Zoosymposia 11: 135–155 (2016)
http://www.mapress.com/j/zs

ISSN 1178-9905 (print edition)
ZOOSYMPOSIA
ISSN 1178-9913 (online edition)

http://dx.doi.org/10.11646/zoosymposia.11.1.15
http://zoobank.org/urn:lsid:zoobank.org:pub:7D17148D-CD76-40C5-A0DD-C1779D253FEE

Toward a comprehensive COI DNA barcode library for Swiss Stoneflies (Insecta: Plecoptera) with special emphasis on the genus *Leuctra*

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Abstract

The Swiss Barcode of Life initiative (SwissBOL) aims to inventory the genetic biodiversity in Switzerland using a short DNA sequence. DNA barcoding provides an additional tool for species identification that complements traditional morphological approaches. We report on the establishment of a DNA barcode library for Plecoptera, taxa that are of great importance as bioindicators of water quality and that often present difficulties in species-level identification for larvae and female specimens. Non-destructive DNA extraction, PCR amplification and sequencing of part of the mitochondrial gene Cytochrome Oxidase I (COI) was conducted for 440 individuals (one to eight per species) belonging to 90 species (of the 112 reported from Switzerland). Intra and interspecific distances were calculated and gene trees reconstructed. In most cases, COI was efficient in delimiting stonefly species. Some doubtful specimens were subsequently re-examined and a few misidentifications were found, especially in some problematic groups in the genus *Leuctra* Stephens, 1836. Larger genetic distances in some species (e.g. *Leuctra nigra* (Olivier 1811)) indicate the possible presence of sibling species, while in a few cases closely related species are genetically difficult to separate (within the *Leuctra fusca* species group).

Key words: Stoneflies, Plecoptera, Switzerland, SwissBOL, DNA barcoding

Introduction

Stoneflies in Switzerland

The study of stoneflies has a long history in Switzerland, and the Swiss fauna is well known. François-Jules Pictet was one of the pioneers who conducted stonefly research in Switzerland, his cumulative work documenting at least 27 species (Pictet 1841). One century later, Jacques Aubert began his comprehensive study of the Swiss stoneflies, listing 94 species in his first catalogue (Aubert 1959). He described several new species and reported others for the first time in Switzerland. In the framework of the Swiss Red List, streams, rivers, springs, pounds and lakes were intensively prospected for stoneflies, mayflies and caddisflies at the end of the last century and at the beginning of the 21st. A Red List based on IUCN criteria was recently established, inventorying the conservation status of the species of stoneflies (Lubini *et al.* 2012a), while a key and a checklist were simultaneously published (Lubini *et al.* 2012b).

Stoneflies in Switzerland currently encompass 112 species. The diversity is rather high, in comparison to surrounding countries, if we consider that Swiss land area is only 41,285 km^2 (smaller than France and Germany, by respective ratios of 15 and 9). This richness can be explained by the central position of Switzerland in the Alps and in Europe, the presence of a second mountain range (Jura) and a floodplain at middle altitude (the Plateau). Moreover, several Mediterranean species reach their northern limit of distribution in southern Switzerland, in tributaries of the Po River (especially in Ticino). This high stonefly diversity also reflects the global richness of the Alpine arc where numerous endemic species occur.

Accepted by Craig Macadam: 4 Oct. 2016; published: 18 Nov. 2016

Nearly 40% of the Swiss stoneflies are included in the Red List: 6.3% are considered as extinct, 9.0% as critically endangered, 12.6% as endangered and 11.7% as vulnerable. Additionally, 17% are considered as near threatened (Lubini *et al.* 2012a). Species living in large rivers at low altitudes are especially imperilled. This is also the case for species colonising springs and clean fresh brooklets that are often exposed to human impacts, such as agricultural pollution or water impoundment, sometimes exacerbated by acid rain events or regional effects of climate changes. Moreover, several species exhibit narrow endemicity or have disjunctive distributions, increasing their risk of extinction.

Plecoptera are a major component of the aquatic biodiversity of streams and rivers in Switzerland. They are sensitive biological indicators of water quality in Switzerland (Stucki 2010) and the world over (DeWalt *et al*. 2015). Unfortunately, larvae and female specimens are often not possible to identify to species level (Lubini *et al.* 2012b). The lack of specificity compromises our ability to accurately assess water quality and to conduct ecological and conservation assessments of individual species. Additional tools are needed to improve the rate of species level identifications for these wide-ranging scientific endeavours. The use of DNA barcoding presents an additional tool for identification purposes (Zhou *et al.* 2010; Gill *et al*. 2015).

DNA Barcoding

DNA barcoding allows for the rapid identification of an individual by matching a DNA fragment to a reference library containing sequences generated from identified specimens (DeWalt 2011). For animals, a 658 base-pair fragment of the mitochondrial gene Cytochrome C Oxydase 1 (COI) is widely used (Ball *et al.* 2005; Hebert *et al.* 2003), and was shown to be an effective DNA barcode in aquatic insects (Zhou *et al.* 2010). A library of DNA barcodes for Swiss biodiversity was established, the Swiss Barcode of Life (SwissBOL), and the present study is part of this larger initiative. A rationale for the project is presented at http://www.swissbol.ch.

Accurate identification of aquatic species is fundamental for freshwater research. However, identifications at the species level can be difficult for non-specialists and may be possible only for male imagos. For most stream biological monitoring purposes, only immature stages of insects are collected, leaving many specimens of stoneflies identifiable only to the genus or species group level (Lubini *et al.* 2012b). This is the case for several Plecoptera genera such as *Nemoura* Latreille, 1796, *Isoperla* Banks, 1906 or *Leuctra* Stephens, 1836; even the female imagos of which may not be identified with confidence. Additionally, there are a large number of cryptic species where even specialists have difficulty identifying male imagos on a consistent basis. In those difficult cases, the use of DNA barcodes may provide a useful tool that is complementary to traditional morphological approaches. In fact, an interactive approach involving traditional morphology and barcoding may prove most useful (Smith *et al*. 2008).

