

DNA barcoding of Corsican mayflies (Ephemeroptera) with implications on biogeography, systematics and biodiversity

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Abstract

Mayflies (Ephemeroptera) are known to generally present a high degree of insular endemism: half of the 28 species known from Corsica and Sardinia are considered as endemic. We sequenced the DNA barcode (a fragment of the mitochondrial CO1 gene) of 349 specimens from 50 localities in Corsica, Sardinia, continental Europe and North Africa. We reconstructed gene trees of eight genera or species groups representing the main mayfly families. Alternative topologies were built to test if our reconstructions suggested a single or multiple Corsican/Sardinian colonization event(s) in each genus or species group. A molecular clock calibrated with different evolution rates was used to try to link speciation processes with geological events. Our results confirm the high degree of endemism of Corsican and Sardinian mayflies and the close relationship between these two faunas. Moreover, we have evidence that the mayfly diversity of the two islands is highly underestimated as at least six new putative species occur on the two islands. We demonstrated that the Corsican and Sardinian mayfly fauna reveals a complex history mainly related to geological events. The Messinian Salinity Crisis, which is thought to have reduced marine barriers, thus facilitating gene flow between insular and continental populations, was detected as the most important event in the speciation of most lineages. Vicariance processes related to the split and rotation of the Corso-Sardinian microplate had a minor impact as they involved only two genera with limited dispersal and ecological range. Colonization events posterior to the Messinian Salinity Crisis had only marginal effects as we had indication of recent gene flow only in two clades. With very limited recent gene flow and a high degree of endemism, mayflies from Corsica and Sardinia present all the criteria for conservation prioritization.

Key words

North-western Mediterranean Sea, paleogeography, Messinian Salinity Crisis.

1. Introduction

The Mediterranean Basin is considered as one of the 25 main hotspots on Earth for conservation prioritization (DE FIGUEROA et al. 2013; MYERS et al. 2000). The high degree of endemism in Corsica, Sardinia and other islands of the Tyrrhenian Sea is one of the main factors of the high level of importance of the biodiversity present in this area (MEDAIL & QUEZEL 1999). Corsica possesses the highest concentration of endemic aquatic insects in Eu-

rope (GIUDICELLI 1975). Different events of the complex paleogeographic history of Corsica and Sardinia may have promoted diversification in these islands (BIDEGARRAY-BATISTA & ARNEDO 2011; MEDAIL & QUEZEL 1999; ROSENBAUM et al. 2002). The Corso-Sardinian microplate belongs to the Hercynian chain. Before the Oligocene, this microplate was located close to the French and Spanish mainland, with the Balearics to the South-West, the

two Kabylian and Peloritan-Calabrian microplates at uncertain positions to the South-East. These microplates broke off continental Europe about 30 Mya (= million years ago) (OGGIANO et al. 2009). The split of Corsica + Sardinia was followed by an anti-clockwise rotation of the microplate, which began 21–20.5 Mya and ended 18.0–17.5 Mya (DE JONG 1998; EDEL et al. 2001; OGGIANO et al. 2009). Importantly, during the rotation, the microplate may have kept periodic connections with the continent, especially through the Calabrian microplate (ROSENBAUM et al. 2002).

During the Messinian Salinity Crisis (MSC, 5.96–5.33 Mya), the Mediterranean Sea was disconnected from the Atlantic Ocean; one of the most important consequences was that the Mediterranean Sea became largely desiccated by evaporation about 5.6 Mya (DE JONG 1998; HSÜ et al. 1977; MANZI et al. 2013). The MSC had important complex impacts on the climate. The desiccation of the sea led to an increase of the temperature (MURPHY et al. 2009), although roughly coincident with a glacial stage (MANZI et al. 2013). The amount of precipitation decreased in the South-West and Central parts of the Mediterranean area while increasing in the Alps (FAUQUETTE et al. 2006; MURPHY et al. 2009). These climatic changes had a major impact on stream flow and water temperature and consequently on aquatic macroinvertebrate composition. Moreover, the desiccation of the Mediterranean Sea re-established connections between the islands, southern Europe and North Africa, as well as between some basins. The refilling of the Mediterranean Sea through the Gibraltar Strait was extremely fast from a few months to two years (GARCIA-CASTELLANOS et al. 2009) and re-established the effective isolation of the island ecosystem (ROSENBAUM et al. 2002). During the Quaternary glacial cycles there were considerable sea level fluctuations, with a lowest point of 120 meters below the present sea level (ROHLING et al. 1998). These oscillations created land bridges between Corsica and Sardinia, and probably also with the continent, via Elba Island; they greatly reduced the effect of the sea barrier by favoring “stepping stone” colonization (STÖCK et al. 2008). As glaciers never completely covered Corsica and Sardinia (KUHLEMANN et al. 2005), the islands potentially acted as refugia.

The main aim of phylogeography is to understand evolutionary processes in their geographical context by analyzing the distribution of genealogical lineages (AVISE 1998; FIORENTINO et al. 2010). Although many studies have been carried out on the phylogeography of West Mediterranean animals (e.g. ALLEGRUCCI et al. 2005; CANESTRELLI et al. 2006; CESARONI et al. 1989; FIORENTINO et al. 2010; HOFMAN et al. 2007; KETMAIER et al. 2006; KETMAIER et al. 2010; STEFANI et al. 2004; TRONTELI et al. 2005), very few of them focused on aquatic insects (FOCHETTI et al. 2009; RIBERA et al. 2011; TRIZZINO et al. 2011) and there are no mayfly studies of which we are aware. Ephemeroptera appeared in the fossil record in the late Carboniferous or in the early Permian (HUBBARD & KUKALOVÁ-PECK 1980; EDMUNDS & MCCAFFERTY 1988; BRITAIN & SARTORI 2009).

Mayflies live in freshwater habitats in all continents, except Antarctica (BARBER-JAMES et al. 2008). They are divided into 42 families, 400 genera and more than 3000 species (BARBER-JAMES et al. 2008). Their larvae are strictly freshwater dependent while subimagos, a maturation stage unique in pterygote insect, and imagos are aerial and only live from a few hours to a few days (BRITAIN & SARTORI 2009). Due to their limited dispersal capacity and particular habitat requirements, current mayfly distribution was assumed to reflect paleogeological events like continental drift and ancient dispersal history (EDMUNDS 1972). Nevertheless, some mayfly taxa have colonized recent volcanic islands such as La Réunion, isolated by more than 700 km from Madagascar, the nearest continental island (GATTOLLIAT 2004). Moreover, recent molecular studies demonstrated that dispersal power of mayflies has been underestimated and that vicariance, radiation and dispersal have to be cautiously taken into account in order to explain their current distribution (RUTSCHMANN et al. 2014; VUATAZ et al. 2013). In Madagascar, the Baetidae fauna proved to be the result of a combination of vicariance, radiation and bidirectional mainland-island dispersal (MONAGHAN et al. 2005). A major radiation occurred in a clade strictly endemic to Madagascar with important evolution processes (GATTOLLIAT & SARTORI 2000; MONAGHAN et al. 2005).

A total of 28 mayfly species are reported from Corsica and/or Sardinia (Table 1). Only genera belonging to the families Baetidae, Leptophlebiidae and Heptageniidae have been taxonomically re-evaluated (BELFIORE 1987a,b,c; SARTORI & JACOB 1986; THOMAS & SOLDÁN 1987; THOMAS & GAZAGNES 1984; SARTORI & THOMAS 1989). Corsican and Sardinian mayflies present important affinities due to their geographical proximity and common history: three-quarters of the species are thought to be shared by both islands. Among these 28 reported species, five are thought to be strictly endemic to Corsica, one strictly endemic to Sardinia and six are Corso-Sardinian endemics.

Although powerful molecular methods are available for better understanding relationships between insular and continental populations, no molecular data are available for Corsican and Sardinian mayflies. In the present study, we sequenced a 658 bp fragment of the mitochondrial protein-coding gene cytochrome c oxidase subunit I (CO1) from 349 specimens belonging to seven genera (three specimens per locality per species). We tested the following issues:

(1) Do the CO1 trees confirm the high level of insular endemism, and is the morphological taxonomy of Corsican and Sardinian mayflies challenged by molecular reconstructions?

We also measured the genetic distances between haplotypes or haplogroups using the Kimura 2-parameter (K2P) model, especially to estimate if clades representing different insular populations as well as insular and continental populations should be considered as conspecific or not.

Table 1. List of mayfly species reported from Corsica and Sardinia (in literature: BUFFAGNI et al. 2003 and <http://www.opie-benthos.fr/opie/insecte.php>). In boldface: species included in the analysis. Continental: species reported from continental Europe and/or North Africa.

| Family | Species | Corsica | Sardinia | Continental |
|------------------------|--|-----------|-----------|-------------|
| Baetidae | <i>Alainites albinatii</i> (Sartori & Thomas, 1989) | × | | |
| Baetidae | <i>Alainites muticus</i> (Linnaeus, 1758) | | × | × |
| Baetidae | <i>Baetis buceratus</i> Eaton, 1870 | × | × | × |
| Baetidae | <i>Baetis cyrneus</i> Thomas & Gazagnes, 1984 | × | × | |
| Baetidae | <i>Baetis fuscatus</i> (Linnaeus, 1761) | × | × | × |
| Baetidae | <i>Baetis ingridae</i> Thomas & Soldan, 1987 | × | | |
| Baetidae | <i>Baetis rhodani</i> (Pictet, 1843) | | × | × |
| Baetidae | <i>Centroptilum luteolum</i> (Müller, 1776) | | × | × |
| Baetidae | <i>Cloeon dipterum</i> (Linnaeus, 1761) | × | × | × |
| Baetidae | <i>Cloeon simile</i> Eaton, 1870 | × | × | × |
| Baetidae | <i>Procloeon bifidum</i> (Bengtsson, 1912) | | × | × |
| Caenidae | <i>Caenis luctuosa</i> (Burmeister, 1839) | | × | × |
| Caenidae | <i>Caenis martae</i> Belfiore, 1984 | × | × | × |
| Caenidae | <i>Caenis strugaensis</i> Ikononov, 1961 | | × | × |
| Ephemerellidae | <i>Serratella ignita</i> (Poda, 1761) | × | × | × |
| Heptageniidae | <i>Ecdyonurus corsicus</i> (Esben-Petersen, 1912) | × | × | |
| Heptageniidae | <i>Ecdyonurus cortensis</i> Belfiore, 1988 | × | × | |
| Heptageniidae | <i>Electrogena fallax</i> (Hagen, 1864) | × | × | |
| Heptageniidae | <i>Electrogena zebrata</i> (Hagen, 1864) | × | × | |
| Heptageniidae | <i>Rhithrogena eatoni</i> Esben-Petersen, 1912 | × | | |
| Heptageniidae | <i>Rhithrogena insularis</i> Esben-Petersen, 1913 | × | | |
| Heptageniidae | <i>Rhithrogena nuragica</i> Belfiore, 1987 | | × | |
| Leptophlebiidae | <i>Choroterpes picteti</i> (Eaton, 1871) | × | | × |
| Leptophlebiidae | <i>Habroleptoides budtzi</i> (Esben-Petersen, 1912) | × | | |
| Leptophlebiidae | <i>Habroleptoides modesta</i> (Hagen, 1864) | × | × | |
| Leptophlebiidae | <i>Habrophlebia consiglioi</i> Biancheri, 1959 | | × | × |
| Leptophlebiidae | <i>Habrophlebia eldae</i> Jacob & Sartori, 1984 | × | × | × |
| Siphonuridae | <i>Siphonurus lacustris</i> Eaton, 1870 | × | × | × |
| | Total | 20 | 22 | |

(2) Are the main branching events of CO1 trees congruent with some Corsican and Sardinian paleogeographic events or are they related to recent colonization? To evaluate the dating of the different branching events, we reconstructed ultrametric CO1 trees under a strict molecular clock model.

2. Material and methods

2.1. Sampling

We sampled 51 running water localities in Corsica, Sardinia and adjacent European continental regions (France: Mercantour, Mont Ventoux; Italy: Appennino, Toscana, Liguria) in May and July 2009 (Fig. 1, Table 2). Mayfly larvae were collected using kick-sampling. The approximately 5,000 collected specimens were preserved in 100% ethanol in the field, sorted and identified under a dissecting microscope in the laboratory, and housed at -18°C at the Museum of Zoology in Lausanne, Switzerland (MZL).

We focused on seven genera belonging to four families: *Serratella* (Ephemerellidae); *Electrogena* and *Ec-*

dyonurus (Heptageniidae); *Habrophlebia* and *Habroleptoides* (Leptophlebiidae); *Alainites* and two species groups in the genus *Baetis* (Baetidae): *Baetis* gr. *rhodani* (including *B. ingridae*) and *Baetis* gr. *alpinus* (including *B. cyrneus*). As a result, half of the mayfly species known from Corsica and Sardinia were included in our analysis (boldface in Table 1). We sequenced, when possible, three specimens per species per locality (Table 2). Additional specimens from Central Europe and Tunisia, stored in the collections of the MZL, were also included. To cover the Alpine area, Morocco and the Canary Islands, we used *Baetis* gr. *alpinus* and *Baetis* gr. *rhodani* unpublished sequences from Dr. Michael T. Monaghan (IGB, Berlin, Germany), as well as sequences from GenBank (accession numbers: AY265231; AY265245; AY265248; AY265252; AY265255; AY265256; AY26525; AY265259; AY265267; AY265272; AY265284; AY265285; AY265289; AY265294; AY265304; AY326909.1 from WILLIAMS et al. 2006; GU682393.1 from IBOLD).

