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Cross-breeding of *Chaetopteryx morettii* and related species, with molecular and eidonomical results (Trichoptera, Limnephilidae)

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Abstract – We test the possibility of a hybrid origin for *Chaetopteryx morettii* using cross-breeding experiments and molecular analysis. We show that species within the *Chaetopteryx villosa* group can hybridize, but that *C. moretti* is not of hybrid origin. We discuss the applicability of our approach for testing ideas on hybrid speciation and detecting hybrids in natural populations, *e.g.*, for conservation purposes.

Key words: Hybridization / population dynamics / speciation / wingless / mtDNA

Introduction

The *Chaetopteryx villosa* group includes *C. villosa* Fabricius 1798, *Chaetopteryx fusca* Brauer 1857, *Chaetopteryx sahlbergi* McLachlan 1876, *Chaetopteryx bosniaca* Marinkovic 1955, *Chaetopteryx atlantica* Malicky 1975, and the morphologically more distant species *Chaetopteryx gessneri* McLachlan 1876, *Chaetopteryx vulture* Malicky 1971, and *Chaetopteryx trinacriae* Botosaneanu, Cianficconi & Moretti 1986. All these species have allopatric distributions: *C. villosa* is widespread in Northern and Central Europe, *C. fusca* lives in eastern Austria and in the adjacent parts of Italy, Slovenia, Hungary, Moravia, Slovakia, and Poland. *C. sahlbergi* lives in parts of the Carpathian mountains and in Scandinavia. *C. bosniaca* is found in the central Balkan peninsula, *C. atlantica* in Portugal and Spain, *C. gessneri* in central and northern Italy and the adjacent parts of France and Switzerland, *C. vulture* in southern Italy, and *C. trinacriae* in Sicily.

The *C. villosa* species group includes species that are eidonomically very close to each other. Traditionally they are considered nominate species but they could also be considered subspecies of one species. This latter idea is supported by the existence of contact zones between nominal forms where the intermediate variability of characters is high and almost every specimen looks different. Examples of such contact zones are the region east of the city of Linz in Upper Austria as well as regions in Poland (Majecka and Szczesny, 2005) between *C. fusca* and *C. villosa,* and the region of Kilpisjärvi in northern Finland between *C. villosa* and *C. sahlbergi.*

The surprising discovery of *Chaetopteryx morettii* by Lodovici and Valle (2007) in the mountains of Veneto (northern Italy) raised the question of its relationships and origin. The known localities lie in a potential contact zone between the known ranges of *C. fusca* and *C. gessneri.* The closest known localities of both these are in the mountains slightly more than 50 km away from known localities of *C. morettii.* We hypothesized that the species may be the result of an earlier contact between *C. fusca* and *C. gessneri* and isolated sufficiently long to develop unique morphological features. Most striking was the fact that the males of *C. morettii* completely lack the parameres, a character otherwise unknown in the genus and unusual in the whole family. Is a hybrid origin of the species possible? The question of speciation through hybridization is one that has intrigued scientists for a long time (Mavárez *et al.*, 2006), including in Trichoptera (*e.g.*, Blahnik, 1995). We used an integrative approach combining rearing experiments and molecular phylogenetics to establish the relationships among species of the *C. villosa* group and test the idea of a hybrid origin of *C. morettii.* If *C. morettii* is of hybrid origin, we would expect successful rearing between *C. fusca* and *C. gessneri* to result in intermediate morphology close to that of *C. morettii.* We would also expect

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a phylogenetic placement of *C. morettii* either between *C. fusca* and *C. gessneri* or a paraphyletic placement of *C. morettii* within both adult species.

Material and methods

Rearing experiments

Adults of *C. morettii, C. fusca,* and *C. gessneri* were cross-bred in the laboratory. To obtain freshly emerged adults, we collected adults in the field and bred pure lines to the next generation. *Chaetopteryx* Stephens 1829 species are easy to breed from the eggs in the laboratory with the method described by Malicky *et al.* (2002). Females were collected in the field and forced to oviposit in captivity. The females of *C. morettii* were collected from the type locality Val Canzoi in the province of Belluno, Veneto (46°06'N, 11°56'E); females of *C. fusca* from Lunz am See, Lower Austria (47°51'N, 15°03'E), and females of *C. gessneri* from the upper reaches of river Sordo west of Norcia, Province of Perugia, Umbria (42°47'N, 13°05'E). The larvae that emerged from these eggs were bred under natural day length and moderately varying temperatures of ca. 10–14 °C. The larvae were fed with soaked autumn-shed leaves of *Alnus glutinosa* (L.) Gaertn. and other trees. The adults emerged in the usual season of October–November.