Environmental DNA (eDNA) consists of extracts of DNA from environmental samples. This method associated with Next Generation Sequencing (NGS) is becoming more and more widely used to measure biodiversity, to detect the presence of target species or to evaluate the evolution of species composition (Thomsen & Willerslev 2015). It is also a promising tool for aquatic insects (Deiner *et al.* 2015) since it allows one to find rare species, sibling species, and species living in poorly prospected or unreachable habitats (Mächler *et al.* 2014; Thomsen *et al.* 2012). For Plecoptera and other orders of aquatic insects, eDNA requires a library of known species sequences.

To our knowledge, only a limited number of sequences for authoritatively identified species are available for Central Europe. An incomplete stonefly DNA barcode library exists for North America (Gill et al. 2015, Zhou *et al.* 2009; Zhou *et al.* 2010), Scandinavia (Boumans & Brittain 2012) and Germany (GBOL: https://www.bolgermany.de). To fill this gap, we sequenced the barcode region of COI for nearly all stoneflies known from Switzerland.

Material and methods

Total genomic DNA was extracted using the DNeasy Blood & Tissue kit (QIAGEN) from 440 specimens stored in the collection of the Museum of Zoology in Lausanne (MZL) and in private collections (Gilles Vinçon, Grenoble, France; Jean-Paul Reding, Neuchâtel, Switzerland). Supplier's instructions were adapted to a non-destructive extraction where the whole individuals were first soaked overnight in the extraction buffer with proteinase K at 56°C. Because chitinous parts are kept intact (Vuataz *et al.* 2011), this procedure allows

re-examination of morphological characters whenever necessary. The mitochondrial gene COI was amplified and sequenced using the primers LCO1490 and HCO2198 (Folmer *et al.* 1994). Polymerase chain reaction (PCR) was performed in 20µL total volume with 0.60U Taq (Roche), 2µL of the 10X buffer containing 20mM MgCl2, 0.8µL of each primer (10mM), 0.4µl of a mix containing 10mM of each dNTP (Roche), and 0.85µL template DNA of unknown concentration. The PCR program comprised a heating step at 95°C for 6 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 42°C for 30 s and elongation at 72°C for 45 s, with a final elongation step of 72°C for 10 min. The PCR products were bi-directionally sequenced on an ABI 377 automated sequencer (Applied Biosystems) following the manufacturer's protocols.

Forward and reverse sequences 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). Sequences will be made publically available on BOLD Systems (in progress). Maximum Likelihood (ML) tree searches were conducted using Treefinder v. March 2011 (Jobb *et al.* 2004). We used the Kimura 2-parameter (K2P) model, as implemented in Mega 5.05 to calculate genetic distances between haplotypes and haplogroups (Tamura *et al.* 2011). We considered 3.5% sequence divergence (K2P=0.035) as a likely maximal value for intraspecific divergence (Hebert *et al.* 2003; Zhou *et al.* 2010); this limit must be considered with caution, as higher intraspecific K2P values are not uncommon within Plecoptera (Mynott *et al*. 2011; Sweeney *et al*. 2011; Boumans & Baumann 2012; Gill *et al*. 2015). We chose not to include additional COI sequences from public databases in our analyses considering the present study as preliminary analysis.

DNA extractions are stored at -80°C in the University of Geneva and will be incorporated into the Swiss DNA Bank, while skins and additional material from the same sample are housed at 5°C in the MZL collection.

Specimens that did not align in trees with conspecifics were re-examined for possible misidentification. If they were misidentified or appeared to be new species, their new status was re-evaluated in the database.

Results

COI sequences were obtained from 270 specimens, representing 90 of the 112 species recorded on Switzerland. Although stored in good conditions at the MZL, 39% of the specimens failed to be sequenced. Specimens had not been collected with the aim of being sequenced and storage conditions following collecting are for most of the cases unknown and have not been controlled (EtOH concentration and quality, storing temperature before incorporation into the MZL collection). Therefore, DNA degradation is here recognized as an important cause of amplification failure. Moreover, the use of Plecoptera specific primers could have helped in enhancing the amplification-sequencing success, but such primers are still lacking from available literature.

The monophyly of the different species, species groups, genera and the two sub-orders were well supported by the reconstruction. Our preliminary results revealed fifteen cases of specimens that did not cluster with their conspecifics. *A posteriori* re-identifications allowed us to correct thirteen misidentified specimens (mainly female imagos or larvae within the genus *Leuctra*). Two of these may be due to contaminations during manipulation in the laboratory. In our final reconstruction, the 268 sequenced individuals clustered in their own clade.

With 37 species, *Leuctra* was the most diversified genus in our data set (Table 1; Fig. 1). We used it to illustrate different cases: *i*) high intraspecific distance between different populations within a species without subspecies (*L. nigra* (Olivier, 1811) K2P value = 10.6%; Table 2 & Fig. 2); *ii*) moderate intraspecific distance between different populations within a species with described subspecies (*L. vinconi* Ravizza & Ravizza-Dematteis, 1993 - maximum K2P value between the two subspecies = 3.2%; Table 2 & Fig. 3); iii) low interspecific distances between well recognised species (*L. meridionalis* Aubert, 1951 and *L. zwicki* Ravizza & Vinçon, 1991 - K2P value = 0.8%; Table 2 & Fig. 4).

TABLE 1. Specimen information - GBIF identifiers, specimen code, species name and locality of sequenced specimens. Stage: L = larva; A = Adult. Sex: M = male; F = female. CX and CY are the Swiss coordinates CH1903. Lge designated the collector of the specimens.