2.2. Laboratory procedures

DNA was extracted from 349 specimens using Qiagen Extraction Kits (Qiagen, Hilden, Germany). We amplified 658 bp of the mitochondrial protein-coding gene

Table 2. List of the sequenced specimens (a single specimen per locality per haplotype is included in the table). Code corresponds to the tip labels of the figures including in supporting information. MZL: material collected by the authors.

| Taxa | Code | Collection locality | Collector | Date | Genbank accession |
|--|--------------|----------------------|-----------|------------|-------------------|
| Baetidae <i>Alainites</i> | | | | | |
| <i>Alainites albinatii</i> | AL-10-CO | Corsica | MZL | May 2009 | HG934991 |
| <i>Alainites albinatii</i> | AL-12-CO | Corsica | MZL | May 2009 | HG934992 |
| <i>Alainites albinatii</i> | AL-12-CO2 | Corsica | MZL | May 2009 | HG934993 |
| <i>Alainites albinatii</i> | AL-15-CO | Corsica | MZL | May 2009 | HG934994 |
| <i>Alainites albinatii</i> | AL-17-CO | Corsica | MZL | May 2009 | HG934995 |
| <i>Alainites</i> cf. <i>muticus</i> | AL-27-SA | Sardinia | MZL | May 2009 | HG934996 |
| <i>Alainites</i> cf. <i>muticus</i> | AL-27-SA2 | Sardinia | MZL | May 2009 | HG934997 |
| <i>Alainites</i> cf. <i>muticus</i> | AL-43-FR(ME) | France, Mercantour | MZL | July 2009 | HG934999 |
| <i>Alainites muticus</i> | AL-36-FR(MV) | France, Mont Ventoux | MZL | July 2009 | HG934998 |
| <i>Alainites muticus</i> | AL-CH | Switzerland | MZL | April 2009 | HG935000 |
| Baetidae <i>Baetis</i> gr. <i>alpinus</i> | | | | | |
| <i>Baetis alpinus</i> | BA-33-IT(AP) | Italia, Appennino | MZL | May 2009 | HG935011 |
| <i>Baetis alpinus</i> | BA-34-IT(AP) | Italia, Appennino | MZL | May 2009 | HG935012 |
| <i>Baetis alpinus</i> | BA-36-FR(MV) | France, Mont Ventoux | MZL | July 2009 | HG935013 |
| <i>Baetis alpinus</i> | BA-40-FR(ME) | France, Mercantour | MZL | July 2009 | HG935014 |
| <i>Baetis alpinus</i> | BA-43-FR(ME) | France, Mercantour | MZL | July 2009 | HG935015 |
| <i>Baetis alpinus</i> | BA-44-FR(ME) | France, Mercantour | MZL | July 2009 | HG935016 |
| <i>Baetis alpinus</i> | BA-45-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935017 |
| <i>Baetis alpinus</i> | BA-47-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935018 |
| <i>Baetis alpinus</i> | BA-48-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935019 |
| <i>Baetis alpinus</i> | BA-50-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935020 |
| <i>Baetis</i> cf. <i>cyrneus</i> | BA-09-CO | Corsica | MZL | May 2009 | HG935001 |
| <i>Baetis</i> cf. <i>cyrneus</i> | BA-10-CO | Corsica | MZL | May 2009 | HG935002 |
| <i>Baetis</i> cf. <i>cyrneus</i> | BA-12-CO | Corsica | MZL | May 2009 | HG935003 |
| <i>Baetis</i> cf. <i>cyrneus</i> | BA-13-CO | Corsica | MZL | May 2009 | HG935004 |
| <i>Baetis</i> cf. <i>cyrneus</i> | BA-14-CO | Corsica | MZL | May 2009 | HG935005 |
| <i>Baetis</i> cf. <i>cyrneus</i> | BA-15-CO | Corsica | MZL | May 2009 | HG935006 |
| <i>Baetis</i> cf. <i>cyrneus</i> | BA-20-SA | Sardinia | MZL | May 2009 | HG935007 |
| <i>Baetis</i> cf. <i>cyrneus</i> | BA-21-SA | Sardinia | MZL | May 2009 | HG935008 |
| <i>Baetis</i> cf. <i>cyrneus</i> | BA-26-SA | Sardinia | MZL | May 2009 | HG935009 |
| <i>Baetis</i> cf. <i>cyrneus</i> | BA-27-SA | Sardinia | MZL | May 2009 | HG935010 |
| <i>Baetis punicus</i> | BA-TU | Tunisia | S. Zrelli | April 2009 | HG935021 |
| Baetidae <i>Baetis</i> gr. <i>rhodani</i> | | | | | |
| <i>Baetis</i> cf. <i>ingridae</i> | BR-08-CO | Corsica | MZL | May 2009 | HG935022 |
| <i>Baetis</i> cf. <i>ingridae</i> | BR-08-CO2 | Corsica | MZL | May 2009 | HG935023 |
| <i>Baetis</i> cf. <i>ingridae</i> | BR-08-CO3 | Corsica | MZL | May 2009 | HG935024 |
| <i>Baetis</i> cf. <i>ingridae</i> | BR-20-CO | Corsica | MZL | May 2009 | HG935025 |
| <i>Baetis</i> cf. <i>ingridae</i> | BR-21-SA | Sardinia | MZL | May 2009 | HG935026 |
| <i>Baetis</i> cf. <i>ingridae</i> | BR-25-SA | Sardinia | MZL | May 2009 | HG935027 |
| <i>Baetis</i> cf. <i>ingridae</i> | BR-26-SA | Sardinia | MZL | May 2009 | HG935028 |
| <i>Baetis</i> cf. <i>ingridae</i> | BR-27-SA | Sardinia | MZL | May 2009 | HG935029 |
| <i>Baetis</i> cf. <i>rhodani</i> | BR-28-IT(TO) | Italia, Toscana | MZL | May 2009 | HG935030 |
| <i>Baetis</i> cf. <i>rhodani</i> | BR-29-IT(TO) | Italia, Toscana | MZL | May 2009 | HG935031 |
| <i>Baetis</i> cf. <i>rhodani</i> | BR-34-FR(AP) | Italia, Appennino | MZL | May 2009 | HG935032 |
| <i>Baetis</i> cf. <i>rhodani</i> | BR-38-FR(MV) | France, Mont Ventoux | MZL | July 2009 | HG935033 |
| <i>Baetis</i> cf. <i>rhodani</i> | BR-42-FR(ME) | France, Mercantour | MZL | July 2009 | HG935034 |
| <i>Baetis</i> cf. <i>rhodani</i> | BR-45-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935035 |
| <i>Baetis</i> cf. <i>rhodani</i> | BR-50-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935036 |
| <i>Baetis</i> cf. <i>rhodani</i> | BR-CH | Switzerland | MZL | April 2009 | HG935037 |
| <i>Baetis</i> cf. <i>rhodani</i> | BR-TU | Tunisia | S. Zrelli | April 2009 | HG935038 |
| Heptageniidae <i>Ecdyonurus</i> | | | | | |
| <i>Ecdyonurus corsicus</i> | EC-10-CO | Corsica | MZL | May 2009 | HG935039 |
| <i>Ecdyonurus corsicus</i> | EC-26-SA | Sardinia | MZL | May 2009 | HG935040 |
| <i>Ecdyonurus</i> sp. | EC-28-IT(TO) | Italia, Toscana | MZL | May 2009 | HG935041 |
| <i>Ecdyonurus</i> sp. | EC-29-IT(TO) | Italia, Toscana | MZL | May 2009 | HG935042 |
| <i>Ecdyonurus</i> sp. | EC-34-IT(AP) | Italia, Appennino | MZL | May 2009 | HG935043 |
| <i>Ecdyonurus</i> sp. | EC-35-FR(MV) | France, Mont Ventoux | MZL | July 2009 | HG935044 |
| <i>Ecdyonurus</i> sp. | EC-37-FR(MV) | France, Mont Ventoux | MZL | July 2009 | HG935045 |
| <i>Ecdyonurus</i> sp. | EC-40-FR(ME) | France, Mercantour | MZL | July 2009 | HG935046 |
| <i>Ecdyonurus</i> sp. | EC-42-FR(ME) | France, Mercantour | MZL | July 2009 | HG935047 |

Table 2 continued.

| Taxa | Code | Collection locality | Collector | Date | Genbank accession |
|--|--------------|----------------------|-----------|------------|-------------------|
| Heptageniidae <i>Ecdyonurus</i> | | | | | |
| <i>Ecdyonurus</i> sp. | EC-43-FR(ME) | France, Mercantour | MZL | July 2009 | HG935048 |
| <i>Ecdyonurus</i> sp. | EC-47-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935049 |
| <i>Ecdyonurus</i> sp. | EC-50-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935050 |
| <i>Ecdyonurus</i> sp. | EC-TU | Tunisia | MZL | July 2009 | HG935051 |
| Heptageniidae <i>Electrogena</i> | | | | | |
| <i>Electrogena fallax</i> | EF-01-CO | Corsica | MZL | May 2009 | HG935052 |
| <i>Electrogena fallax</i> | EF-04-CO | Corsica | MZL | May 2009 | HG935053 |
| <i>Electrogena fallax</i> | EF-11-CO | Corsica | MZL | May 2009 | HG935054 |
| <i>Electrogena fallax</i> | EF-12-CO | Corsica | MZL | May 2009 | HG935055 |
| <i>Electrogena fallax</i> | EF-14-CO | Corsica | MZL | May 2009 | HG935056 |
| <i>Electrogena lateralis</i> | ELLA-CH | Switzerland | A. Wagner | May 2007 | HG935060 |
| <i>Electrogena lateralis</i> | ELLA-FR | France, Savoie | A. Wagner | March 2006 | HG935061 |
| <i>Electrogena lateralis</i> | ELLA-FR2 | France, Ardèche | A. Wagner | May 2005 | HG935062 |
| <i>Electrogena lateralis</i> | ELLA-FR3 | France, Isère | A. Wagner | June 2006 | HG935063 |
| <i>Electrogena</i> sp. | EL-31-IT(AP) | Italia, Appennino | MZL | May 2009 | HG935057 |
| <i>Electrogena</i> sp. | EL-32-IT(AP) | Italia, Appennino | MZL | May 2009 | HG935058 |
| <i>Electrogena</i> sp. | EL-33-IT(AP) | Italia, Appennino | MZL | May 2009 | HG935059 |
| <i>Electrogena zebrata</i> | EZ-04-CO | Corsica | MZL | May 2009 | HG935064 |
| <i>Electrogena zebrata</i> | EZ-07-CO | Corsica | MZL | May 2009 | HG935065 |
| <i>Electrogena zebrata</i> | EZ-21-SA | Sardinia | MZL | May 2009 | HG935066 |
| <i>Electrogena zebrata</i> | EZ-25-SA | Sardinia | MZL | May 2009 | HG935067 |
| <i>Electrogena zebrata</i> | EZ-26-SA | Sardinia | MZL | May 2009 | HG935068 |
| <i>Electrogena zebrata</i> | EZ-27-SA | Sardinia | MZL | May 2009 | HG935069 |
| Leptophlebiidae <i>Habroleptoides</i> | | | | | |
| <i>Habroleptoides budtzi</i> | HL-01-CO | Corsica | MZL | May 2009 | HG935070 |
| <i>Habroleptoides budtzi</i> | HL-06-CO | Corsica | MZL | May 2009 | HG935071 |
| <i>Habroleptoides budtzi</i> | HL-07-CO | Corsica | MZL | May 2009 | HG935072 |
| <i>Habroleptoides budtzi</i> | HL-08-CO | Corsica | MZL | May 2009 | HG935073 |
| <i>Habroleptoides budtzi</i> | HL-11-CO | Corsica | MZL | May 2009 | HG935074 |
| <i>Habroleptoides</i> cf. <i>umbratilis</i> | HL-33-IT(AP) | Italia, Appennino | MZL | May 2009 | HG935080 |
| <i>Habroleptoides</i> cf. <i>umbratilis</i> | HL-40-FR(ME) | France, Mercantour | MZL | July 2009 | HG935081 |
| <i>Habroleptoides</i> cf. <i>umbratilis</i> | HL-42-FR(ME) | France, Mercantour | MZL | July 2009 | HG935075 |
| <i>Habroleptoides</i> cf. <i>umbratilis</i> | HL-49-IT(LI) | Italy, Liguria | MZL | July 2009 | HG935076 |
| <i>Habroleptoides confusa</i> | HL-CH | Switzerland | A. Wagner | April 2007 | HG935077 |
| <i>Habroleptoides confusa</i> | HL-CH2 | Switzerland | A. Wagner | May 2007 | HG935078 |
| <i>Habroleptoides modesta</i> | HL-17-CO | Corsica | MZL | May 2009 | HG935079 |
| <i>Habroleptoides</i> sp. | HL-TU | Tunisia | S. Zrelli | April 2010 | HG935082 |
| Leptophlebiidae <i>Habrophlebia</i> | | | | | |
| <i>Habrophlebia eldae</i> | HP-04-CO | Corsica | MZL | May 2009 | HG935083 |
| <i>Habrophlebia eldae</i> | HP-28-IT(TO) | Italia, Toscana | MZL | May 2009 | HG935084 |
| <i>Habrophlebia eldae</i> | HP-30-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935085 |
| <i>Habrophlebia eldae</i> | HP-31-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935086 |
| <i>Habrophlebia eldae</i> | HP-40-FR(ME) | France, Mercantour | MZL | July 2009 | HG935087 |
| <i>Habrophlebia eldae</i> | HP-45-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935088 |
| <i>Habrophlebia eldae</i> | HP-46-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935089 |
| <i>Habrophlebia eldae</i> | HP-50-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935090 |
| <i>Habrophlebia eldae</i> | HP-HU | Hungary | A. Wagner | July 2007 | HG935091 |
| <i>Habrophlebia lauta</i> | HPLA-CH | Switzerland | A. Wagner | May 2007 | HG935092 |
| EphemereIIDae <i>Serratella</i> | | | | | |
| <i>Serratella</i> cf. <i>ignita</i> | SE-36-FR(MV) | France, Mont Ventoux | MZL | July 2009 | HG935102 |
| <i>Serratella ignita</i> | SE-06-CO | Corsica | MZL | May 2009 | HG935093 |
| <i>Serratella ignita</i> | SE-08-CO | Corsica | MZL | May 2009 | HG935094 |
| <i>Serratella ignita</i> | SE-08-CO2 | Corsica | MZL | May 2009 | HG935095 |
| <i>Serratella ignita</i> | SE-08-CO3 | Corsica | MZL | May 2009 | HG935096 |
| <i>Serratella ignita</i> | SE-16-CO | Corsica | MZL | May 2009 | HG935097 |
| <i>Serratella ignita</i> | SE-23-SA | Sardinia | MZL | May 2009 | HG935098 |
| <i>Serratella ignita</i> | SE-26-SA | Sardinia | MZL | May 2009 | HG935099 |
| <i>Serratella ignita</i> | SE-27-SA | Sardinia | MZL | May 2009 | HG935100 |
| <i>Serratella ignita</i> | SE-28-IT(TO) | Italia, Toscana | MZL | May 2009 | HG935101 |
| <i>Serratella ignita</i> | SE-49-IT(LI) | Italia, Liguria | MZL | July 2009 | HG935103 |