Freshly emerged males and females from these broods were set together in the desired combinations in small boxes with high air humidity and were fed with sugar solution to prolong their lifetime. All three species immediately went into copula that lasted several days, as usual in Chaetopterygini. The females were then separated and kept in small boxes under high air humidity over wet moss. The egg batches quickly enlarged upon rehydration and were then separated and regularly controlled. The larvae were bred as outlined above. The broods were kept at the same temperature as above, but under (not strictly controlled) long day conditions, *i.e.*, about 14 h of light per day. In an attempt to speed up the development they were fed with fresh leaves of *Taraxacum officinale* Wigg., which is a food of high nutrient value.

Molecular methods

We examined sequence data from the partial mitochondrial cytochrome oxidase 1 (mtCOI) and partial nuclear wingless (nWG) gene regions to test if offspring from hybridization experiments were truly hybrids and not the result of parthenogenesis. Genes were chosen because they reflect both maternal and biparental lineages and have proved successful at resolving lower level phylogenetic relationships and intraspecific variation (Pauls *et al.*, 2008, Pauls, unpublished). Methods for DNA extraction, PCR amplification mtCOI and nWG genes followed Pauls *et al.* (2006, 2008). Sequences were generated at the University of Minnesota BioMedical Genomics Center. Electropherograms were assembled, edited, and aligned in Geneious v4.7.6 (Biomatters, Auckland). We visually assessed and summarized the occurrence of ambiguous sites in Geneious. In this study, we assessed ambiguous sites as indicators of multiple alleles.

To test the potential hybrid origin of *C. moretti*, we additionally performed a phylogenetic assessment of relationships among the *C. villosa* group taxa using *Chaetopteryx rugulosa* and *Chaetopterygopsis maclachlani* Stein 1874 as outgroups to obtain signal from relatively close and relatively distant taxa. We used Bayesian/Markov Chain Monte Carlo (B/MCMC) phylogenetic reconstructions in MrBayes v3.1 (Ronquist and Huelsenbeck, 2003) independently for both genes and for a combined data set. The best-fitting model of nucleotide substitution for Bayesian analysis was selected using the Akaike Information Criterion (AIC) in Modeltest 3.7 Posada and Crandall (1998). We performed two runs with eight chains each for 5×10^6 generations. We selected 3×10^6 generations as burn-in based on post-analysis evaluation of the likelihood scores using Tracer and the average standard deviation of split frequencies between the two runs (< 0.01).

Results

Rearing experiments

Eggs were obtained from the mating combinations *fusca* ♂ × *fusca* ♀, *morettii* ♂ × *morettii* ♀, *fusca* ♂ × *morettii* ♀, *morettii* ♂ × *fusca* ♀, *morettii* ♂ × *gessneri* ♀, *fusca* ♂ × *gessneri* ♀, and *gessneri* ♂ × *morettii* ♀. Some batches of eggs perished from infection by fungi. Larvae emerged from the eggs of the mating combinations *fusca* ♂ × *fusca* ♀, *morettii* ♂ × *morettii* ♀, *fusca* ♂ × *morettii* ♀, *morettii* ♂ × *fusca* ♀ and *morettii* ♂ × *gessneri* ♀. No embryonal development occurred in the eggs from *fusca* ♂ × *gessneri* ♀ and *gessneri* ♂ × *morettii* ♀ matings. The *morettii* ♂ × *gessneri* ♀ mating only produced few larvae that were not bred but only preserved for DNA analysis.

Eidonomical differences of adults: the differences between these species are minimal, therefore only the male genitalia were compared. *C. fusca* is a small species (forewing length 8–12 mm), *C. gessneri* is larger (11–16 mm), and *C. morettii* is in between. However, because the size of the adults is highly variable in all of them, the differences in size were not used for species separation.