GBIF-CH Process. ID	Number SwissBol	Species	Stage	Sex	Canton or Country	CX	CY	Alt. (m)	Leg	Year
GBIFCH00280328	366	*Amphinemura standfussi*	A	F	GR	822950	204555	1840	P. Stucki	2006
GBIFCH00279818	368	*Amphinemura sulcicollis*	A	M	SG	731883	243576	760	V. Lubini	2009
GBIFCH00279862	463	*Amphinemura sulcicollis*	L		VD	569100	125875	700	S. Knispel	2012
GBIFCH00279828	135	*Amphinemura triangularis*	L		LU	641198	213401	700	V. Lubini	2012
GBIFCH00280188	136	*Amphinemura triangularis*	L		AG	637194	238503	420	S. Knispel	2012
GBIFCH00280351	431	*Brachyptera risi*	A	F	LU	638000	219000	636	S. Knispel	2010
GBIFCH00280321	61	*Brachyptera risi*	A	F	ZH	684843	246075	418	V. Lubini	2009
GBIFCH00280231	63	*Brachyptera risi*	L		SG	728600	241960	700	V. Lubini	2009
GBIFCH00279878	266	*Brachyptera seticornis*	A	F	LU	647365	198007	1170	R. Ryf	2009
GBIFCH00280195	455	*Brachyptera seticornis*	L		LU	636415	191370	835	V. Lubini	2013
GBIFCH00280013	267	*Brachyptera trifasciata*	L		SG	685629	238700	480	V. Lubini	2009
GBIFCH00279790	299	*Capnia nigra*	A	M	VS	647012	134106	748	M. Blur	2009
GBIFCH00280306	415	*Capnia nigra*	A	F	BE	590780	152560	1240	P. Stucki	2006
GBIFCH00279917	85	*Capnia nigra*	L		SG	756000	215800	480	V. Lubini	2009
GBIFCH00280014	300	*Capnia vidua*	A	F	VS	632580	142615	1770	P. Stucki	2008
GBIFCH00280091	301	*Capnia vidua*	A	M	BE			1230	M. Blur	2010
GBIFCH00279800	302	*Capnia vidua*	A	F	LU	766490	154440	1680	V. Lubini	2011
GBIFCH00280220	303	*Capnioneura nemuroides*	A	M	VD	560000	147000	1054	S. Knispel	2010
GBIFCH00280334	304	*Capnioneura nemuroides*	L		BE	590780	152560	1240	P. Stucki	2006
GBIFCH00279962	88	*Capnioneura nemuroides*	A	F	LU	647365	198007	1170	M. Blur	2009
GBIFCH00280317	222	*Chloroperla susemicheli*	A	F	LU	766490	154440	180	V. Lubini	2010
GBIFCH00280057	223	*Chloroperla susemicheli*	L		GR	732136	163324	1517	V. Lubini	2009
GBIFCH00280277	91	*Chloroperla susemicheli*	L		VD	573590	119240	1550	S. Knispel	2008

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TABLE 1. (Continued)

GBIF-CH Process. ID	Number SwissBol	Species	Stage	Sex	Canton or Country	CX	CY	Alt. (m)	Leg	Year
GBIFCH00280238	226	*Chloroperla tripunctata*	A	F	ZH	702524	245014	537	V. Lubini	2008
GBIFCH00279833	436	*Chloroperla tripunctata*	A	F	SG	734794	230437	800	V. Lubini	2013
GBIFCH00280352	420	*Dictyogenus alpinum*	L		GR	685725	245891	1900	V. Lubini	2011
GBIFCH00280052	82	*Dictyogenus alpinus*	A	F	BE	602500	156500	1260	P. Stucki	2010
GBIFCH00279916	84	*Dictyogenus alpinus*	L		VS	632580	142615	1770	P. Stucki	2008
GBIFCH00279841	494	*Dictyogenus cf fontium*	A	F	Italy			1375	G. Vinçon	2014
GBIFCH00279845	495	*Dictyogenus cf fontium*	A	M	Italy			1375	G. Vinçon	2014
GBIFCH00280144	147	*Dictyogenus fontium*	L		GR	718282	163532	2320	V. Lubini	2012
GBIFCH00279832	247	*Dictyogenus fontium*	L		VD	574200	120800	1420	M. Sartori	2008
GBIFCH00280133	452	*Dictyogenus fontium*	L		GR	811028	169370	2087	V. Lubini	2011
GBIFCH00280084	130	*Dinocras cephalotes*	A	M	ZH	687937	272115	350	V. Lubini	2009
GBIFCH00280059	276	*Dinocras cephalotes*	L		SG	729883	134682	680	V. Lubini	2011
GBIFCH00279958	235	*Dinocras ferreri*	L		TI	719495	93175	440	V. Lubini	2007
GBIFCH00279868	237	*Dinocras megacephala*	L		SG	729883	134682	680	V. Lubini	2011
GBIFCH00277504	238	*Dinocras megacephala*	A	M	SG	730897	240338	880	V. Lubini	2009
GBIFCH00277473	451	*Dinocras megacephala*	L		SG	729883	234682	680	V. Lubini	2013
GBIFCH00280237	248	*Isoperla carbonaria*	L		TI	707125	124440	585	H. Vicentini	2006
GBIFCH00280002	249	*Isoperla carbonaria*	L		TI	707125	124440	585	H. Vicentini	2006
GBIFCH00279957	250	*Isoperla carbonaria*	L		TI	704465	128910	755	H. Vicentini	2006
GBIFCH00278543	433	*Isoperla cf rivulorum*	L		GR	724509	166591	1640	V. Lubini	2007
GBIFCH00280053	133	*Isoperla grammatica*	L		AG	634429	239712	405	S. Knispel	2012
GBIFCH00280226	281	*Isoperla grammatica*	L		BE	606030	183255	550	P. Stucki	2007
GBIFCH00279846	453	*Isoperla obscura*	A	M	GR	729810	123070	1050	D. Bolt	2011
GBIFCH00279912	253	*Isoperla rivulorum*	L		GL	730570	205547	840	V. Lubini	2010
GBIFCH00280047	254	*Isoperla rivulorum*	L		VD	574820	122360	1265	M. Sartori	2008