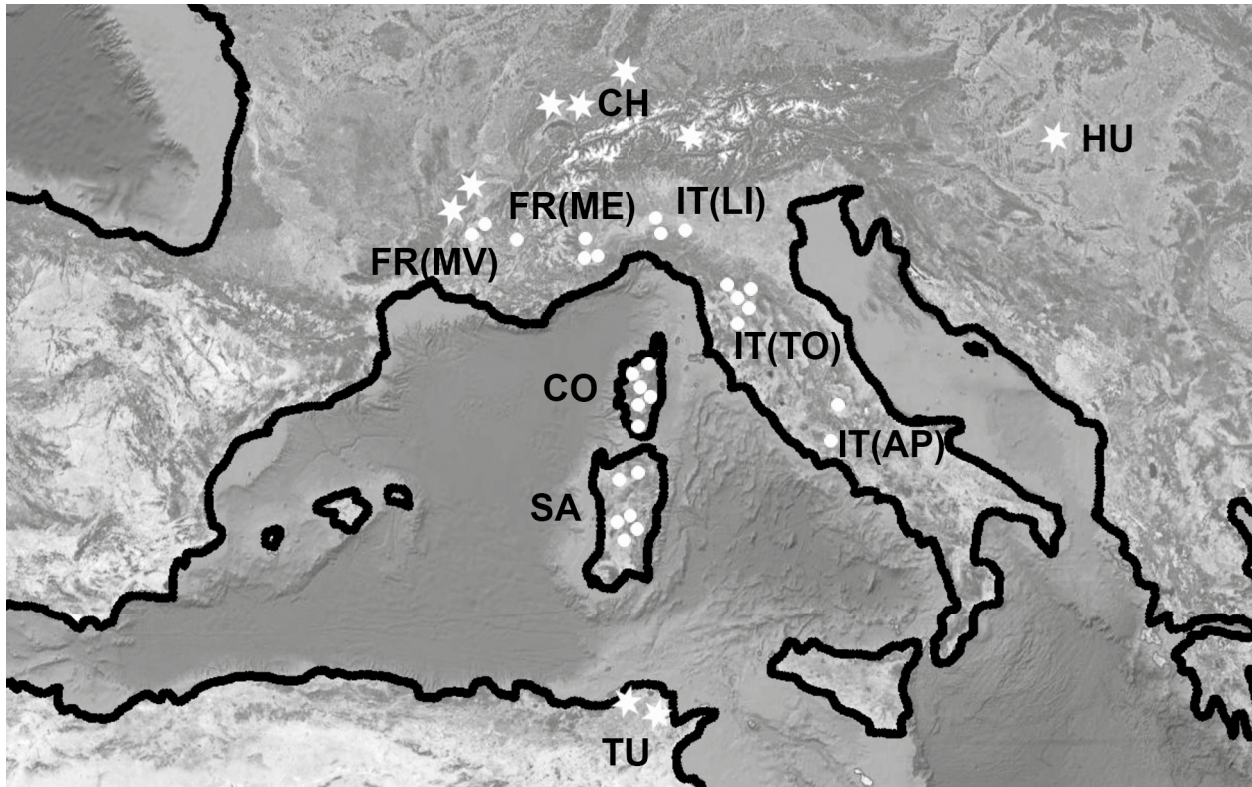


Fig. 1. Map of sampling localities: circles: material collected in 2009; stars: additional material from the MZL collections (GenBank material not reported on the map). CO = Corsica; SA = Sardinia; FR(ME) = France Mercantour; FR(MV) = France Mont Ventoux; IT(AP) = Italy Appennino; IT(LI) = Italy Liguria; IT(TO) = Italy Toscana; CH = Switzerland; HU = Hungary; TU = Tunisia.

cytochrome c oxidase subunit I (COI) using the primers LCO1490 and HCO2198 (FOLMER et al. 1994). The fragments were amplified by PCR in 25 μ l of solution containing 2.0 μ l of DNA. The reaction conditions were: 5 min at 94°C, then 30 s at 94°C, 30 s at 48°C and 60 s at 72°C (40 cycles), and a final elongation step at 72°C during 10 min. PCR products were checked by electrophoresis on 1.5% agarose gels. The PCR products were then purified using Qiagen Purification kits and sequenced in both directions using Big Dye 3.1 on an ABI 3100 automated capillary sequencer (Applied Biosystems, Foster City, CA) at the Center for Integrative Genomics (CIG), University of Lausanne.

2.3. Molecular reconstruction

Forward and reverse sequencing reads were assembled and corrected using CodonCode Aligner (CodonCode Corporation, Dedham, MA) and aligned using CLUSTALW as implemented in Mega 5.05 (TAMURA et al. 2011). Collapse 1.2 (POSADA 2004) was used to remove identical haplotypes. All sequences are available from EMBL database (HG934991–HG935103). We used the Kimura 2-parameter (K2P) model as implemented in Mega 5.05 to calculate genetic distances between haplotypes and haplogroups. According to the maximum intraspecific (3.4%) and minimum interspe-

cific (3.3%) sequence divergence previously observed in mayflies (BALL et al. 2005; KJAERSTAD et al. 2012; WEBB et al. 2007; WEBB et al. 2007, 2012; ZHOU et al. 2009, 2010), we considered 3% sequence divergence ($K2P = 0.03$) as the likely maximal value for intraspecific divergence (HEBERT et al. 2003).

Maximum Likelihood (ML) and Bayesian Inference (BI) tree searches were independently conducted on each genus or species group using Treefinder v. March 2011 (JOBBERG et al. 2004) and MrBayes 3.1.2 (RONQUIST & HUELSENBECK 2003), respectively. As the different codon positions present different substitution rates, two partitions were considered: one for the first and second codon positions and one for the third positions. The most suitable evolutionary models were chosen following the second-order Akaike information criterion (AICc) implemented in MrAIC 1.4. (NYLANDER 2004). ML bootstrap analyses of 1000 replicates were conducted with all model parameters set to optimum and all other options set to default. Two independent analyses of four MCMC chains run for two million generations with trees sampled every 1000 generations were used for BI. The stationary nucleotide frequencies, the alpha shape parameter of the gamma distribution, the relative rate of substitution and the proportion of invariant sites were unlinked across partitions. The first 10% were discarded as a burnin after visually verifying that likelihood curves had flattened-out and that the independent runs converged using Tracer 1.5 (DRUMMOND & RAMBAUT 2007). Specimens belonging

to genera from the same family were used as outgroup taxa for each tree reconstruction.

To estimate the age of divergence of the different clades, we reconstructed ultrametric COI trees under a strict molecular clock model implemented in BEAST 1.6.1 (DRUMMOND & RAMBAUT 2007). Because no external evidence like fossils or other studies is available for node calibration, we used a standard arthropod mitochondrial rate of evolution $2.3\% \text{ My}^{-1}$ (BROWER 1994), which has been proven to be appropriate in a recent study on mayflies (RUTSCHMANN et al. 2014), as well as two contrasting COI rates reported for insects (1.5% and 3.5%; see PONS & VOGLER 2005; PAPADOPOULOU et al. 2010; FARRELL 2001; PEREIRA-DA-CONCEICAO et al. 2012). We also refrained from calibrating nodes using paleogeological processes, because recent molecular studies demonstrated overseas dispersal in mayflies (MONAGHAN et al. 2005; VUATAZ et al. 2013).

2.4. Tests of topology

In part of our COI reconstructions, morphological species are represented by two or more insular clades. When these insular clades are not monophyletic, we compared the obtained reconstruction with alternative topologies grouping all insular clades in a single monophyletic lineage. The different topologies were compared using the Shimodaira-Hasegawa (SH) test (SHIMODAIRA & HASEGAWA 1999) and the approximately unbiased (AU) test (SHIMODAIRA 2002) as implemented in Treefinder (JOBBERG et al. 2004). Alternative topologies were created by manually modifying the ML topologies in TreeView 1.6.6 (PAGE 1996).

3. Results

The sequence alignment was 658 bp long and had no indels (insertion/deletion); it contained 148 haplotypes (10 *Alainites*, 30 *Baetis* gr. *alpinus*, 43 *Baetis* gr. *rhodani*, 13 *Ecdyonurus*, 18 *Electrogena*, 13 *Habroleptoides*, 10 *Habrophlebia* and 11 *Serratella* haplotypes). The selected evolutionary model for positions 1 & 2 and for position 3 was GTR+I+G for *Alainites*, *Baetis* gr. *alpinus*, *Ecdyonurus*, *Habrophlebia* and *Serratella*; while it was HKY+I+G for *Electrogena*, *Baetis* gr. *rhodani* and *Habroleptoides*. BI and ML tree topologies were very similar, with BI posterior probability (PP) and ML bootstrap support (BS) higher than 0.9 and 90% respectively for most nodes. The support of the few incongruent nodes was weak, with PP and BS lower than 0.6 and 60, respectively.

The *Alainites* reconstruction yielded three clades (one Corsican, one Sardinian, one continental) and one singleton (continental), with unresolved relationships be-

tween them (Figs. 2A, S1). The K2P distances between the three clades are higher than 0.20. The split between the different clades was estimated at 15–22 Mya (Fig. 2A) with a 2.3% rate.

The *Baetis* gr. *alpinus* reconstruction yielded seven clades (one Corsican, one Sardinian, one Corso-Sardinian and four continental) and two singletons (Corsican and continental), with unresolved relationships between them (Figs. 2B, S2). Most clades originated 13–20 Mya (Fig. 2B), except one more recent Corso-Sardinian split around 7 Mya. The K2P distances between the different clades and singletons were 0.14–0.18.

The *Baetis* gr. *rhodani* reconstruction yielded nine clades and four singletons (Figs. 2C, S3). We used Williams' original designation for clades Haplo I–VII, which only included continental haplotypes (WILLIAMS et al. 2006; Haplo II not represented in our sample). Haplotypes from Corsica and Sardinia were included in three new clades called Haplo VIII, IX and X. Haplo VIII and IX were composed of haplogroups from both islands, while Haplo X only included specimens from Corsica. Williams' clades were well supported except Haplo III, which formed a weakly supported clade with Haplo VIII. Haplo IX and Haplo X formed a strongly supported monophyletic clade. The Corso-Sardinian clade including Haplo IX and X originated around 13 Mya (Fig. 2C), and the splitting of these two haplogroups around 6.3 Mya. Haplo VIII diverged from the closest continental clade only around 2.9 Mya. The K2P distances between the different clades and singletons ranged between 0.12 and 0.27.

The well-resolved *Ecdyonurus* reconstruction constituted three clades (one Corso-Sardinian and two continental) and one continental singleton (Figs. 3A, S4). The Corso-Sardinian clade originated around 9 Mya (Fig. 3A) and split around 2.0 Mya. The K2P distances between the Corsican and Sardinian clades are equal to 0.04, while distances between the Corso-Sardinian clade and other continental clades are higher than 0.14.