There are two important differences between the males of *C. fusca* and *C. morettii*. First, the intermediate appendages are bent upwards and pointed in both species, but in *C. fusca* they have an additional large spine bent outward, which is lacking in *C. morettii* (Fig. 1, s). Second, *C. fusca* has large, well developed and distally pilose parameres which are totally lacking in *C. morettii* (Fig. 1, p). Both species have a lateral indentation of segment IX which is deeper in *C. morettii* where it forms a small pocket in lateral view (Fig. 1, i). The ventral part of

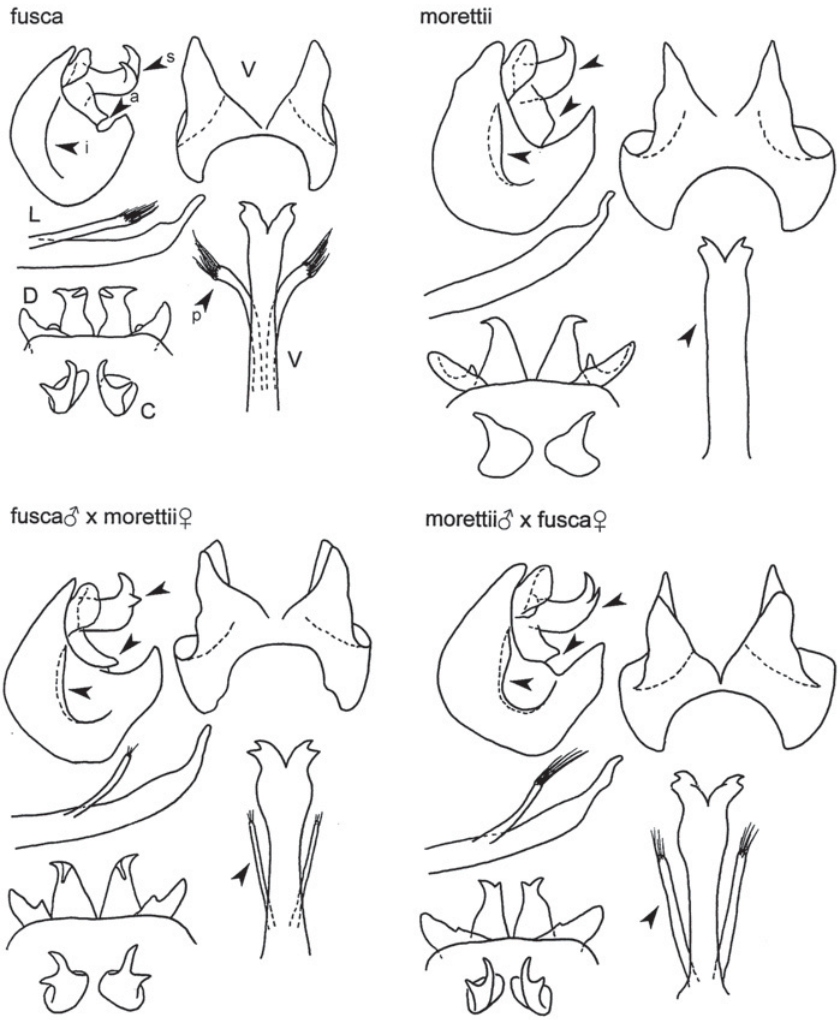

Fig. 1. Male genitalia of *C. fusca*, *C. morettii*, and reciprocal hybrids. L, lateral views of abdominal segments IX and X (top) and the phallus with parameres (bottom); D, dorsal view of segments IX and X; V, ventral view of inferior appendages (top) and phallus with parameres (bottom); C, caudal view of the intermediate appendages. Arrows point to segregating characters (s: spines on intermediate appendages; p: parameres; i: indentation of segment IX; a: ventral part of intermediate appendages).

the intermediate appendages is rather bulky in *C. morettii,* but more slender in *C. fusca* (Fig. 1, a).

The hybrids are intermediate in all cases, but different among the combinations. In both combinations small parameres are present. They are distinctly smaller than in *C. fusca,* and smaller and more slender in *fusca* ♂ × *morettii* ♀ ($N = 25$ ♂♂) than in *morettii* ♂ × *fusca* ♀ ($N = 6$ ♂♂). The lateral spine of the intermediate appendages is present, but smaller than in *C. fusca,* and different between the two combinations (Fig. 1). The lateral indentation of segment IX is similar to that of *C. morettii* in both combinations. These characters are constant in both combinations.