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TABLE 1. (Continued)

GBIF-CH Process. ID	Number SwissBol	Species	Stage	Sex	Canton or Country	CX	CY	Alt. (m)	Leg	Year
GBIFCH00279963	94	*Leuctra albida*	A	F	SG	729125	250080	720	V. Lubini	2009
GBIFCH00280323	95	*Leuctra albida*	A	M	VD	521100	161535	650	P. Stucki	2010
GBIFCH00280142	96	*Leuctra albida*	A	F	BE	587190	137405	1175	P. Stucki	2005
GBIFCH00279210	152	*Leuctra alpina*	A	F	GR	730844	162613	1857	V. Lubini	2012
GBIFCH00279446	215	*Leuctra alpina*	A	M	VS	580230	80510	2260	P. Stucki	2010
GBIFCH00278424	305	*Leuctra alpina*	A	F	BE	669300	180500	1845	S. Knispel	2009
GBIFCH00279443	359	*Leuctra alpina*	A	M	ZH	702700	257880	580	V. Lubini	2006
GBIFCH00280060	503	*Leuctra ameliae*	A	F	Italy			1900	G. Vinçon	2014
GBIFCH00279630	218	*Leuctra armata*	A	F	GR	772805	142345	2200	V. Lubini	2010
GBIFCH00278553	456	*Leuctra armata*	A	F	GR	764945	132236	1285	V. Lubini	2013
GBIFCH00279844	467	*Leuctra armata*	A	F	GR	728528	160129	1900	V. Lubini	2013
GBIFCH00279843	468	*Leuctra armata*	L		GR	816124	170489	1969	V. Lubini	2012
GBIFCH00278522	307	*Leuctra aurita*	A	M	GR	768989	205678	700	V. Lubini	2006
GBIFCH00280267	535	*Leuctra autumnalis*	A	F	SO	601.818	233.756	830	J.-P.Reding	2010
GBIFCH00280085	536	*Leuctra autumnalis*	A	F	SO	601.819	233.757	830	J.-P.Reding	2010
GBIFCH00280175	537	*Leuctra autumnalis*	A	M	SO	601.820	233.758	830	J.-P.Reding	2010
GBIFCH00279813	308	*Leuctra braueri*	L		AG	641200	237900	640	H. Vicentini	2007
GBIFCH00279911	309	*Leuctra braueri*	L		ZH	705406	250598	710	V. Lubini	2007
GBIFCH00280338	310	*Leuctra braueri*	L		VD	541045	159310	828	S. Knispel	2010
GBIFCH00279995	510	*Leuctra brevipennis*	A	M	Italy			1500	G. Vinçon	2014
GBIFCH00280177	511	*Leuctra brevipennis*	A	F	Italy			1500	G. Vinçon	2014
GBIFCH00280222	512	*Leuctra brevipennis*	A	F	Italy			1500	G. Vinçon	2014
GBIFCH00279840	504	*Leuctra caprai*	A	F	Italy			1900	G. Vinçon	2014
GBIFCH00279856	505	*Leuctra caprai*	A	M	Italy			1900	G. Vinçon	2014
GBIFCH00279908	506	*Leuctra caprai*	A	M	Italy			1900	G. Vinçon	2014

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TABLE 1. (Continued)

GBIF-CH Process. ID	Number SwissBol	Species	Stage	Sex	Canton or Country	CX	CY	Alt. (m)	Leg	Year
GBIFCH00278397	329	*Leuctra cf cingulata*	L		NE	561840	216095	1025	P. Stucki	2009
GBIFCH00278544	345	*Leuctra cf helvetica*	A	F	GR	766490	154440	1680	V. Lubini	2011
GBIFCH00278515	311	*Leuctra cingulata*	A	M	SG	734794	230437	760	V. Lubini	2011
GBIFCH00279382	312	*Leuctra cingulata*	A	F	BL	623000	246000	690	E. Sandoz	2009
GBIFCH00279219	313	*Leuctra cingulata*	A	F	GR	713000	174000	1224	S. Knispel	2009
GBIFCH00279853	507	*Leuctra dolasilla*	A	F	Italy			1900	G. Vinçon	2014
GBIFCH00279905	508	*Leuctra dolasilla*	A	F	Italy			1900	G. Vinçon	2014
GBIFCH00280086	509	*Leuctra dolasilla*	A	F	Italy			1900	G. Vinçon	2014
GBIFCH00279874	148	*Leuctra fusca*	A	M	LU	635879	214737	712	V. Lubini	2012
GBIFCH00279797	314	*Leuctra fusca*	A	F	BE	606030	183255	550	P. Stucki	2007
GBIFCH00279795	315	*Leuctra fusca*	A	M	ZH	687937	272115	350	V. Lubini	2008
GBIFCH00279879	316	*Leuctra geniculata*	L		AG	649300	246800	400	V. Lubini	2008
GBIFCH00280051	74	*Leuctra geniculata*	L		JU	579750	244790	440	P. Stucki	2002
GBIFCH00280141	75	*Leuctra geniculata*	A	F	AG	649300	246800	400	V. Lubini	2005
GBIFCH00280265	528	*Leuctra gr vinconi n. sp.*	A	F	Italy			1900	G. Vinçon	2014
GBIFCH00279949	529	*Leuctra gr vinconi n. sp.*	A	M	Italy			1900	G. Vinçon	2014
GBIFCH00280129	530	*Leuctra gr vinconi n. sp.*	A	F	Italy			1900	G. Vinçon	2014
GBIFCH00280337	317	*Leuctra handlirschi*	A	F	VD	524590	172190	682	P. Stucki	2010
GBIFCH00280289	318	*Leuctra handlirschi*	A	F	BL	623000	246000	690	L. Sandoz	2009
GBIFCH00280311	319	*Leuctra handlirschi*	A	F	ZH	708000	246139	690	U. Mürle	2009
GBIFCH00280287	320	*Leuctra hexacantha*	A	F	JU	563463	235469	610	P. Stucki	2007
GBIFCH00280305	324	*Leuctra hexacantha*	A	F	VD	566570	130500	684	S. Knispel	2006
GBIFCH00279808	325	*Leuctra hexacantha*	A	M	TI	719930	125110	270	H. Vicentini	2006
GBIFCH00280315	432	*Leuctra hexacantha*	A	F	VD	513235	147842	565	S. Knispel	2006
GBIFCH00280313	321	*Leuctra hippopus*	A	F	FR	566000	171000	793	S. Knispel	2010