The well-resolved *Electrogena* reconstruction constituted five clades (two Corsican, one Sardinian and two continental) (Figs. 3B, S5). Haplotypes from Corsica belonged to two distinct strongly supported clades, morphologically corresponding to *Electrogena zebrata* (Hagen, 1864) and *Electrogena fallax* (Hagen, 1864). Sardinian haplotypes constituted a single clade, morphologically identified as *E. zebrata*, which was highly supported as sister group of the Corsican *E. zebrata* clade (Figs. 3B, S5). *E. zebrata* originated more than 30 Mya (Fig. 3B) and the Corso-Sardinian splitting occurred around 6.2 Mya. The K2P distances between the Corsican and Sardinian clades of *E. zebrata* are lower than 0.10, while distances between the Corso-Sardinian clade and continental clades are higher than 0.22. *E. fallax* diverged from the most closely related continental clade around 15 Mya and split in two clades around 4.9 Mya. The K2P distances between the two Corsican clades of *E. fallax* is lower than 0.11 while distances to the continental clades are higher than 0.24.

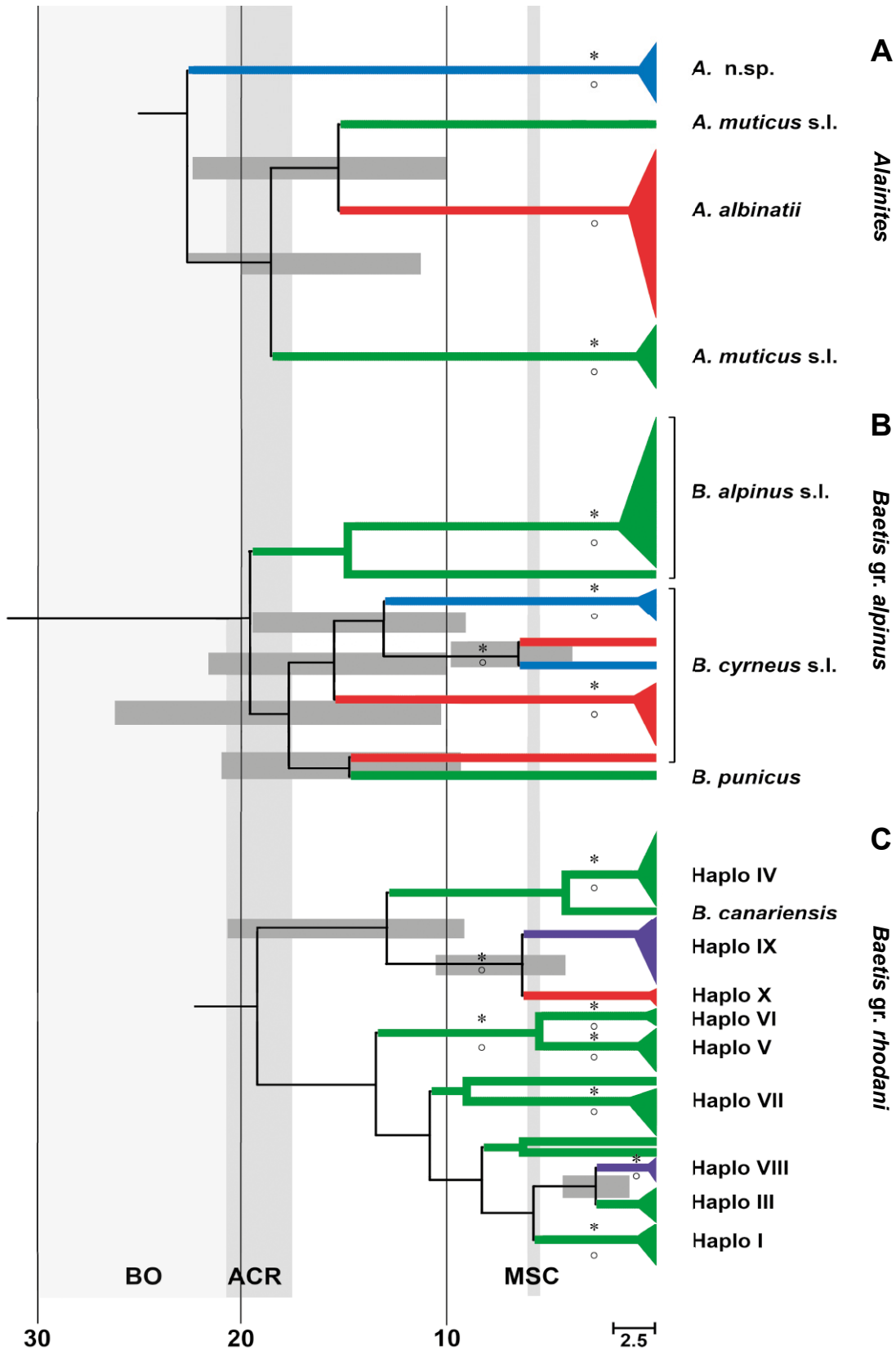


Fig. 2. Ultrametric Bayesian maximum clade credibility trees of Baetidae taxa obtained with BEAST using a strict molecular clock and a mutation rate of 2.3% per My. **A:** *Alainites*. **B:** *Baetis* gr. *alpinus*. **C:** *Baetis* gr. *rhodani*. — Star: likelihood bootstrap support (BS) > 90; circle: Bayesian posterior probability (PP) > 0.9. Terminals: width of clade ends proportional to number of included specimens, unwidened ends represent a single specimen (singleton). Horizontal grey bars indicate calculated age of nodes with a rate of 1.5% per My (left ends, maximum age) and 3.5% per My (right ends, minimum age). Scale = 2.5 My. Red = Corsica; blue = Sardinia; purple = Corsica + Sardinia; green = continental. Grey vertical areas represent important geological or historical events: BO = break-off of the Corso-Sardinian microplate; ACR = anti-clockwise rotation of the Corso-Sardinian microplate; MSC = Messinian Salinity Crisis.

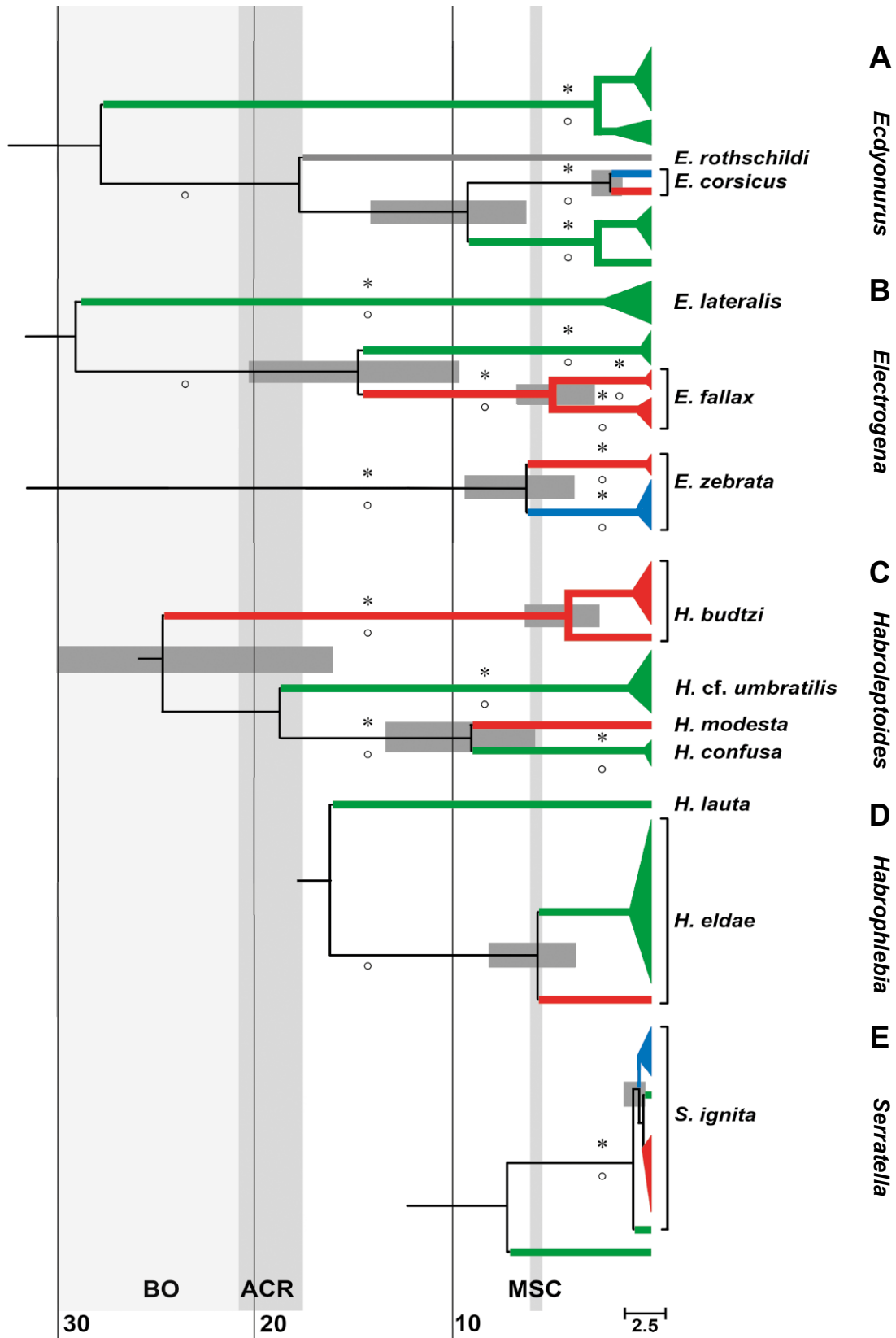


Fig. 3. Ultrametric Bayesian maximum clade credibility trees of non-Baetidae taxa obtained with BEAST using a strict molecular clock and a mutation rate of 2.3% per My. **A:** *Ecdyonurus*. **B:** *Electrogena*. **C:** *Habroleptoides*. **D:** *Habrophlebia*. **E:** *Serratella*. — Star: likelihood bootstrap support (BS) > 90; circle: Bayesian posterior probability (PP) > 0.9. Terminals: width of clade ends proportional to number of included specimens, unwidened ends represent a single specimen (singleton). Horizontal grey bars indicate calculated age of nodes with a rate of 1.5% per My (left ends, maximum age) and 3.5% per My (right ends, minimum age). Scale = 2.5 My. Red = Corsica; blue = Sardinia; green = continental. Grey vertical areas represent important geological or historical events: BO = break-off of the Corso-Sardinian microplate; ACR = anti-clockwise rotation of the Corso-Sardinian microplate; MSC = Messinian Salinity Crisis.

The *Habroleptoides* reconstruction yielded three clades (one Corsican and two continental) and two singletons (Corsican and Tunisia) (Fig. 3C). Haplotypes from Corsica belonged to two distinct strongly supported clades, morphologically identified as *Habroleptoides budtzi* (Esben-Petersen, 1912) and *Habroleptoides modesta* (Hagen, 1864). The Swiss clade morphologically corresponded to *Habroleptoides confusa* Sartori & Jacob 1986, closely related to *H. modesta* in the reconstruction (Figs. 3C, S6) with K2P distance equal to 0.16. *Habroleptoides budtzi* originated around 25 Mya (Fig. 3C), and *H. modesta* diverged from the continental clade around 9 Mya.

The *Habrophlebia* reconstruction yielded one clade (continental) and two singletons (Corsican and continental) (Figs. 3D, S7). The continental clade and the Corsican haplotype formed a well-supported clade, morphologically identified as *Habrophlebia eldae* Jacob & Sartori, 1984. The splitting between Corsican and continental *H. eldae* originated around 5.6 Mya (Fig. 3D). The K2P distances between the Corsican and continental haplotypes of *H. eldae* is lower than 0.07 and the K2P distance among the continental clades is lower than 0.01.

The *Serratella* reconstruction yielded three closely related clades (Corsican, Sardinian and continental) and one earlier-diverging singleton (continental) (Figs. 3E, S8). The splitting between Corsican, Sardinian and continental clades occurred almost simultaneously around 0.4 Mya, with K2P distances lower than 0.01.

The ML topologies suggesting two independent clades in Corsica (and partly Sardinia) for *Electrogena* (corresponding to *E. fallax* and *E. zebrata*), for *Habroleptoides* (corresponding to *H. budtzi* and *H. modesta*), and for *B. gr. rhodani* were significantly better than alternative topologies grouping all insular clades under exclusion of continental ones in a single monophyletic lineage (SH and AU tests, p-values < 0.01). The ML topologies suggesting exclusive monophyly of all Corsican and Sardinian *Ecdyonurus* was significantly better than alternative topologies (SH and AU tests, p-values < 0.01). The result is trivial for *Habrophlebia*, for which our sampling only included an insular singleton. For *B. gr. alpinus*, *Alainites* and *Serratella*, there was equivocal support for every topology considered.