Molecular analysis

We generated 28 new mtCOI sequences and 44 new nWG sequences (see Table 1). The alignments were 495 and 403 bp in length for mtCOI and nWG, respectively. One hundred and twenty-seven sites were variable in the mtCOI sequences and 41 sites in the nWG alignment. Evaluation of ambiguous nucleotide positions in nWG sequences of hybrid specimens is summarized in Table 2. Nine sites in the nWG alignment were variable and informative regarding the hybridization among species, *i.e.*, consistent within species but different among wild caught specimens of different species. In cases where the parental taxa unambiguously differed at a certain nucleotide position, we would expect the hybrid offspring to show an ambiguity at that nucleotide for the two nucleotides of the siring taxa. One example of this is at position 299 (Table 2). At this position *C. fusca* and *C. gessneri* always carried a cytidine (C). *C. morettii* on the other hand always carried an adenosine (A). The hybrid offspring between *C. morettii* and either *C. fusca* or *C. gessneri* carry one allele with an A and a second allele with a C at position 299. In the electropherogram of the

Table 1. Specimens used for genetic analysis.

Species	Stage/Sex	Specimen ID	mtCOI GenBank accession	nWG GenBank accession	Combined phylogeny
C. maclachlani	♂	SMFTRI00014886	EU215081[a]	EU215123[a]	X
	♂	SMFTRI00014887	JN596157	JN596200	X
	♂	SMFTRI00014888	JN596158	JN596201	X
	♂	SMFTRI00014889	JN596159	JN596202	X
C. rugulosa	♂	SMFTRI00014890	EU215083[a]	EU215125[a]	X
C. fusca	♂	SMFTRI00014891		JN596228	
	♂	SMFTRI00014892	JN596160	JN596185	X
	♂	SMFTRI00014893	JN596161	JN596186	X
	♂	SMFTRI00014894		JN596187	
	♂	SMFTRI00014895	JN596162	JN596188	X
	♂	SMFTRI00014896		JN596189	
	♂	SMFTRI00014897	JN596163	JN596190	X
	♂	SMFTRI00014898		JN596191	
	L	SMFTRI00014899	JN596164	JN596218	X
C. gessneri	♂	SMFTRI00014900		JN596192	
	♂	SMFTRI00014901		JN596193	
	♂	SMFTRI00014902		JN596194	
	♂	SMFTRI00014903		JN596195	
	♂	SMFTRI00014904	JN596181	JN596196	X
	♂	SMFTRI00014905	JN596182	JN596197	X
	♂	SMFTRI00014906		JN596198	
	♂	SMFTRI00014907	JN596180	JN596199	X
C. morettii	♂	SMFTRI00014909		JN596203	
	♂	SMFTRI00014911		JN596204	
	♂	SMFTRI00014912	JN596171	JN596205	X
	♂	SMFTRI00014913	JN596172	JN596206	X
	L	SMFTRI00014914	JN596173	JN596219	X
	♂	SMFTRI00014915	JN596177	JN596216	X
	♀	SMFTRI00014916	JN596176	JN596217	X
C. villosa	♂	SMFTRI00014918		JN596207	
	♂	SMFTRI00014919		JN596208	
	♂	SMFTRI00014920		JN596209	
	♂	SMFTRI00014921	JN596178	JN596210	X
	♂	SMFTRI00014922	JN596179	JN596211	X
	♂	SMFTRI00014924		JN596212	
C. fusca ♂ × *C. morettii* ♀	♂	SMFTRI00014925	JN596170	JN596213	X
	♀	SMFTRI00014926		JN596214	
	L	SMFTRI00014927		JN596227	
	L	SMFTRI00014928	JN596174	JN596220	X
	L	SMFTRI00014929	JN596175		
C. morettii ♂ × *C. fusca* ♀	♀	SMFTRI00014930	JN596168	JN596215	X
	♂	SMFTRI00014931	JN596169		
	L	SMFTRI00014932	JN596165	JN596221	X
	L	SMFTRI00014933	JN596166	JN596222	X
	L	SMFTRI00014934	JN596167	JN596223	X
C. morettii ♂ × *C. gessneri* ♀	L	SMFTRI00014935	JN596183	JN596224	X
	L	UMSP000113617	JN596184	JN596225	X
	L	UMSP000113618		JN596226	