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TABLE 1. (Continued)

GBIF-CH Process. ID	Number SwissBol	Species	Stage	Sex	Canton or Country	CX	CY	Alt. (m)	Leg	Year
GBIFCH00280288	322	*Leuctra hippopus*	A	M	VD	526260	163485	540	P. Stucki	2010
GBIFCH00280295	323	*Leuctra hippopus*	A	M	ZH	686523	245278	500	V. Lubini	2009
GBIFCH00279784	149	*Leuctra inermis*	L		LU	635879	214737	712	V. Lubini	2012
GBIFCH00280310	326	*Leuctra inermis*	A	M	BE	652432	177270	580	V. Lubini	2007
GBIFCH00279809	414	*Leuctra inermis*	L		GR	729410	127720	765	H. Vicentini	2006
GBIFCH00278408	328	*Leuctra leptogaster*	A	F	SG	734794	230437	760	V. Lubini	2011
GBIFCH00277601	421	*Leuctra leptogaster*	A	F	SG	752647	209920	540	V. Lubini	2006
GBIFCH00277529	423	*Leuctra leptogaster*	A	M	LU	636415	191370	835	V. Lubini	2013
GBIFCH00277457	331	*Leuctra major*	A	F	UR	688437	170670	975	H. Vicentini	2006
GBIFCH00277472	332	*Leuctra major*	A	F	GR	731024	189486	1902	N. Nembrini	2009
GBIFCH00279849	470	*Leuctra major*	A	F	LU	647838	202724	740	V. Lubini	2013
GBIFCH00279793	334	*Leuctra meridionalis*	A	F	TI	701340	120035	360	H. Vicentini	2006
GBIFCH00279794	335	*Leuctra meridionalis*	A	F	TI	704465	128910	755	H. Vicentini	2006
GBIFCH00280341	336	*Leuctra meridionalis*	A	F	TI	719930	125110	270	H. Vicentini	2006
GBIFCH00280219	513	*Leuctra meridionalis*	A	M	Italy			1500	G. Vinçon	2014
GBIFCH00279951	514	*Leuctra meridionalis*	A	F	Italy			1500	G. Vinçon	2014
GBIFCH00279952	515	*Leuctra meridionalis*	A	F	Italy			1500	G. Vinçon	2014
GBIFCH00277690	337	*Leuctra mortoni*	A	F	SG	734794	230437	760	V. Lubini	2011
GBIFCH00279387	338	*Leuctra mortoni*	A	F	SG	752647	209920	540	V. Lubini	2006
GBIFCH00278446	422	*Leuctra mortoni*	A	M	BE	623575	164284	1010	P. Stucki	2007
GBIFCH00278558	459	*Leuctra mortoni*	A	F	LU	647445	206547	640	V. Lubini	2013
GBIFCH00278580	306	*Leuctra moselyi*	A	M	GR	729410	127720	765	H. Vicentini	2006
GBIFCH00277528	339	*Leuctra moselyi*	A	F	SG	734794	230437	760	V. Lubini	2011
GBIFCH00277612	340	*Leuctra moselyi*	A	F	BE	623575	164284	1010	P. Stucki	2007

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TABLE 1. (Continued)

GBIF-CH Process. ID	Number SwissBol	Species	Stage	Sex	Canton or Country	CX	CY	Alt. (m)	Leg	Year
GBIFCH00277464	341	*Leuctra moselyi*	A	M	GR	701284	170078	1340	V. Lubini	2008
GBIFCH00277517	342	*Leuctra nigra*	A	F	VD	544450	158260	864	S. Knispel	2010
GBIFCH00277566	343	*Leuctra nigra*	A	F	SG	734067	242801	800	V. Lubini	2009
GBIFCH00277633	344	*Leuctra nigra*	A	F	TI	713007	110476	680	R. Tester Ryf	2009
GBIFCH00277668	458	*Leuctra niveola*	A	M	SG	734794	230437	800	V. Lubini	2013
GBIFCH00280335	346	*Leuctra prima*	A	M	VD	544450	158260	864	S. Knispel	2010
GBIFCH00279969	347	*Leuctra prima*	A	F	BE	632000	195000	835	M. Blur	2010
GBIFCH00280307	348	*Leuctra prima*	A	F	BL	617000	214000	600	F. Sandoz	2009
GBIFCH00280264	532	*Leuctra pseudorosinae*	A	M	VD	530.388	189.018	1200	J.-P.Reding	2014
GBIFCH00280174	533	*Leuctra pseudorosinae*	A	F	VD	530.388	189.019	1200	J.-P.Reding	2014
GBIFCH00279996	534	*Leuctra pseudorosinae*	A	M	VD	530.388	189.020	1200	J.-P.Reding	2014
GBIFCH00280300	349	*Leuctra pseudosignifera*	A	F	VD	560000	147000	1054	S. Knispel	2010
GBIFCH00279815	350	*Leuctra pseudosignifera*	A	F	SZ	710770	222870	670	H. Vicentini	2006
GBIFCH00280285	351	*Leuctra pseudosignifera*	A	F	SO	606550	234600	1000	V. Lubini	2006
GBIFCH00280087	155	*Leuctra rauscheri*	L		GL	731990	200795	873	V. Lubini	2011
GBIFCH00280314	352	*Leuctra rauscheri*	A	M	BE	603000	157000	1260	P. Stücki	2010
GBIFCH00280298	353	*Leuctra rauscheri*	A	M	VS	584000	584000	1133	S. Knispel	2010
GBIFCH00277474	354	*Leuctra rosinae*	A	M	BE	590000	147000	1250	S. Knispel	2010
GBIFCH00277455	355	*Leuctra rosinae*	A	F	VS	632580	142615	1770	P. Stucki	2007
GBIFCH00280345	356	*Leuctra schmidi*	A	M	VD	575495	128070	1620	V. Lubini	2006
GBIFCH00280343	357	*Leuctra schmidi*	A	F	VS	574285	97720	1500	P. Stucki	2005
GBIFCH00280355	443	*Leuctra schmidi*	A	M	VD	575575	127810	1660	P. Stucki	2006
GBIFCH00279742	139	*Leuctra sesvenna*	A	F	GR	718282	163532	2320	V. Lubini	2012