4. Discussion

4.1. Biodiversity, endemism and systematic implications

Serratella ignita (Poda, 1761) and *Habrophlebia eldae* are reported from continental Europe as well as from Corsica and Sardinia (Table 1). No insular endemic species is reported for *Serratella*. Another species of *Habrophlebia*, *H. consigloi* Biancheri, 1959, occurs in

Sardinia and in Tunisia (ZRELLI et al. 2011), but was not included in the present study. We obtained short genetic distances between insular and continental *Serratella ignita* haplotypes, supporting their conspecificity. A single haplotype from southern France (SE-36-FR(MV)) significantly diverged, although the genus is supposed to be monospecific in Europe and no morphological differences were observed, suggesting continental cryptic species in *Serratella*. The genetic distance between Corsican and continental *Habrophlebia* haplotypes was relatively significant, while the distance among continental haplotypes was low despite a wide sampling range (from southern France to Hungary). Although no obvious morphological differences between insular and continental populations were observed, our results suggest a distinct, endemic *Habrophlebia* species in Corsica. However, incomplete sampling could also explain these significant distances, and additional populations from Corsica and Sardinia are needed to confirm our findings.

In contrast, Corsican and/or Sardinian endemic species were described in all the other studied taxa. Our results were in agreement with a Corso-Sardinian endemic species of *Ecdyonurus*, morphologically corresponding to *E. corsicus* (Esben-Petersen, 1912). No specimen of the Corsican endemic species *Ecdyonurus cortensis* Belfiore, 1988 was successfully amplified. *Ecdyonurus* is a very diverse genus in Western Palaearctic and two subgenera were recently established, *Ecdyonurus* s.str. and *Helvetoraeticus* (BAUERNFEIND & SOLDÁN 2012). Our reconstruction is in agreement with this division as the haplotypes were clearly separated into two lineages corresponding to the two subgenera; *E. corsicus* clustered with the European haplotypes of the subgenus *Ecdyonurus* and the Tunisian species *Ecdyonurus (E.) rothschildi* Navás, 1929.

Two Corso-Sardinian endemic species of *Electrogena* (*E. zebrata* and *E. fallax*) were also well supported by our reconstruction; each species constituting a monophyletic lineage clearly separated from the continental clades (Fig. S5). Within *E. fallax*, the Corsican haplotypes were split in two well separated haplogroups suggesting distinct species. The geographic distributions of the two haplogroups do not overlap suggesting a possible vicariance process on Corsica; however additional populations, including haplotypes from Sardinia, are needed to further address this question. Haplotypes of *E. zebrata* significantly diverged from other species of *Electrogena* shown in the reconstruction (Fig. S5), supporting its controversial generic placement. This species was assigned to five different genera since its original description (BAUERNFEIND & SOLDÁN 2012) and is now attributed either to *Afronurus* (HEFTI & TOMKA 1989) or *Electrogena* (GAINO & BELFIORE 1987; KLUGE 2004). More *Electrogena* and *Afronurus* species are required for a better understanding of generic placement of *E. zebrata*. *Afronurus kugleri* Demoulin, 1973 from Israel morphologically and geographically represents the potential closest relative to *Electrogena zebrata* and thus is a crucial taxon for solving this riddle (SARTORI 1992).

Our results are also in agreement with a Corsican endemic species of *Alainites* (*A. albinatii* (Sartori & Thomas, 1989)), which has not been reported from Sardinia (Table 1). Although the continental species *A. muticus* (Linnaeus, 1758) is supposed to occur in Sardinia (BUFFAGNI et al. 2003), the genetic distance between our Sardinian and continental lineages did not support this hypothesis. Moreover, morphological characters generally used for specific identification (number of pairs of abdominal gills, structure of the expansion of the paraproct) notably differ between Sardinian and European continental populations of *A. muticus*, the Sardinian population appearing morphologically closer to the North African species *A. sadati* Thomas, 1984 (ZRELLI et al. 2012; SARTORI & THOMAS 1989). Consequently, the Sardinian clade most probably constitutes a new species endemic to this island. A recent study of the East European Baetinae revealed the presence of cryptic species within *A. muticus* (SROKA 2012), also supporting our findings. Interestingly, *Alainites* is the only mayfly genus for which strong affinities between at least part of the Corsican and Sardinian populations were not found.

Baetis gr. *alpinus* is one of the most diversified groups in Corsica and Sardinia, with four insular lineages in our reconstruction (Fig. S2). Their K2P distances clearly corresponded to interspecific divergences. We can thus expect that four different cryptic species occur in Corsica and Sardinia, although a single species, *B. cyrneus* Thomas & Gazagnes, 1984, is currently recognized (Table 1). Our preliminary morphological analysis did not allow us to find diagnostic characters to separate any of the four putative species. This result is not surprising as the morphological differences between the species of the *B.* gr. *alpinus* are recognized as tenuous (THOMAS & GAZAGNES 1983, 1984). Cryptic species have been well documented within the genus *Baetis* (SROKA 2012; WILLIAMS et al. 2006), but it is the first time that such a high diversity is observed for *B.* gr. *alpinus* within such a restricted area.

Baetis gr. *rhodani*, considered by some authors as the subgenus *Rhodobaetis* (JACOB 2003; SOLDÁN & GODUNKO 2006; SROKA et al. 2012), is one of the most common taxa in all kinds of West Palearctic streams. This eurythermic and eurytopic group of species is generally eudominant except at high altitude where it is substituted by *B.* gr. *alpinus*. In ecological and faunistic studies, *Baetis* larvae with spines on the external gill margins have generally been identified as *B. rhodani* (Pictet, 1843). Recent molecular studies greatly challenged this concept of *B. rhodani*: WILLIAMS et al. (2006) found seven significantly divergent haplogroups in Western Europe, constituting many putative species. Some of them are widely distributed while others have restricted geographic distributions; LUCENTINI et al. (2011) revealed four additional lineages presumably endemic to the Italian Peninsula; high genetic distances between East and Central European populations of *Baetis rhodani* s.l. also suggested cryptic species (SROKA 2012). Recent study of the mayflies from Canary Islands also revealed insular endemic species of

the subgenus *Rhodobaetis* (RUTSCHMANN et al. 2014). Our reconstruction revealed two separate insular clades (the Corso-Sardinian Haplo IX and the Corsican Haplo X; Fig. S3) that are sister to each other, while the Corso-Sardinian Haplo VIII formed a monophyletic lineage with Haplo III (sensu WILLIAMS et al. 2006), a widely distributed haplogroup in Central and North Europe. According to current taxonomy, *Baetis ingriddae* Thomas & Soldan, 1987 is the single species of the *rhodani* group occurring in Corsica, while the widespread species *B. rhodani* is the single record for Sardinia (Table 1). While our results clearly showed that three lineages, constituting as many putative species, occur on the two islands, it was not possible to assign one of them to *B. ingriddae*. Important morphological variability was noticed in the collected material but it remains extremely difficult to evaluate the specific validity of this variability and its stability. Only direct morphological comparison with the type material could solve this riddle. Interestingly, as it was already noticed in other populations of *B. rhodani* s.l. (LUCENTINI et al. 2011; WILLIAMS et al. 2006), at least two haplogroups can co-occur in the same locality (for example Haplo VIII and IX in Porto, Corsica, 10/V/2009) at the same larval stage (last larval instar).

Two species of *Habroleptoides* are reported from Corsica and/or Sardinia: *H. modesta* and *H. budtzi* (Table 1). They are recognized as Corso-Sardinian endemic (BUFFAGNI et al. 2003); despite we could not sample either of the species in Sardinia. *H. budtzi* was more frequent and more widely distributed in our sample (Fig. S6). Haplotype HL-07-CO diverged from the other *H. budtzi* haplotypes, most probably because of undersampling. *H. modesta* was represented by a single haplotype in our sampling; it appeared as sister-group of the widely distributed continental species *H. confusa*. This relationship is not surprising as *H. modesta* and *H. confusa* were considered conspecific until recently (SARTORI & JACOB 1986).

Although there are important limitations of using single-locus data, they can provide useful information on evolutionary histories and diversity, particularly for large-scale studies (MONAGHAN et al. 2009; FUJISAWA & BARRACLOUGH 2013). As for many other animal groups, COI has been successfully used in mayfly barcoding (BALL et al. 2005; KJAERSTAD et al. 2012; WEBB et al. 2007, 2012; ZHOU et al. 2009, 2010) and phylogenetic studies (LUCENTINI et al. 2011; RUTSCHMANN et al. 2014; SROKA 2012; WILLIAMS et al. 2006). However, morphological support is essential to validate any molecular-based putative species, which is particularly true for single-locus data, although the lack of reliable morphological characters to support putative species constitutes one of the main difficulties in mayfly species delimitation (SROKA 2012).

4.2. Biogeography

Our dated reconstructions clearly showed that current Corsican and Sardinian mayfly diversity and endemism

cannot be explained by a single process or event, but rather arose from old vicariance, radiation and more recent colonization promoted by the break-off (BO) of the Corso-Sardinian microplate and anti-clockwise rotation (ACR), the Messinian Salinity Crisis (MSC) and the Quaternary glaciations (QG).

Although it is widely recognized that rates of evolution vary among lineages and through time (PAPADOPOULOU et al. 2010), standard molecular clocks often represent the only possible approach for dating phylogenies when fossils are lacking and paleogeological events cannot confidently be exploited. As reported for a growing number of groups (KODANDARAMAIAH 2011), recent molecular studies suggested mayfly overseas dispersal (MONAGHAN et al. 2005; VUATAZ et al. 2013). Consequently, we considered that using microplates splitting for tree nodes calibration would have been too arbitrary as recently confirmed by aberrant results obtained in a study within another merolimnic insect order (FOCHETTI et al. 2009).

In *Alainites* (Fig. 2A) and *Habroleptoidea* (Fig. 3C), the origin of main clades matched timeframe of the BO and ACR, suggesting the diversification within these genera was promoted by this important geological event. Both *Alainites* and *Habroleptoidea* present restricted ecological niches (SARTORI & LANDOLT 1999; BAUERNFEIND & SOLDÁN 2012) which is known to strongly influence the dispersal power of the species (RIBERA et al. 2010).

The MSC had considerable impact on the circum-Mediterranean flora and fauna (HSÜ et al. 1977). It corresponded to a major diversification period for Corsica and Sardinia (KETMAIER et al. 2006; TRIZZINO et al. 2011), as well as for the northeastern Mediterranean region (SOLÀ et al. 2013). The drying of the Mediterranean Sea created connections between insular and continental limnic systems, and probably promoted strictly freshwater invertebrates' dispersal on and between Mediterranean islands (SOLÀ et al. 2013). Hence, the rapid refilling of the Mediterranean Sea may have recreated the isolation of insular populations by stopping gene flow between insular and continental populations of *Habrophlebia eldae*, between Corsican and Sardinian populations of *Electrogena zebrata*, as well as some clades of *Baetis* gr. *rhodani* (Figs. 2, 3). According to Vuataz et al. (in prep.), the origin of the Corso-Sardinian species of the mayfly genus *Rhithrogena* also dated from the MSC.

Quaternary glaciations are recognized as important promoters of population divergence and speciation for many organisms (e.g. HEWITT 2004; ALLEGRUCCI et al. 2005), including mayflies (Vuataz et al. in prep.). However, probably because Corsica and Sardinia were never completely covered by ice (KUHLEMANN et al. 2005), glacial impact on the fauna of the two islands was probably moderate. According to our dating analyses, QG promoted intraspecific divergence in *Serratella ignita* (Fig. 3E) and in *Baetis* gr. *rhodani* (Fig. 2C). Both taxa are among the most common and widely distributed species and are generally eudominant except at high altitude. This is in accordance with the conclusion of DAPPORTO

& DENNIS (2008): species are more likely to colonize islands when they are frequent at sources and live at low altitude. *Baetis* gr. *rhodani* and *Serratella ignita* are also among the first mayfly species to colonize new aquatic habitats influenced by human activities (LANDA & SOLDÁN 1986). While *Baetis* gr. *rhodani* is present on the principal islands and archipelagos of south-western Europe such as the Balearic Islands, Canary Islands or Madeira (ALBA-TERCEDOR & JAIMEZ-CUELLAR 2003; GATTOOLIAT et al. 2008; MÜLLER-LIEBENAU 1971), *S. ignita*, as other Ephemerellidae, is widely distributed in continental Europe but generally not present on isolated islands (ALBA-TERCEDOR & JAIMEZ-CUELLAR 2003; GATTOOLIAT et al. 2008; MÜLLER-LIEBENAU 1971). The almost contemporary gene flow in *S. ignita* between Corsica, Sardinia and continental Europe is therefore rather surprising.

Recent insect studies indicated links between the Corso-Sardinian area and North Africa (e.g. DAPPORTO 2011). Sardinia is almost equidistant from the Italian Peninsula and Tunisia (around 200 km), but 300 km from Sicily. Moreover, Corsica, Sardinia and Kabylie have a common geological history as they separated almost simultaneously from continental Europe (ROSENBAUM et al. 2002; DE JONG 1998). Connections across the strait of Sicily may also have induced relationships between the North African fauna and the Corsican, Sardinian and Sicilian ones (STÖCK et al. 2008). The first Sardo-Tunisian mayfly distribution was recently reported for *Habrophlebia consiglioi* (ZRELLI et al. 2011). Here we included Tunisian specimens of *Ecdyonurus*, *Baetis* gr. *alpinus* and *Baetis* gr. *rhodani*. However, the Tunisian haplotype of *Ecdyonurus* was not the closest to the Corsican or Sardinian ones (Fig. S4), while the relationships between Tunisian haplotypes of *Baetis* gr. *alpinus* and *B. gr. rhodani* with Corsican and Sardinian ones were unresolved (Figs. S2, S3). Including more Tunisian species like *Habrophlebia consiglioi* and *Alainites sadati* is necessary for better understanding relationships between Corsica, Sardinia and North Africa.