[a]Taken from Pauls *et al.* (2008).

sequencing trace, both alleles are observed at position 299 making the base at this position ambiguous between A and C, which by IUPAC convention is defined as amino (M) (NC-IUB, 2010). All variable positions that differed between parental species resulted in consistently ambiguous patterns of the hybrid offspring (Table 2). As a control we also examined the F1 offspring sired from *C. morettii* ♂ and ♀, which always showed only alleles found in *C. morettii* adults caught in the wild (Table 2).

Besides *C. fusca*, *C. gessneri*, *C. morettii*, *C. villosa*, and hybrids among these taxa, we also included one specimen of *C. rugulosa* and four specimens of *C. maclachlani* Stein 1874 as outgroup taxa in our phylogeny (Table 1). ModelTest 3.7 (Posada and Crandall, 1998) selected the

Table 2. Variable base position in wingless gene (WG) that shows two alleles in hybrid specimens. Shown are the states observed in parent species *C. fusca*, *C. gessneri*, and *C. moretti*, as well as the nucleotide ambiguity observed in hybrid offspring *fusca* ♂ × *moretti* ♀, *moretti* ♂ × *fusca* ♀, *moretti* ♂ × *C. gessneri* ♀.

	Nucleotide position[a]								
Species	90	110	113	137	140	170	248	260	299
fusca	C	C	C	C/S	G	G	G	C/Y	C
gessneri	A/M	T	C	G	G	G	G	C	C
moretti	C	C	C/Y	G	A	A/G/R	A	C	A
fusca ♂ × *moretti* ♀	C	C	C/Y	G	R	G/R	R	C	M
moretti ♂ × *fusca* ♀	C	C	C	S	R	G	R	C/Y	M
moretti ♂ × *gessneri* ♀	M	Y	C	G	R	G	R	C	M
moretti F1	C	C	Y	G	A	G/R	A	C	A

[a]M = A/C; R = A/G; S = C/G; Y = C/T.

models GTR + G and HKY + I for the mtCOI and wingless data, respectively. Standard deviation of likelihood splits after 3×10^6 generations was 0.005 for mtCOI and 0.007 for WG, and 0.005 for the combined run. The results of the combined run are presented in Figure 2. The topology of the mtCOI analysis was identical to the combined run (not shown); the topology of the wingless run showed an unresolved comb for all our ingroup sequences (not shown). This suggests that the topology of the combined analysis is informed almost entirely from the mtCOI data. In the topology, we can see that the examined species of *Chaetopteryx* form a highly supported clade with regard to *C. maclachlani* (pp = 1.0). Within genus *Chaetopteryx*, *C. rugulosa* is basal to the other taxa, which again form a highly supported clade (pp = 1.0). Within this clade we can clearly trace the maternal inheritance of the mtCOI gene in the hybrid specimens. *C. morettii* and *fusca* ♂ × *morettii* ♀ hybrids form one distinct clade (pp = 1.0). *C. gessneri* and *morettii* ♂ × *gessneri* ♀ hybrids form two supported clades (pp = 1.0), but the relationship among them is unresolved. The hybrids, however, do not group with the genotypes of the father, suggesting that the females from which they were sired carry a different haplotype than the *C. gessneri* males we sequenced. *C. fusca*, *C. villosa*, and *morettii* ♂ × *fusca* ♀ hybrids form a clade (pp = 1.0) that follow the expectations of maternal inheritance of mitochondrial genes. While *C. villosa* is basal to *C. fusca* and *morettii* ♂ × *fusca* ♀ hybrids, this relationship is only supported with a pp = 0.94.

Discussion

In eidonomical respect, it appears that the characters are intermediate but with a slight tendency to maternal species. The lack in emergence success of larvae of *gessneri* ♂ × *morettii* ♀ matings, and the poor success of *morettii* ♂ × *gessneri* ♀ matings suggests reduced fitness and in the latter case even post-mating incompatibility. The fact that the hybridogenous eggs with *gessneri* failed to develop or only very few larvae emerged supports the conclusion that *C. gessneri* is more distantly related, while *fusca* and *morettii* are more closely related and may be genetically more compatible. While our approach cannot discount this possibility, the molecular phylogenetic analysis does not support this conclusion.