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TABLE 1. (Continued)

GBIF-CH Process. ID	Number SwissBol	Species	Stage	Sex	Canton or Country	CX	CY	Alt. (m)	Leg	Year
GBIFCH00277527	360	*Leuctra subalpina*	A	F	JU	588564	239063	640	P. Stucki	2007
GBIFCH00278561	361	*Leuctra subalpina*	A		VD	524580	172075	680	P. Stucki	2007
GBIFCH00278396	419	*Leuctra subalpina*	A	M	JU	571350	238900	600	P. Stucki	2006
GBIFCH00279787	219	*Leuctra teriolensis*	A	M	VS	580500	79790	2400	P. Stucki	2010
GBIFCH00280136	362	*Leuctra teriolensis*	A	F	UR	678421	167454	1830	V. Lubini	2010
GBIFCH00279834	363	*Leuctra teriolensis*	A	M	LU	651050	244600	1165	M. Blur	2009
GBIFCH00279950	525	*Leuctra vinconi aubertorum*	A	M	TI				G. Vinçon	2014
GBIFCH00280130	526	*Leuctra vinconi aubertorum*	A	F	TI				G. Vinçon	2014
GBIFCH00280132	527	*Leuctra vinconi aubertorum*	A	M	TI				G. Vinçon	2014
GBIFCH00280176	516	*Leuctra vinconi vinconi*	A	M	Italy				G. Vinçon	2014
GBIFCH00280041	517	*Leuctra vinconi vinconi*	A	F	Italy				G. Vinçon	2014
GBIFCH00279997	518	*Leuctra vinconi vinconi*	A	F	Italy				G. Vinçon	2014
GBIFCH00280221	519	*Leuctra vinconi vinconi*	A	F	Italy			1500	G. Vinçon	2014
GBIFCH00280131	520	*Leuctra vinconi vinconi*	A	F	Italy			1500	G. Vinçon	2014
GBIFCH00279994	521	*Leuctra vinconi vinconi*	A	M	Italy			1500	G. Vinçon	2014
GBIFCH00279904	523	*Leuctra vinconi vinconi*	A	M	Italy				G. Vinçon	2014
GBIFCH00280040	524	*Leuctra vinconi vinconi*	A	F	Italy				G. Vinçon	2014
GBIFCH00280043	491	*Leuctra zwicki*	A	M	France (06)			600	G. Vinçon	2014
GBIFCH00279847	492	*Leuctra zwicki*	A	M	France (06)			600	G. Vinçon	2014
GBIFCH00279838	493	*Leuctra zwicki*	A	F	France (06)			600	G. Vinçon	2014
GBIFCH00280330	365	*Nemoura avicularis*	A	M	NE	549321	207143	1000	P. Stucki	2009
GBIFCH00280296	428	*Nemoura avicularis*	L		NE	549321	207143	1000	P. Stucki	2009
GBIFCH00279839	445	*Nemoura cambrica*	A	M	VD	543650	158850	855	S. Knispel	2010

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TABLE 1. (Continued)

GBIF-CH Process. ID	Number SwissBol	Species	Stage	Sex	Canton or Country	CX	CY	Alt. (m)	Leg	Year
GBIFCH00280134	446	*Nemoura cambrica*	A	M	LU	638	219	636	S. Knispel	2010
GBIFCH00280089	447	*Nemoura cambrica*	A	M	SG	729125	250080	720	V. Lubini	2009
GBIFCH00278416	379	*Nemoura cf minima*	L		ZH	697235	257796	455	V. Lubini	2010
GBIFCH00278394	383	*Nemoura cf obtusa*	L		GL	730160	204059	790	V. Lubini	2010
GBIFCH00279811	444	*Nemoura cinerea*	L		NE	547184	205183	995	P. Stucki	2009
GBIFCH00280098	76	*Nemoura cinerea*	A	F	ZH	707200	245800	622	V. Lubini	2008
GBIFCH00280096	77	*Nemoura cinerea*	L		GR	718400	175200	1920	V. Lubini	2001
GBIFCH00279807	372	*Nemoura dubitans*	A	F	ZH	702751	244753	540	V. Lubini	2008
GBIFCH00279873	128	*Nemoura flexuosa*	L		ZH	699219	248930	515	V. Lubini	2012
GBIFCH00279859	472	*Nemoura flexuosa*	L		GE	486715	117735	440	S. Knispel	2011
GBIFCH00279998	473	*Nemoura flexuosa*	L		VD	538930	176970	460	S. Knispel	2011
GBIFCH00280339	375	*Nemoura marginata*	A	M	BE	614	203	660	E. Sandoz	2010
GBIFCH00280356	376	*Nemoura marginata*	A	M	VD	542018	159064	801	S. Knispel	2010
GBIFCH00280353	377	*Nemoura marginata*	A	M	LU	638	219	636	S. Knispel	2010
GBIFCH00279955	378	*Nemoura minima*	A	M	VD	560	147	1054	S. Knispel	2010
GBIFCH00279968	380	*Nemoura minima*	L		SG	731275	246750	730	V. Lubini	2009
GBIFCH00279918	134	*Nemoura mortoni*	L		GR	719936	164484	2200	V. Lubini	2012
GBIFCH00279913	214	*Nemoura mortoni*	L		VS	580500	79790	2400	P. Stucki	2010
GBIFCH00280180	381	*Nemoura mortoni*	L		BE	665065	177085	1230	P. Stucki	2009
GBIFCH00280361	382	*Nemoura obtusa*	A	M	GR	734100	174703	890	V. Lubini	2007
GBIFCH00278406	384	*Nemoura obtusa*	A	M	SZ	708400	205300	1560	V. Lubini	2009
GBIFCH00279855	474	*Nemoura obtusa*	L		GR	777452	141709	2010	V. Lubini	2013
GBIFCH00280350	389	*Nemoura sciurus*	L		VD	544450	158260	864	S. Knispel	2010
GBIFCH00280360	390	*Nemoura sciurus*	L		VD	542018	159064	801	S. Knispel	2010

