dipterum* L. 1761
211 (Baetidae; Sequence Read Archive SRP050093) and expressed sequence tags (EST) of *Baetis*
212 sp. (Baetidae; FN198828-FN203024). The sequence reads of *C. dipterum* were trimmed and
213 *de novo* assembled using NEWBLER v.2.5.3 (454 Life Science Corporation) under the default
214 settings for large datasets. Ortholog sequences prediction of both data sets was performed
215 with HAMSTR v.9 using the insecta_hmmer3-2 core reference taxa set (http://www.deep-
216 phylogeny.org/hamstr/download/datasets/hmmer3/insecta_hmmer3-2.tar.gz, last accessed
217 December 20, 2015), including 1,579 orthologous genes. We ran the program DISCOMARK
218 with default settings ('python run_project.py -i input/Cloeon -i input/Baetis -r
219 input/reference/Cloeon.fa -a input/co2go.ixosc.csv -d output/cloeon_baetis'), using the
220 predicted orthologs from HAMSTR and the whole-genome *Cloeon*-data as reference (step 4).
221 The Pearson correlation between the number of SNPs between primer pairs and
222 corresponding estimated product length was calculated using the function cor within the stats
223 package for R (R Development Core Team, 2016). A t-test for significance was performed
224 using the function cor.test.

225 From the total of designed primer pairs (77 markers, 338 primer pairs, see results) we
226 selected eight and amplified them for four species of the *C. dipterum* species complex (Table
227 1) in the laboratory. We used standardized polymerase chain reactions (PCR; 35-40 PCR
228 cycles with annealing temperature of 55°C), followed by Sanger sequencing. Forward and
229 reverse sequences were assembled and edited with GENEIOUS R7 v.7.1.3 (Biomatters Ltd.),
230 indicating ambiguous positions following the IUPAC nucleotide codes. Heterozygous
231 sequences were decoded with CODONCODEALIGNER v.3.5.6 (CodonCode Corporation) using
232 the find and split heterozygous function. Multiple sequence alignments were created for all

233 sequences per marker. The predicted orthologous sequences of *Baetis* sp. were used as
234 reference to infer the exon-intron splicing boundaries (canonical and non-canonical splice site
235 pairs). The final sequence alignments were checked for the occurrence of stop codons and
236 indels, and split into exon and intron parts using a custom Python script
237 (https://github.com/srutschmann/python_scripts, last accessed March 28, 2016). Sequence
238 alignments were phased using the program PHASE v.2.1.1 (Stephens *et al.* 2001; Stephens
239 and Donnelly 2003) with a cutoff value of 0.6 (Harrigan *et al.* 2008; Garrick *et al.* 2010),
240 whereby input and output files were formatted using the Perl scripts included in SEQPHASE
241 (Flot 2010). Heterozygous sites that could not be resolved were coded as ambiguity codes for
242 subsequent analyses. After phasing, all alignments were re-aligned with MAFFT. The number
243 of variable and informative sites, and the nucleotide diversity per exon alignment was
244 calculated with a custom script.

245 To investigate the heterogeneity of each marker´s DNA sequences, we reconstructed
246 haplotype networks, using FITCHI (Matschiner 2015). As input for each marker we inferred a
247 gene tree using the program RAXML v.8 (Stamatakis 2014) with the GTRCAT model and
248 1,000 bootstrap replicates under the rapid bootstrap algorithm. The phylogenetic relationships
249 were calculated with Bayesian inference, using MRBAYES v.3.2.3 (Ronquist *et al.* 2012)
250 based on a concatenated nDNA matrix that consisted of the exon sequences from all 15
251 nDNA markers. The best-fitting model of molecular evolution for each sequence alignment
252 was selected via a BIC criterion in JMODELTEST v.2.1 (Guindon and Gascuel 2003; Darriba *et*
253 *al.* 2012). We calculated 10^6 generations with random seed, a burn-in of 25% and four
254 MCMC chains. As an outgroup we used the predicted orthologous sequences of *Baetis* sp..