4.3. Conservation strategy

Our results have important implications for insular biodiversity conservation. We showed that all the morphologically recognized Corsican or Corso-Sardinian endemic species were also genetically supported. We also demonstrated that two Sardinian lineages supposed to represent continental species (*Baetis rhodani* and *Alainites muticus*) are most probably endemic undescribed species. Significant genetic distances between haplogroups within *Electrogena fallax* and *Electrogena zebrata* suggested the presence of morphologically cryptic species, although it could also be an undersampling artifact. These putative species were presumably allopatric in Corsica (North-East vs the rest of the island in *E. fallax*) or endemic to each island (in *E. zebrata*). We found three well-separated insular clades of *Baetis* gr. *alpinus*, which rep-

resented three putative species in each group. In *Baetis* gr. *rhodani*, we recognized three independent clades and putative species related to two independent colonization events of Corsica and Sardinia. Reliable morphological characters to separate the different putative species are essential. A global study including the type material is required before species names can be attributed and the putative species morphologically diagnosed.

The formally described Corsican or Corso-Sardinian endemic ephemeropteran species represent app. half of the mayfly species known from the islands. Our results strongly suggest this proportion is underestimated. Most mayfly species occurring on Corsica and Sardinia are typical island species without recent gene flow with their continental relatives. Therefore, they satisfy all the criteria for conservation priorities (EKEN et al. 2004). As also reported from the Canary Islands (RUTSCHMANN et al. 2014), the molecular approach almost doubled the previously reported total mayfly diversity on Corsica and Sardinia in the studied groups; moreover this diversity is mainly due to island-endemics, which is of major importance for conservation.

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Electronic Supplement File

at <http://www.senckenberg.de/arthropod-systematics> (“Contents”)

File 1: gattolliat&al-corsicanmayflies-asp2015-electronicsupplement-1.pdf. – Maximum Likelihood (ML) consensus trees reconstructed for various ephemeropteran taxa. Deeper nodes are labelled above branches with Maximum Likelihood bootstrap support (BS) and under branches with Bayesian posterior probability (PP) only for BS > 50 and PP > 0.5. Two digit code: sample number. Tip labels: the first two letters indicate the taxon name (details below). Last group of letters: origin of the material (CO = Corsica; SA = Sardinia; FR(ME) = France Mercantour; FR(MV) = France Mont Ventoux; IT(AP) = Italy Appennino; IT(LI) = Italy Liguria; IT(TO) = Italy Toscana; CAN = Canary Islands; CH = Switzerland; FR = France; HU = Hungary; TU = Tunisia). – **Fig. S1.** *Alainites* spp. (Baetidae). Tip labels: AL = *Alainites*; BA = *Baetis* gr. *alpinus*. – **Fig. S2.** *Baetis* gr. *alpinus* (Baetidae). Tip labels: BA = *Baetis* gr. *alpinus*; BR = *Baetis* gr. *rhodani*. Other tip labels refer to additional material (GenBank and unpublished data). – **Fig. S3.** *Baetis* gr. *rhodani* (Baetidae). Tip labels: BR = *Baetis* gr. *rhodani*; BA = *Baetis* gr. *alpinus*. Tips without labels refer to additional material (GenBank and unpublished data). – **Fig. S4.** *Ecdyonurus* spp. (Heptageniidae). Tip labels: EC = *Ecdyonurus* spp.; EL = *Electro-*

gena sp.; EZ = *Electrogena zebrata*. – **Fig. S5.** *Electrogena* spp. (Heptageniidae). Tip labels: EF = *Electrogena fallax*; EZ = *Electrogena zebrata*; ELLA = *Electrogena lateralis*; EL = *Electrogena* spp. – **Fig. S6.** *Habroleptoides* spp. (Leptophlebiidae). Tip labels: HL = *Habroleptoides* spp.; HP = *Habrophlebia* spp. – **Fig. S7.** *Habrophlebia* spp. (Leptophlebiidae). Tip labels: HP = *Habrophlebia eldae*; HPLA = *Habrophlebia lauta*; HL = *Habroleptoides* sp. – **Fig. S8.** *Serratella ignita* (Ephemerellidae). Tip labels: SE = *Serratella ignita*; DR = *Drunella* sp.; EP = *Ephemerella* sp.

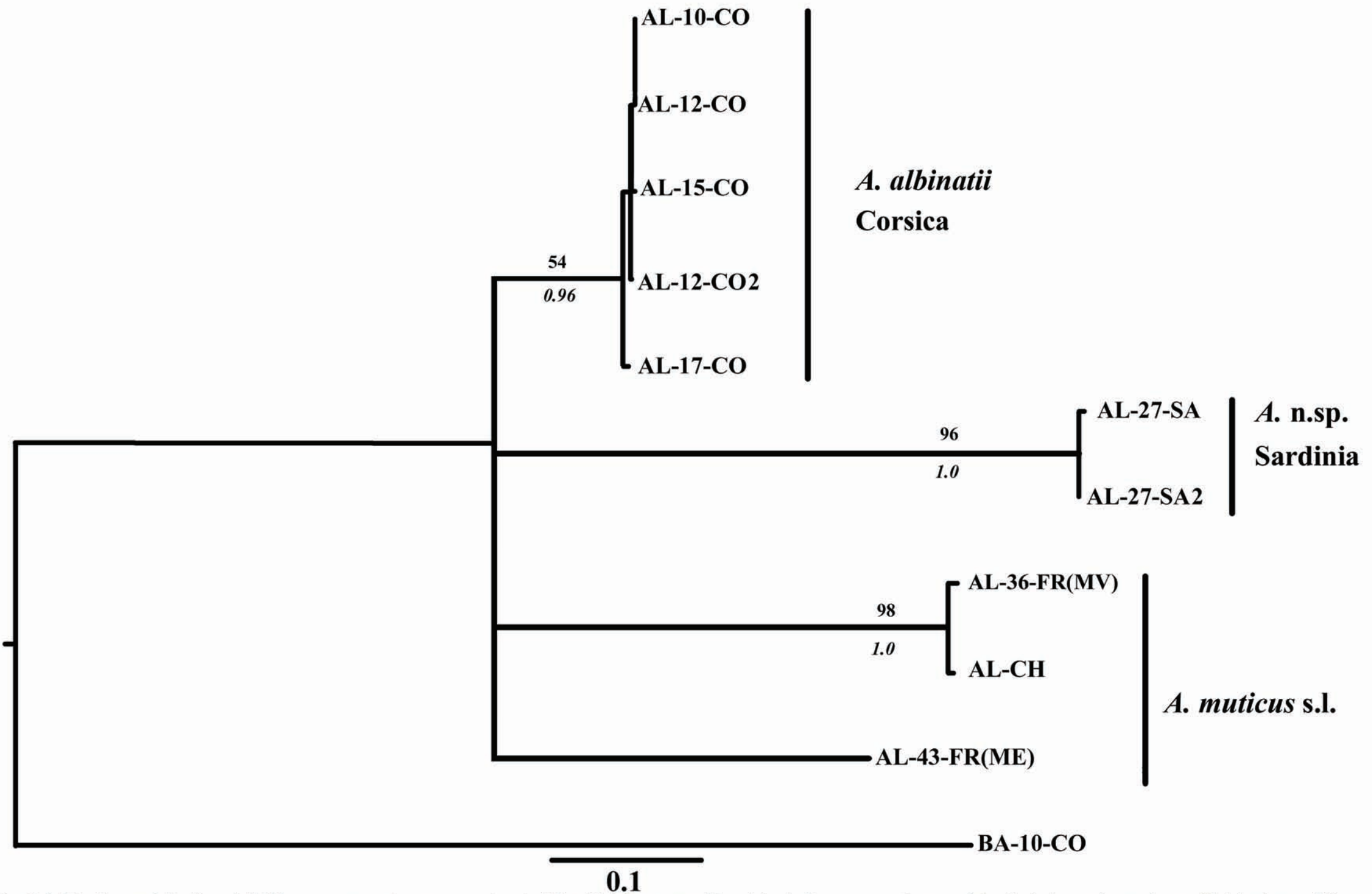


Fig. S1. Maximum Likelihood (ML) consensus tree reconstructed for *Alainites* spp. (Baetidae). Deeper nodes are labelled above branches with Maximum Likelihood bootstrap support (BS) and under branches with Bayesian posterior probability (PP) only for BS > 50 and PP > 0.5. Tip labels: the first two letters indicate the taxon name (AL = *Alainites*; BA = *Baetis* gr. *alpinus*). Two digit code: sample number. Last group of letters: origin of the material (CO = Corsica; SA = Sardinia; FR(ME) = France Mercantour; FR(MV) = France Mont Ventoux; IT(AP) = Italy Appeninno; IT(LI) = Italy Liguria; IT(TO) = Italy Toscana; CAN = Canary Islands; CH = Switzerland; FR = France; HU = Hungary; TU = Tunisia).

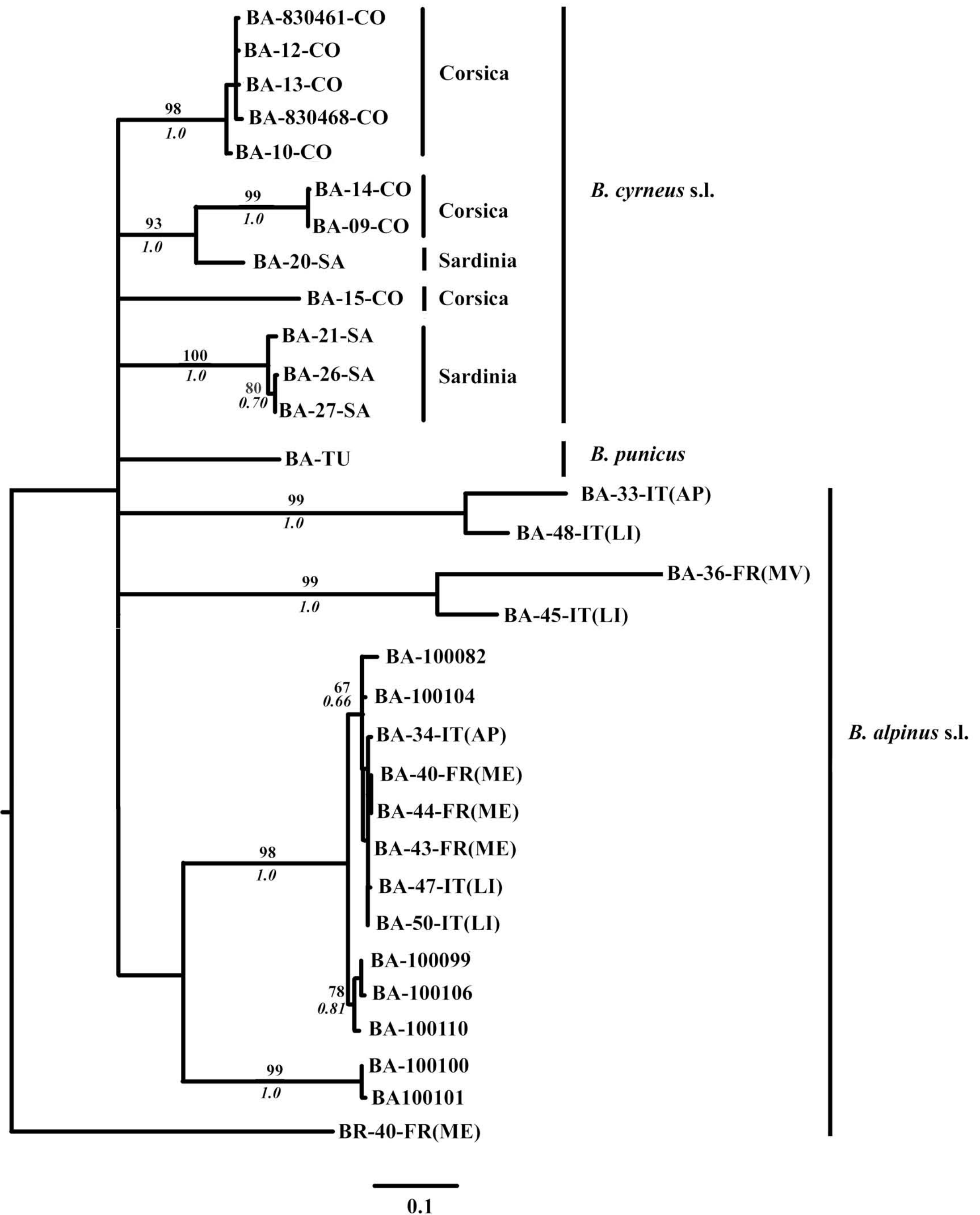


Fig. S2. Maximum Likelihood (ML) consensus tree reconstructed for *Baetis* gr. *alpinus* (Baetidae). Deeper nodes are labelled above branches with Maximum Likelihood bootstrap support (BS) and under branches with Bayesian posterior probability (PP) only for BS > 50 and PP > 0.5. Tip labels: the first two letters indicate the taxon name (BA = *Baetis* gr. *alpinus*; BR = *Baetis* gr. *rhodani*). For other codes see Fig. S1. Other tip labels refer to additional material (GenBank and unpublished data).