The phylogenetic analysis shows no evidence for a hybrid origin of *C. moretti*. Three patterns would suggest a hybrid origin: (1) an intermediate placement between parent species, as observed for the *moretti* ♂ × *gessneri* ♀ offspring; (2) a grouping of *C. moretti* specimens within both the parent species clades if females of both species were involved in the original hybrid population, or (3) placement of *C. moretti* within or significantly closer to one of the parent clades if only one species provided the females in the original hybrid population. Instead we find that *C. moretti* is clearly basal to both *C. gessneri* and *C. fusca*.

We are aware that the two approaches used in this study produced contradictory results. From the traditional cross-breeding method that resulted in many healthy offspring specimens from the combination of *C. fusca* and *C. morettii*, and poor or no offspring from the combination of *C. gessneri* with the two others, one would conclude that *fusca* and *morettii* are genetically closer than to *gessneri*. However, the molecular analysis shows that *fusca* and *gessneri* are closer and *morettii* is more distant. This discrepancy may be the starting point for a fundamental discussion on the methodology, but we content ourselves with pointing at the facts.

The results of the genetic analysis and phylogeny clearly demonstrate that the offspring hatching from eggs laid by females involved in the hybrid mating experiments were actually hybrid offspring and not the result of parthenogenesis. Parthenogenesis or facultative parthenogenesis is known from other limnephilid species, for example in the genus *Apatania* (Malicky, 2005; Salokannel *et al.*, 2010). Besides clarifying the results in our experiment, the molecular results of our study show that the molecular markers we used in the study can be useful at identifying hybrid specimens in the wild or tracing the origin of taxa that putatively evolved through hybridization.

C. morettii and *C. fusca* have allopatric distributions without any known contact zone which explains that hybrids are unknown from field collections. However, in a situation of possible contact, intermediate specimens