255 *Distantly related species - insect order Ephemeroptera.* In this test case, we used contigs
256 derived from whole-genome sequencing projects of the species *Baetis* sp. (Baetidae;
257 BioProject PRJNA219528), *Ephemera danica* MÜLLER 1764 (Ephemeridae; BioProject
258 PRJNA219552), *Eurylophella* sp. (Ephemerellidae; BioProject PRJNA219556), and
259 *Isonychia bicolor* WALKER 1853 (Isonychiidae; BioProject PRJNA219568). The contigs from
260 each species were used for ortholog predicting with HAMSTR v.13.2.4
261 (http://sourceforge.net/projects/hamstr/files/hamstr.v13.2.4.tar.gz, last accessed December 20,
262 2015). We ran DISCOMARK with the default settings, using the *Baetis* sp. data as reference
263 ('python run_project.py -i input/Baetis -i input/Ephemera -i input/Eurylophella -i
264 input/Isonychia -r input/references/Baetis.fa -a input/co2go.ixosc.csv -d output/mayflies').

265 **Results**

266 *Closely related species - species complex of Cloeon dipterum s.l.*

267 DISCOMARK identified a total of 804 nDNA markers and 77 alignments with 338 primer pairs
268 for orthologous sequences of both species (*Baetis* sp. and *C. dipterum* s.l.). Ortholog
269 prediction yielded 403 orthologous sequences for the *Baetis* sp. EST-data and 1,211 for *C.*
270 *dipterum*. For the individual species, DISCOMARK identified 790 markers for *C. dipterum* and
271 123 for *Baetis* sp. The lengths of the markers including both species were between 201 and
272 925 bp with median length of 451.5 bp. The number of SNPs per marker ranged from zero to
273 37 (median: 5) with an average of one SNP per 68 bp. Marker length and number of SNPs
274 were correlated with a Pearson's correlation coefficient of 0.35 (Pearson's product-moment
275 correlation $P < 0.001$). The total run time for this data set on a local Linux machine (quad-
276 core Intel i5, 8 GB RAM) was 24 min.

277 The haplotype networks based on the eight selected markers showed a clear structure for
278 all markers, including two markers with shared haplotypes for the two species from the U.S.
279 and Madeira (Fig. 3 and Fig. S1, Supporting information). The length of the concatenated
280 sequence alignment of the eight markers was 3,530 bp (2,526 bp exon sequence, Table S1,
281 Supporting information). The exon sequence matrix contained 78 variable sites, 27
282 informative sites, and was 92.6% complete. The nucleotide diversity ranged between 0.009
283 and 0.028 (median: 0.013). Phylogenetic tree reconstruction based on these eight markers
284 resulted in a phylogeny with fully resolved nodes (Bayesian posterior probability (PP) ≥ 95%;
285 Fig. 3). The species *C. dipterum* sp1 was found as outgroup to a clade containing the species
286 *C. dipterum* sp2 from Switzerland and the two species from the U.S and Madeira. The latter
287 two species formed a monophyletic clade.

288 *Distantly related species - insect order Ephemeroptera*

289 In total, we found 22 orthologs with a total of 48 primer pairs for all four species (Table S2,
290 Supporting information). The input files per species (i.e. putative orthologous sequences)
291 ranged from 1,445 to 1,523. We detected 41 markers that covered three of the species (99
292 primer pairs), 81 markers covering two species (210 primer pairs), and 117 markers that
293 covered any single species (478 primer pairs). For the individual species, *Baetis* sp. had the
294 most markers available (214) of the single- and multi-species markers. There were 138
295 markers for *Eurylophella* sp., 107 markers for *I. bicolor*, and 88 markers for *E. danica*. The
296 lengths for all markers covering all four species varied between 216 and 997 bp with median
297 of 398.5 bp, containing between 39 and 298 SNPs per marker (Fig. 2,) with a SNP every 4.1
298 bp on average. Marker length and number of SNPs were correlated with a Pearson's
299 correlation coefficient of 0.97 (Pearson's product-moment correlation $P < 0.001$). Run time

300 for this data set on a Linux client (quad-core Intel i5, 8 GB RAM) was 46 min.