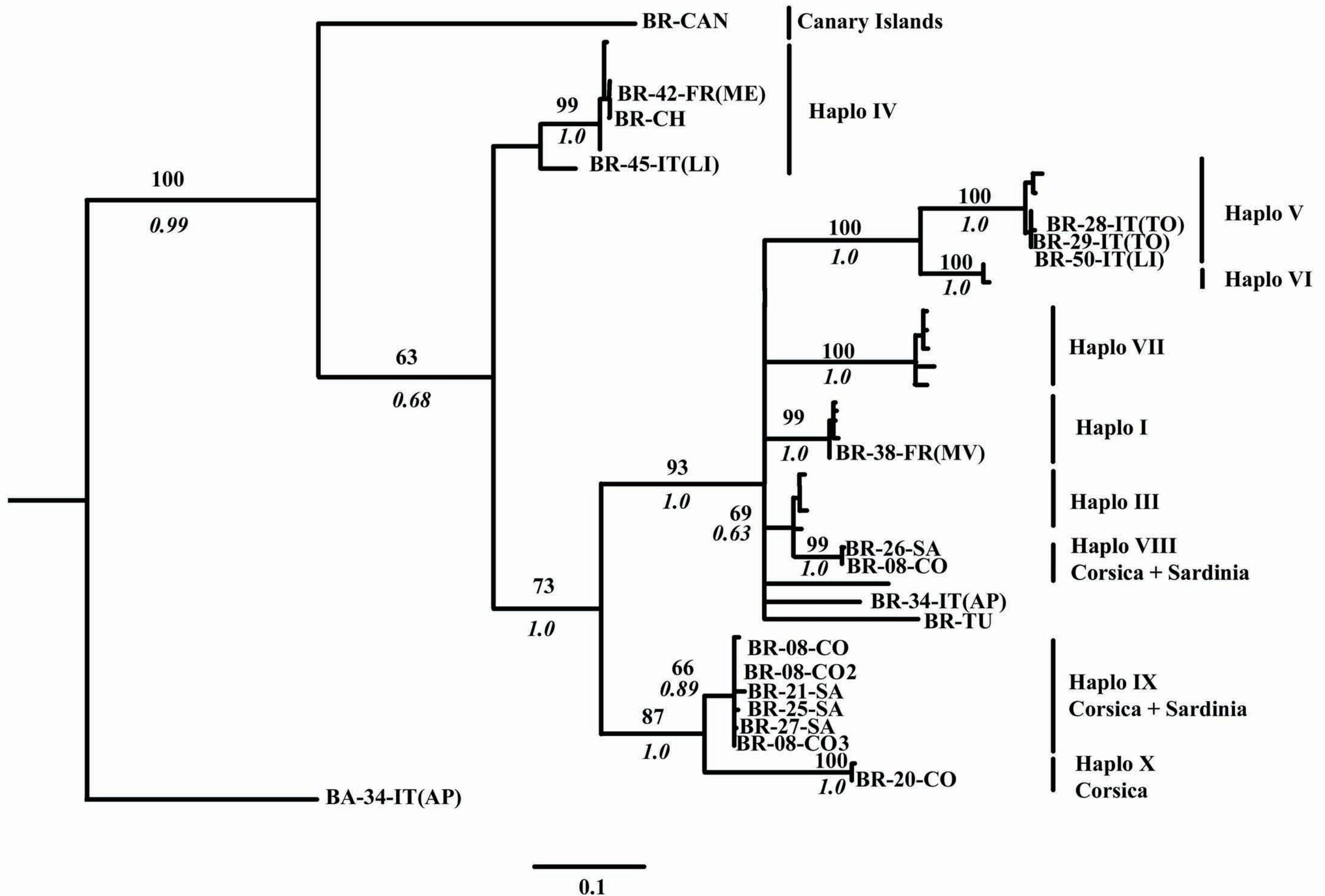


Fig. S3. Maximum Likelihood (ML) consensus tree reconstructed for *Baetis gr. rhodani* (Baetidae). Deeper nodes are labelled above the branch with Maximum Likelihood bootstrap support (BS) and under the branch with Bayesian posterior probability (PP) only for BS > 50 and PP > 0.5. Tips labels are generated as follows: Two first letters indicated the taxon name (BR = *Baetis gr. rhodani*; BA = *Baetis gr. alpinus*). Other codes see Fig. S1. Tip without labels refer to additional material (GenBank and unpublished data).

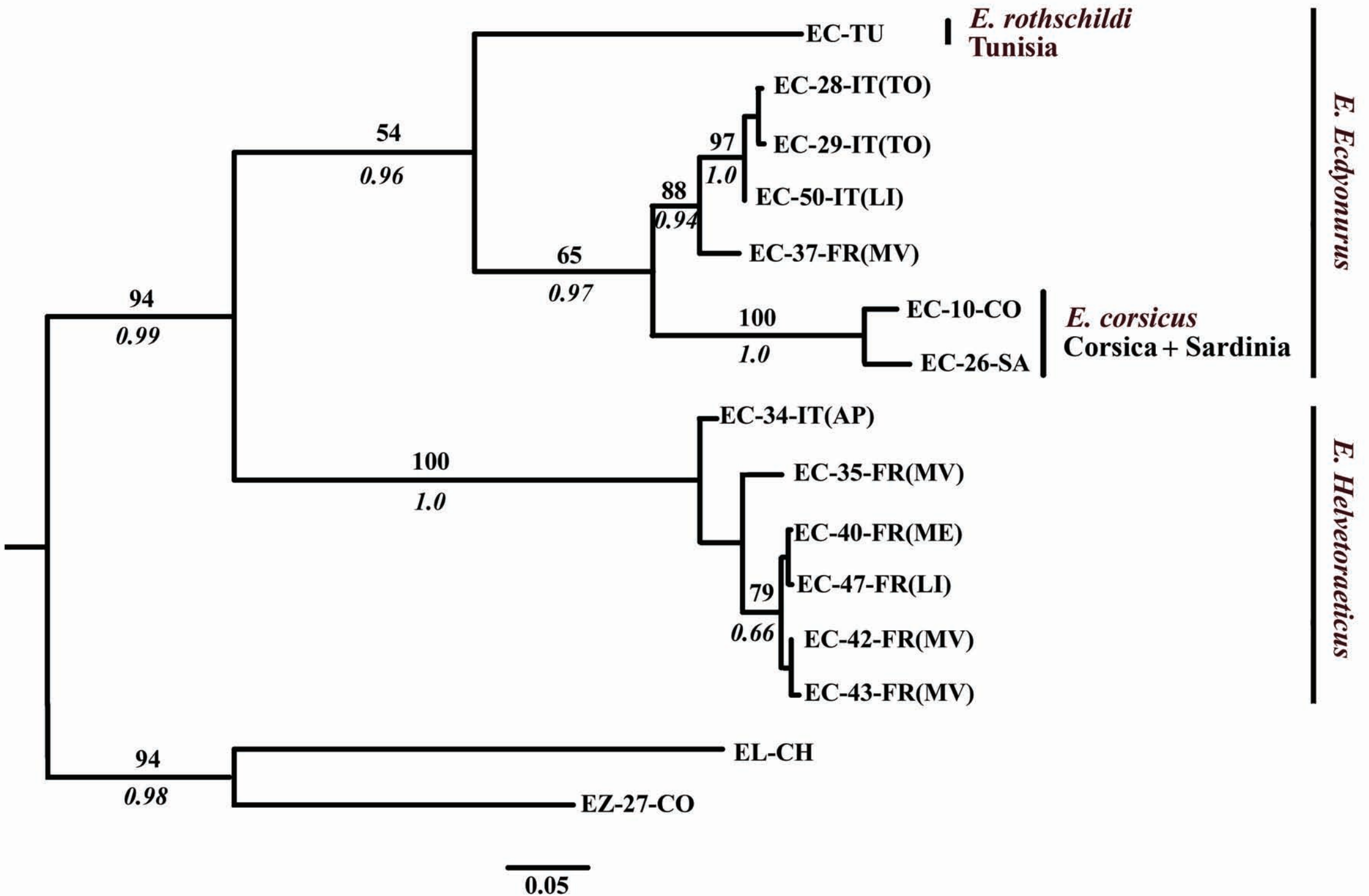


Fig. S4. Maximum Likelihood (ML) consensus tree reconstructed for *Ecdyonurus* spp. (Heptageniidae). Deeper nodes are labelled above the branch with Maximum Likelihood bootstrap support (BS) and under the branch with Bayesian posterior probability (PP) only for BS > 50 and PP > 0.5. Tips labels are generated as followed: Two first letters indicated the taxon name (EC = *Ecdyonurus* spp.; EL = *Electrogena* sp.; EZ = *Electrogena zebrata*). Other codes see Fig. S1.

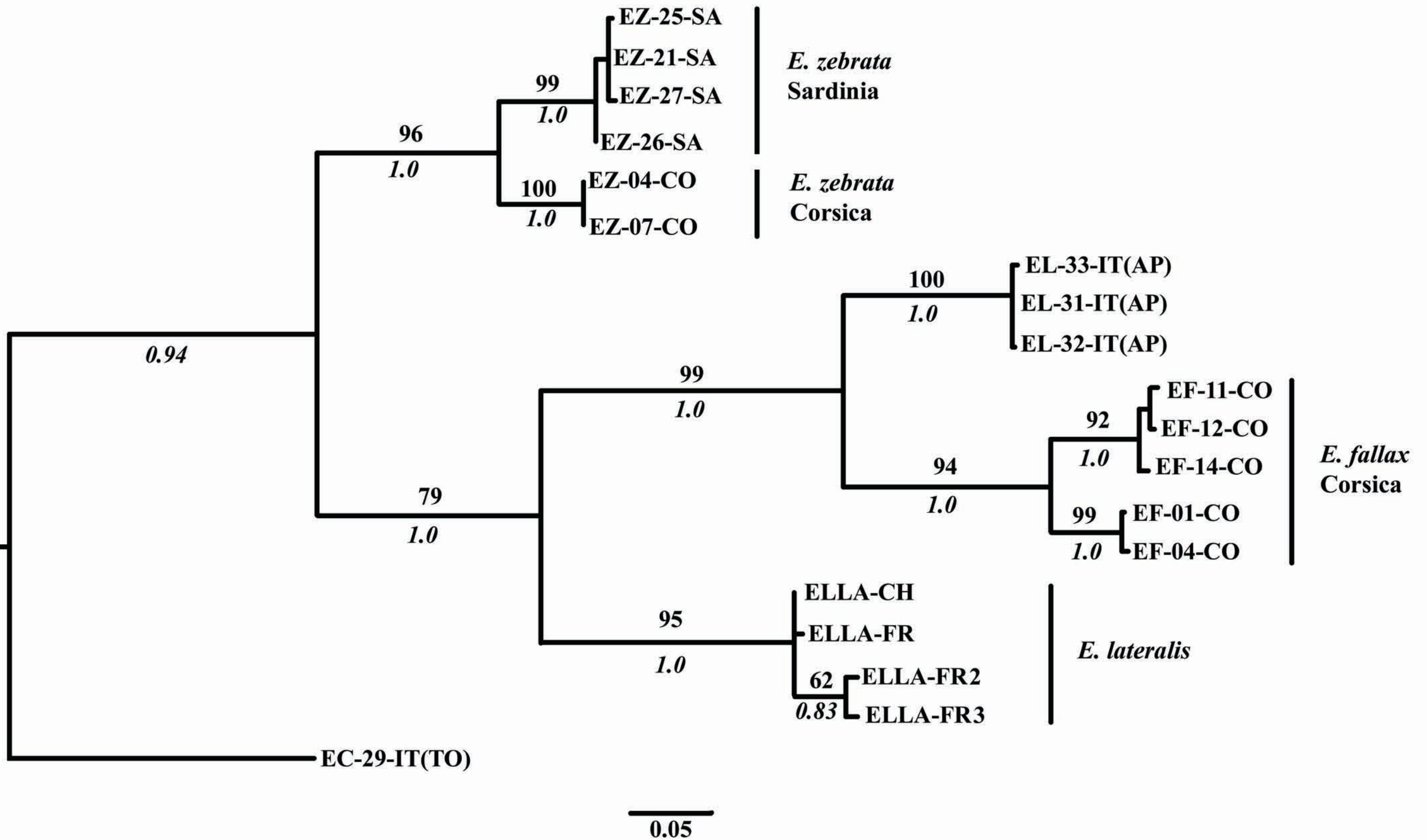


Fig. S5. Maximum Likelihood (ML) consensus tree reconstructed for *Electrogena* spp. (Heptageniidae). Deeper nodes are labelled above branch with Maximum Likelihood bootstrap support (BS) and under the branch with Bayesian posterior probability (PP) only for BS > 50 and PP > 0.5. Tips labels are generated as followed: Two first letters indicated the taxon name (EF = *Electrogena fallax*; EZ = *Electrogena zebrata*; ELLA = *Electrogena lateralis*; EL = *Electrogena* spp.). Other codes see Fig. S1.