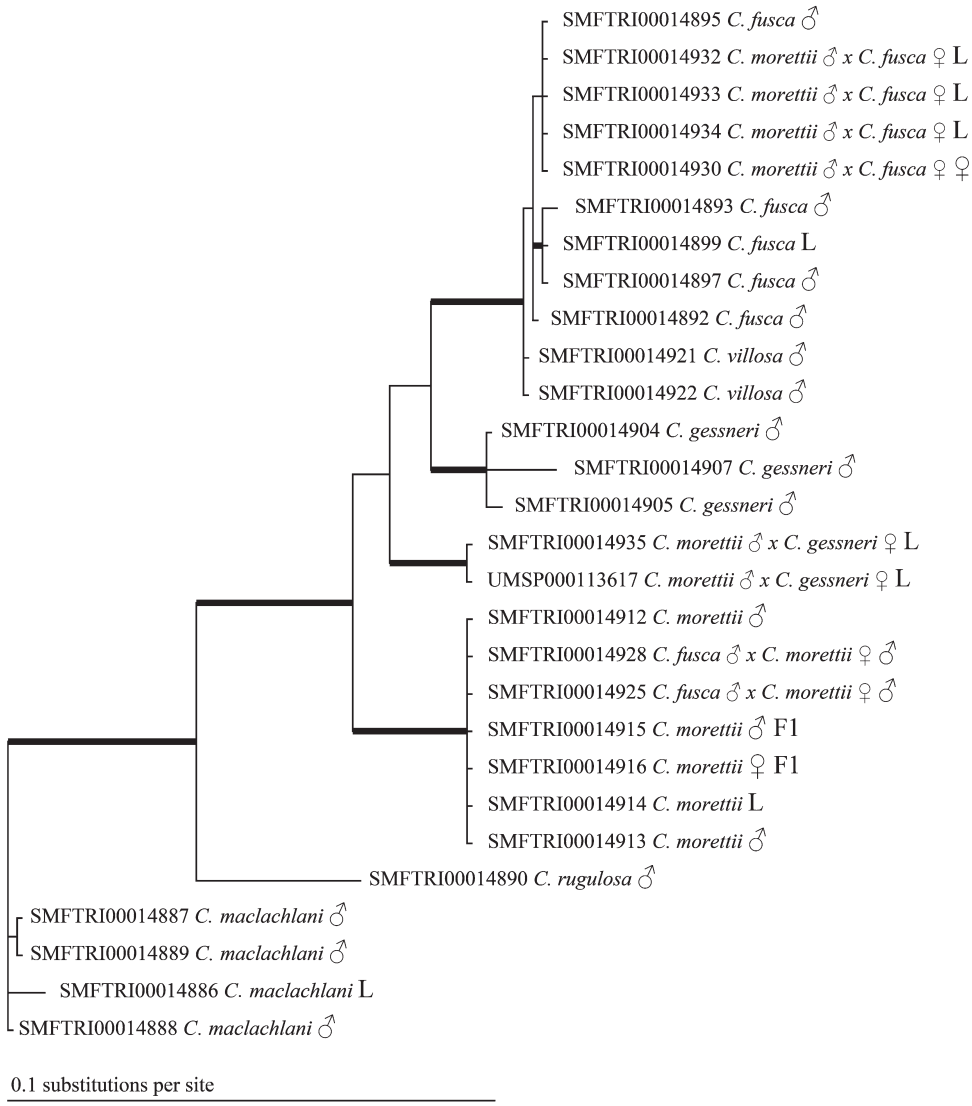

Fig. 2. Bayesian phylogenetic inference of combined mtCOI and nWG sequence data of five species of *Chaetopteryx*, their hybrids and *C. maclachlani*. Rooted 50% majority rule consensus tree based on a Bayesian sampling of 4000 trees. Bold branches indicate nodes with significant posterior probabilities (> 0.94). Taxon labels indicate specimen ID (see Table 1), the species or hybrid taxon, and sex (♂, ♀) of adults. Larvae are designated by "L".

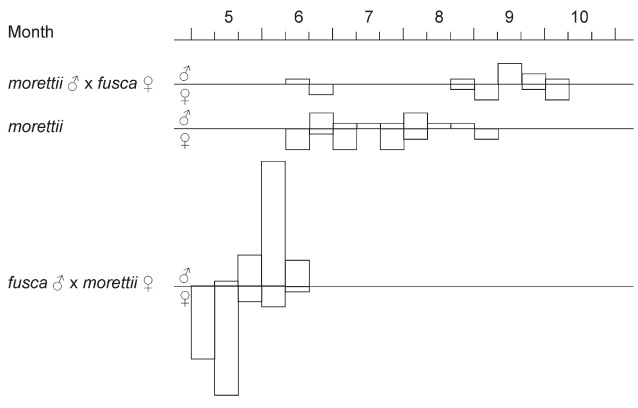

Fig. 3. Emergence patterns of *C. morettii* and hybrids under long-day conditions. Lowest bar represents 1 individual.

could be expected in the field. The molecular approach we applied is simple but effective for detecting recent hybrids in nature, *e.g.*, in contact zones between closely related species. The decreasing cost of DNA sequencing and the increasing availability of suitably variable nuclear markers for shallow phylogenetic and population level analyses will make it increasingly easy to detect hybrids. This is an important development in conservation, where hybridization between native and invasive species is often a matter of concern (*e.g.*, Durand *et al.*, 2002; Brumfield, 2010). The approach can also be applied to test potential cases of hybrid speciation (Durand *et al.*, 2002).

It is noteworthy though not central for this study that the development of the three strains was different under the same conditions (Fig. 3). These differences were probably influenced by the artificial long-day conditions and the unusually nutritious food. Adults of the mating

combination *fusca* ♂ × *morettii* ♀ started to emerge at the end of April and finished in mid-June. Adults of the combination *morettii* ♂ × *fusca* ♀ started emerging in June, but the bulk emerged from the end of August to the beginning of October. Adults of *C. moretti*, however, emerged from mid-June to the beginning of September. The reasons for these differences are unknown, but the observations are worth mentioning. Differences in emergence and phenology in general can offer evidence for speciation through resource partitioning and temporal disjunction, which is an important mode of sympatric speciation (Gullan and Cranston, 2010). This could also be a mechanism in other *Chaetopteryx* species and other caddisfly taxa where contact zones exist and hybridization occurs, though explicit tests are currently outstanding.

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