301 **Discussion**

302 To our knowledge, the program DISCOMARK is the first stand-alone program with the aim of
303 designing primer pairs based on multiple sequence alignments on a genome-wide scale. The
304 visual output gives guidance on the suitability of each marker (i.e. variability within and
305 between species measured as number of SNPs, and information about the included species of
306 each marker. Using this approach, primers can be specifically chosen to match the
307 'phylogenetic scale' (i.e. for closely related species many markers with intermediate number
308 of SNPs and for distantly related species fewer markers with generally higher number of
309 SNPs can be selected. The automatic processing, including combining, aligning, trimming
310 and blasting sequences of any nucleotide FASTA sequences together with the produced
311 graphical output significantly facilitate the design of primer pairs for a large number of nDNA
312 markers. Nevertheless, users retain a high degree of flexibility by the stepwise nature of the
313 workflow. DISCOMARK is free, open-source software to assist the development of markers for
314 non-model species on the genome scale. We demonstrated the efficacy of our approach for
315 closely related species as well as for members of divergent families within an order of insects.
316 Using a reference genome enabled resolution of intron-exon boundaries but is not a strict
317 requirement for marker design.

318 *Markers development within the order Ephemeroptera*

319 The usage of DISCOMARK adds an extensive set of new potential nDNA markers to the ones
320 that have been used to date for mayfly phylogenies based on individual genes (histone 3,
321 elongation factor 1 alpha, phosphenolpyruvate carboxykinase (Vuataz *et al.* 2011; Pereira-da-

322 Conceicoa *et al.* 2012; Vuataz *et al.* 2013). Most recent phylogenetic reconstructions are still
323 mostly based on the information of mitochondrial DNA markers (e.g. Rutschmann *et al.*
324 2014; Macher *et al.* 2016). The availability of more genome data will be very valuable in
325 order to increase the number of markers suitable for phylogenetic studies. The use of the
326 larger marker set for *C. dipterum* developed here resulted in a fully resolved phylogenetic tree
327 in contrast to Rutschmann *et al.* (2014). The availability of more markers promote fine-scaled
328 phylogenetic studies, which are needed to resolve the phylogenetic relationships of so-called
329 morphologically cryptic species that can not be resolved with standard markers (Dijkstra *et al.*
330 2014).

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Data Accessibility

The program, user manual and example data sets are freely available at: https://github.com/hdetering/discomark (last accessed March 28, 2016). Scripts used for the analyses are available at: https://github.com/srutschmann/python_scripts (last accessed March 28, 2016). All DNA sequences from this study are available under GenBank accessions: KU987258-KU987260, KU987265- KU987268, KU987273- KU987276, KU987285-KU987288. GenBank accession numbers for sequences included in previous studies are the following: KU971838-KU971840, KU971851, KU972090-KU972092, KU972104, KU972490-KU972492, KU972503, KU973060-KU973061, KU973074.

Author Contributions

S.R., H.D., S.S., and M.T.M. conceived the study. S.R. coordinated the project and performed the empirical analyses. H.D. implemented the program in Python. S.R. and H.D. drafted the manuscript. S.S. gave guidance for the ortholog prediction. J.F. provided the code of the PriFi web tool. All authors gave helpful comments to the manuscript and approved the final version.

Tables

Table 1 List of species used for the usage examples of the closely related species; *Cloeon dipterum* s.l. species complex.

Species	Voucher	Acc. no. *cox1*	Geographical origin
Cloeon dipterum sp1	SR21B07	KJ631626	Switzerland
Cloeon dipterum sp2	SR21B06	KJ631625	Switzerland
Cloeon dipterum sp3	US	KU757184	U.S.
Cloeon peregrinator	SR23A10	KU757122	Madeira

Figures

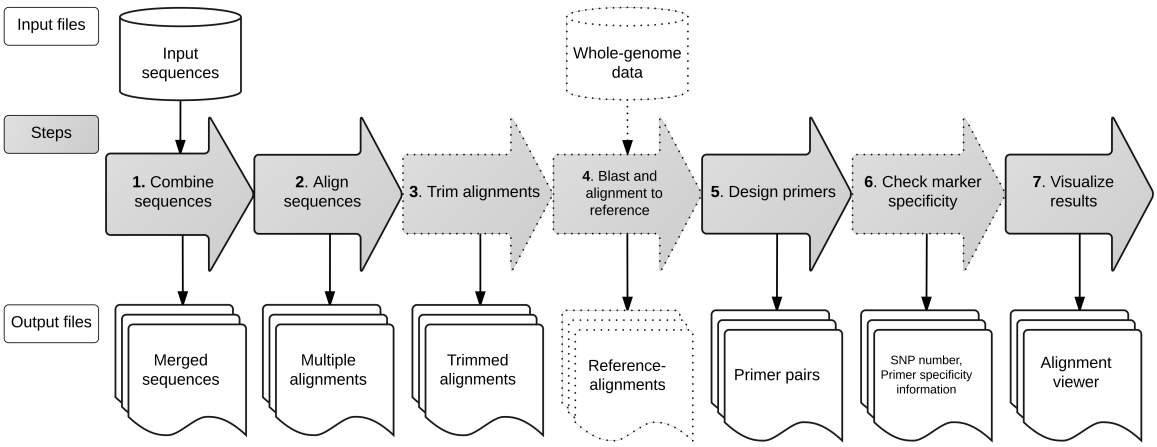

Fig. 1 Overview of the DISCOMARK workflow and processing steps. Arrows with a broken outline indicate optional steps (for details see Materials and Methods section).