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TABLE 1. (Continued)

GBIF-CH Process. ID	Number SwissBol	Species	Stage	Sex	Canton or Country	CX	CY	Alt. (m)	Leg	Year
GBIFCH00280348	391	*Nemoura sciurus*	L		VD	543650	158850	855	S. Knispel	2010
GBIFCH00280234	153	*Nemoura sinuata*	A	F	GR	727513	158131	1940	V. Lubini	2012
GBIFCH00280048	213	*Nemoura sinuata*	A	M	BE	667310	178980	1460	P. Stucki	2010
GBIFCH00279854	475	*Nemoura sinuata*	A	F	BE	632000	155000	2024	S. Knispel	2010
GBIFCH00279829	141	*Nemoura uncinata*	L		SG	742613	253449	583	V. Lubini	2012
GBIFCH00280354	386	*Nemoura uncinata*	A	M	SG	730425	246100	800	C. Meier	2009
GBIFCH00279835	476	*Nemoura uncinata*	L		SG	729883	234682	680	V. Lubini	2013
GBIFCH00280223	460	*Nemoura undulata*	A	F	GR	804604	171842	1820	S. Knispel	2012
GBIFCH00279880	461	*Nemoura undulata*	L		GR	714425	171525	1916	V. Lubini	2012
GBIFCH00280054	151	*Nemurella pictetii*	L		GR	729325	160880	1957	V. Lubini	2012
GBIFCH00279803	387	*Nemurella pictetii*	A	F	GL	724170	215900	445	V. Lubini	2007
GBIFCH00280292	388	*Nemurella pictetii*	A	F	VD	543650	158850	855	S. Knispel	2010
GBIFCH00279244	145	*Perla cf marginata*	L		AG	637194	238503	420	S. Knispel	2012
GBIFCH00280147	241	*Perla grandis*	L		BE	665065	177085	1230	P. Stucki	2009
GBIFCH00280318	242	*Perla grandis*	L		TI	734270	128415	395	H. Vicentini	2006
GBIFCH00277541	438	*Perla grandis*	L		UR	693176	180290	800	V. Lubini	2010
GBIFCH00278579	243	*Perla marginata*	L		AG	672210	248670	425	V. Lubini	2008
GBIFCH00279737	244	*Perla marginata*	A	F	SH	677000	289600	450	V. Lubini	2010
GBIFCH00279548	143	*Perlodes intricatus*	L		GR	806256	187652	2010	V. Lubini	2012
GBIFCH00277572	212	*Perlodes intricatus*	L		GR	791193	181468	2280	V. Lubini	2010
GBIFCH00277687	256	*Perlodes intricatus*	A	F	VS	632580	142615	1770	P. Stucki	2007
GBIFCH00277562	258	*Perlodes intricatus*	A	M	TI	683195	143823	1940	V. Lubini	2008
GBIFCH00280182	260	*Perlodes jurassicus*	L		SO	614490	244025	700	P. Stucki	2006
GBIFCH00280193	280	*Perlodes jurassicus*	L		VD	520900	163600	680	P. Mulattieri	2011

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TABLE 1. (Continued)

GBIF-CH Process. ID	Number SwissBol	Species	Stage	Sex	Canton or Country	CX	CY	Alt. (m)	Leg	Year
GBIFCH00279906	261_B	*Perlodes jurassicus*	L		JU	583640	237660	580	P. Stucki	2006
GBIFCH00279669	137	*Perlodes microcephalus*	L		SG	742613	253449	583	V. Lubini	2012
GBIFCH00278510	263	*Perlodes microcephalus*	A	F	ZH	711660	236460	610	D. Bolt	2007
GBIFCH00280358	392	*Protonemura algovia*	L		GR	817160	164657	2016	V. Lubini	2011
GBIFCH00279925	477	*Protonemura algovia*	A	F	GR	816320	170250	1960	J. Ortlepp	2012
GBIFCH00279430	216	*Protonemura auberti*	A	M	GR	761793	186312	1600	V. Lubini	2010
GBIFCH00277560	395	*Protonemura auberti*	A	F	SG	725782	222000	510	U. Mürle	2009
GBIFCH00279783	126	*Protonemura brevistyla*	L		GR	720595	165648	2212	V. Lubini	2012
GBIFCH00279782	127	*Protonemura brevistyla*	A	M	SZ	701625	202785	735	H. Vicentini	2006
GBIFCH00280346	396	*Protonemura brevistyla*	A	M	TI	683470	144695	1940	V. Lubini	2008
GBIFCH00277494	411	*Protonemura cf praecox*	L		GR	731546	176363	1580	V. Lubini	2008
GBIFCH00278500	412	*Protonemura cf praecox*	A	F	AG	646364	255248	510	V. Lubini	2007
GBIFCH00279674	154	*Protonemura cf risi*	L		ZH	679241	242065	545	V. Lubini	2012
GBIFCH00279953	502	*Protonemura cf. auberti*	A	F	Italy			1400	G. Vinçon	2009
GBIFCH00279864	496	*Protonemura gr. auberti*	A	F	Italy				G. Vinçon	2014
GBIFCH00279954	498	*Protonemura gr. auberti*	A	F	Italy			1375	G. Vinçon	2014
GBIFCH00279852	499	*Protonemura gr. auberti*	A	F	Italy			1375	G. Vinçon	2014
GBIFCH00280008	131	*Protonemura intricata*	L		LU	641198	213401	700	V. Lubini	2012
GBIFCH00280303	397	*Protonemura intricata*	L		SG	731275	246750	730	V. Lubini	2009
GBIFCH00280225	398	*Protonemura intricata*	L		AG	637200	238500	425	H. Vicentini	2009
GBIFCH00280363	220	*Protonemura lateralis*	L		GR	793409	179735	2280	V. Lubini	2010
GBIFCH00280003	221	*Protonemura lateralis*	L		VS	580230	80510	2260	P. Stucki	2010
GBIFCH00279816	399	*Protonemura lateralis*	L		TI	683195	143823	1940	V. Lubini	2008