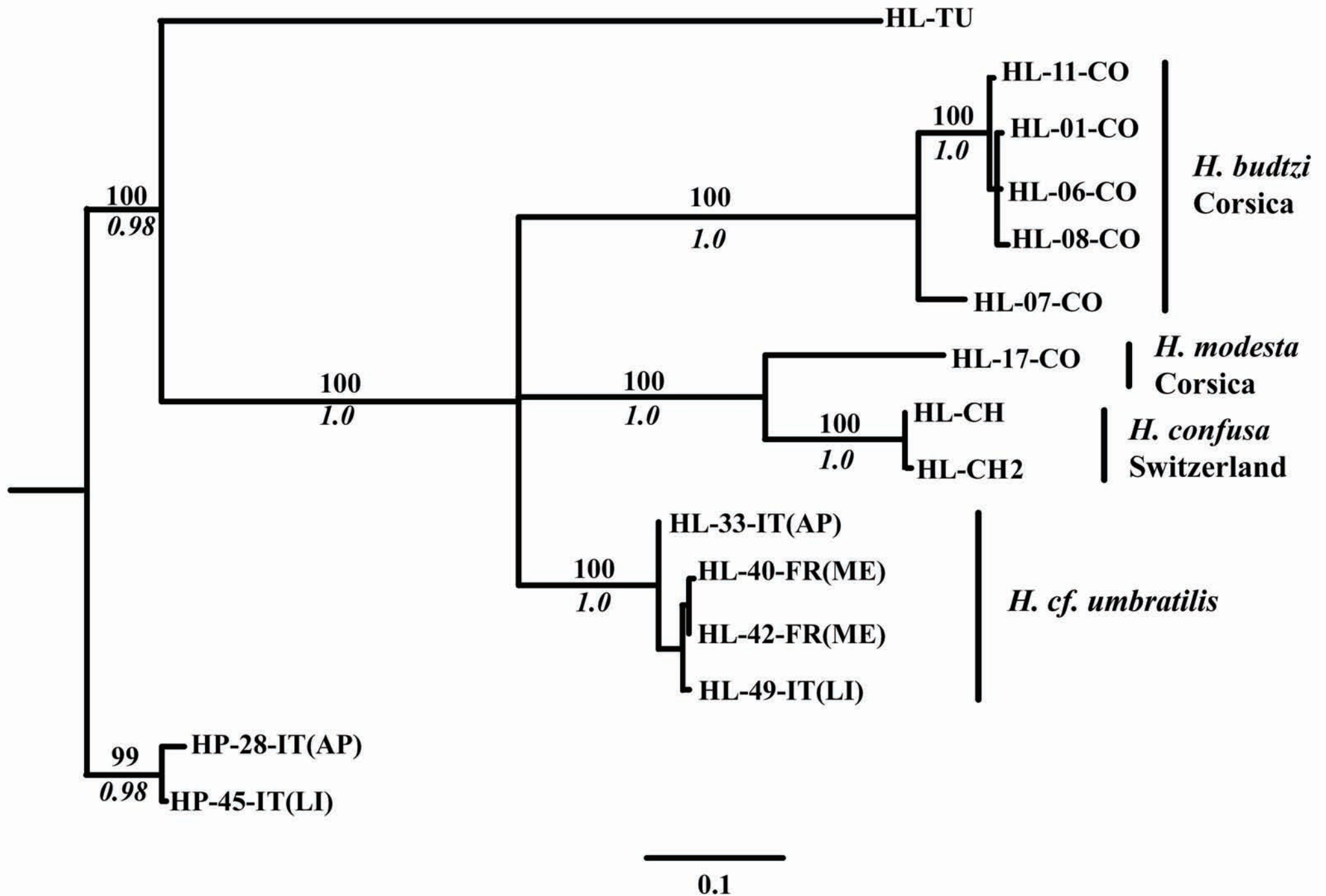


Fig. S6. Maximum Likelihood (ML) consensus tree reconstructed for *Habroleptoides* spp. (Leptophlebiidae). Deeper nodes are labelled above the branch with Maximum Likelihood bootstrap support (BS) and under the branch with Bayesian posterior probability (PP) only for BS > 50 and PP > 0.5. Tips labels are generated as followed: Two first letters indicated the taxon name (HL = *Habroleptoides* spp.; HP = *Habrophlebia* spp.). Other codes see Fig. S1.

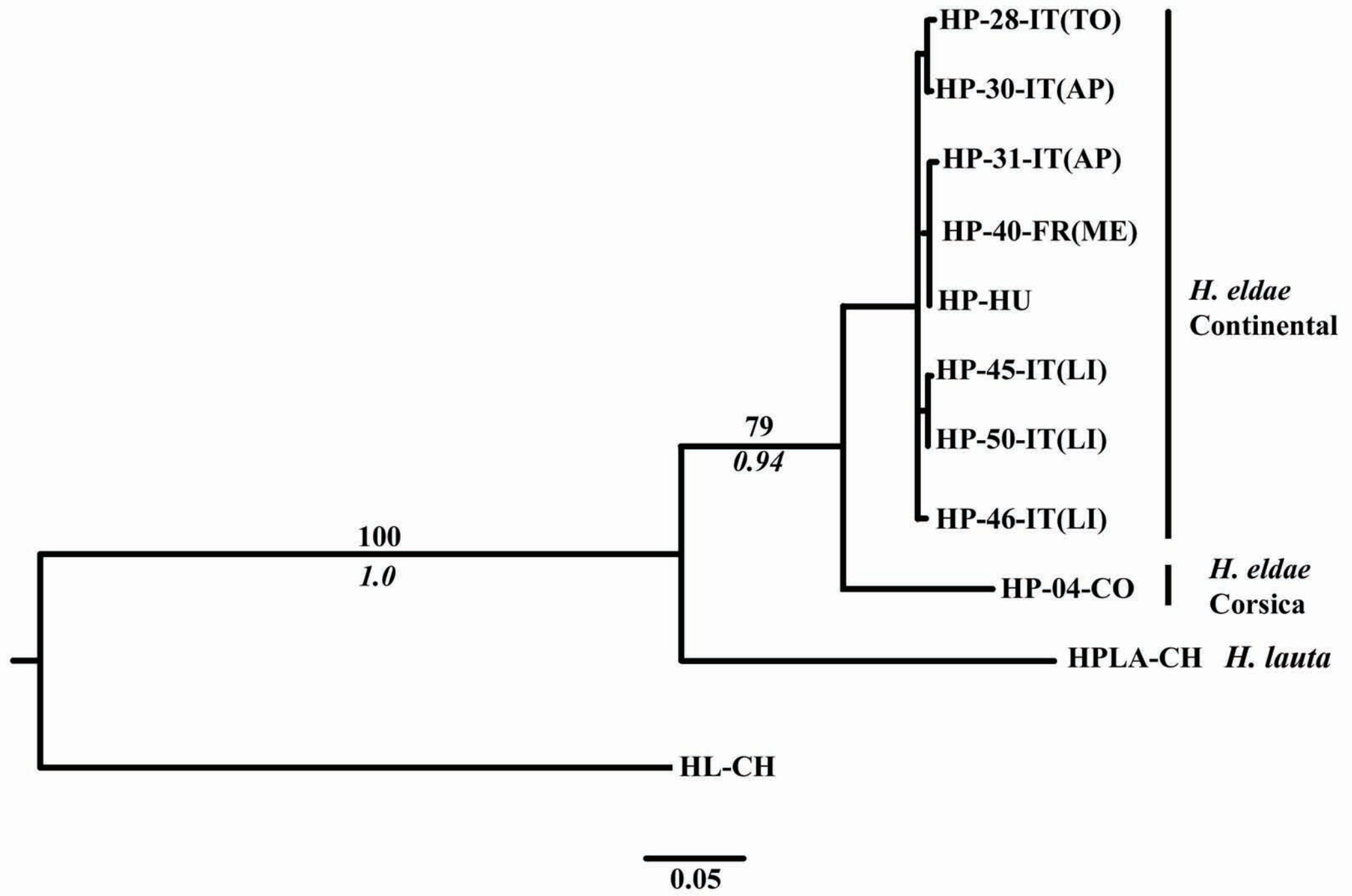


Fig. S7. Maximum Likelihood (ML) consensus tree reconstructed for Habrophlebia spp. (Leptophlebiidae). Deeper nodes are labelled above the branch with Maximum Likelihood bootstrap support (BS) and under the branch with Bayesian posterior probability (PP) only for BS > 50 and PP > 0.5. Tips labels are generated as followed: Two first letters indicated the taxon name (HP = Habrophlebia eldae; HPLA = Habrophlebia lauta; HL = Habroleptoides sp.). Other codes see Fig. S1.

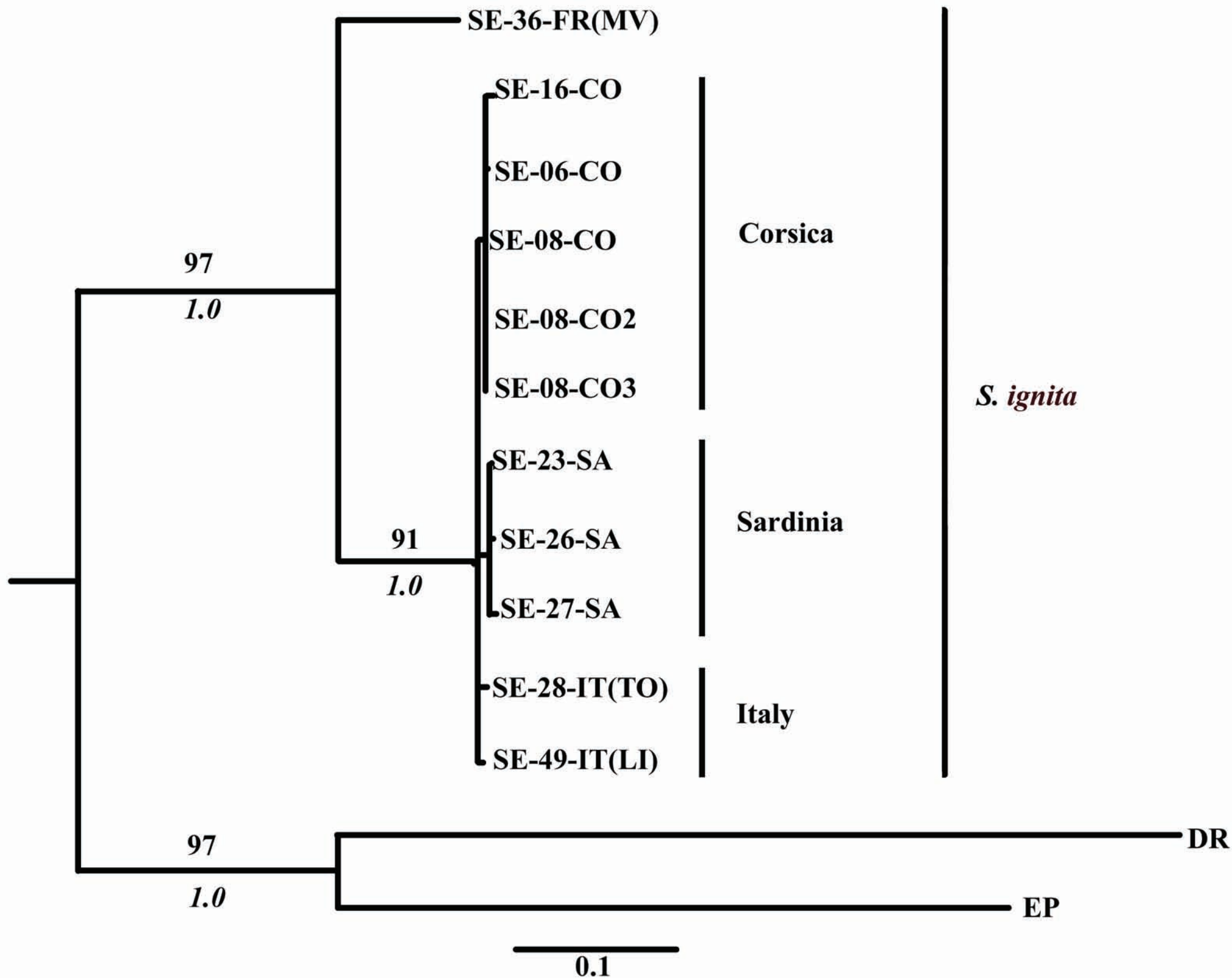


Fig. S8. Maximum Likelihood (ML) consensus tree reconstructed for *Serratella ignita* (Ephemerellidae). Deeper nodes are labelled above the branch with Maximum Likelihood bootstrap support (BS) and under the branch with Bayesian posterior probability (PP) only for BS > 50 and PP > 0.5. Tips labels are generated as followed: Two first letters indicated the taxon name (SE = *Serratella ignita*; DR = *Drunella* sp.; EP = *Ephemerella* sp.). Other codes see Fig. S1.

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