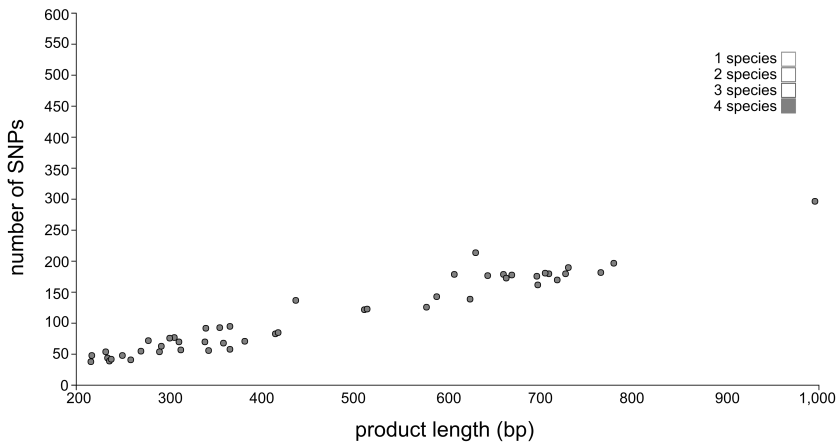

Fig. 2 Visualization of DISCOMARK results: Scatter plot displaying the number of single nucleotide polymorphisms (SNPs) versus product length for each marker of the four mayfly species: *Baetis* sp., *Eurylophella* sp., *Ephemera danica*, and *Isonychia bicolor*. Shown are the markers for all four species (for details see Materials and Methods section).

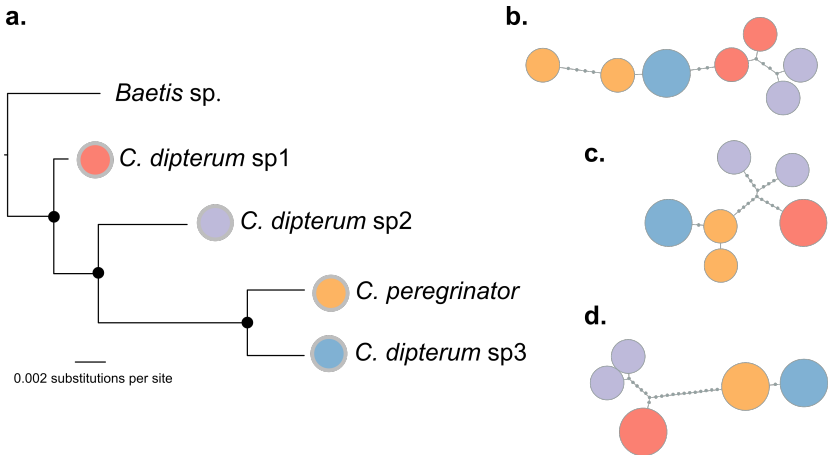

Fig. 3 Phylogenetic reconstruction and haplotype networks for the empirical data. **a**, Phylogenetic reconstruction of four representatives of the species complex *Cloeon dipterum* s.l., including *C. peregrinator*, based on the exon sequences of the eight newly developed nuclear DNA markers (2,526 base pairs). Bayesian inference was used to reconstruct the tree based on the concatenated supermatrix alignment. Bayesian posterior probabilities ≥ 95% are indicated by filled circles. *Baetis* was used as an outgroup. Scale bar represents substitutions per site. **b-d**, Haplotype networks of three amplified markers, **b**, marker 412045, **c**, marker 412741, **d**, marker 412048 (full set of haplotype networks is available in Fig. S1, Supporting information). Circles are proportional to haplotype frequencies. Small circles along the branch indicate missing or unsampled haplotypes. Colors correspond to the four putative species.