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TABLE 1. (Continued)

GBIF-CH Process. ID	Number SwissBol	Species	Stage	Sex	Canton or Country	CX	CY	Alt. (m)	Leg	Year
GBIFCH00280044	479	*Protonemura meyeri*	L		SG	722000	227576	431	U. Mürle	2012
GBIFCH00280333	403	*Protonemura nimborella*	A	F	NW	702170	188232	1936	V. Lubini	2011
GBIFCH00280316	405	*Protonemura nimborella*	A	M	VD	577925	126390	1930	V. Lubini	2006
GBIFCH00279848	482	*Protonemura nimborella*	A	F	VD	575575	127810	1660	P. Stucki	2006
GBIFCH00280233	140	*Protonemura nimborum*	L		GR	813827	181674	1640	V. Lubini	2012
GBIFCH00279910	406	*Protonemura nimborum*	A	M	VS	632580	142615	1770	P. Stucki	2008
GBIFCH00280105	483	*Protonemura nimborum*	L		GR	812280	171747	1803	S. Knispel	2012
GBIFCH00280357	409	*Protonemura nitida*	A	F	GR	713000	174000	1224	S. Knispel	2009
GBIFCH00280349	410	*Protonemura nitida*	A	M	SG	734794	230437	760	V. Lubini	2011
GBIFCH00279842	484	*Protonemura nitida*	A	M	GE	491355	128250	538	S. Knispel	2011
GBIFCH00277640	424	*Protonemura risi*	A	M	VD	541045	159310	828	S. Knispel	2010
GBIFCH00277523	425	*Protonemura risi*	A	M	SO	606550	234600	820	V. Lubini	2006
GBIFCH00279923	268	*Rhabdiopteryx alpina*	A	F	GR			1835	A. Zurwerra	2009
GBIFCH00280058	269	*Rhabdiopteryx alpina*	A	F	GR	707793	166000	1465	A. Zurwerra	2009
GBIFCH00279806	285	*Rhabdiopteryx harperi*	A	M	VS	632580	142615	1770	P. Stucki	2008
GBIFCH00279810	286	*Rhabdiopteryx harperi*	L		GR	685725	245891	1900	V. Lubini	2011
GBIFCH00280344	287	*Rhabdiopteryx harperi*	A	M	GR	726442	167293	1380	V. Lubini	2007
GBIFCH00280099	146	*Rhabdiopteryx neglecta*	L		GR	813827	181674	1640	V. Lubini	2012
GBIFCH00280331	289	*Rhabdiopteryx neglecta*	L		GL	730160	204059	790	V. Lubini	2010
GBIFCH00277542	454	*Rhabdiopteryx neglecta*	A	M	LU	647393	184434	1229	V. Lubini	2013
GBIFCH00280102	227	*Siphonoperla montana*	L		GR	685725	245891	1900	V. Lubini	2011
GBIFCH00279820	270	*Siphonoperla montana*	A	F	VS	632580	142615	1770	P. Stucki	2007
GBIFCH00280359	271	*Siphonoperla montana*	A	M	BE	669300	180500	1845	S. Knispel	2009
GBIFCH00280189	144	*Siphonoperla torrentium*	L		LU	635879	214737	712	V. Lubini	2012

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TABLE 1. (Continued)

GBIF-CH Process. ID	Number SwissBol	Species	Stage	Sex	Canton or Country	CX	CY	Alt. (m)	Leg	Year
GBIFCH00280148	230	*Siphonoperla torrentium*	A	M	VD	521375	172182	900	P. Stucki	2010
GBIFCH00279817	416	*Siphonoperla torrentium*	L		SG	728600	247950	640	V. Lubini	2009
GBIFCH00280104	290	*Taeniopteryx hubaulti*	A	M	SG	733237	237750	849	V. Lubini	2009
GBIFCH00280329	291	*Taeniopteryx hubaulti*	A	M	SG	729050	242790	650	V. Lubini	2009
GBIFCH00280045	442	*Taeniopteryx hubaulti*	A	F	SG	729883	234682	680	V. Lubini	2013
GBIFCH00280090	441	*Taeniopteryx kuehtreiberi*	L		SG	734794	230437	800	V. Lubini	2013
GBIFCH00280340	293	*Taeniopteryx kuhtreiberi*	A	M	FR	585850	161280	950	P. Derleth	2011
GBIFCH00279956	296	*Zwicknia ledoarei*	A	M	TG	719576	275844	490	V. Lubini	2006

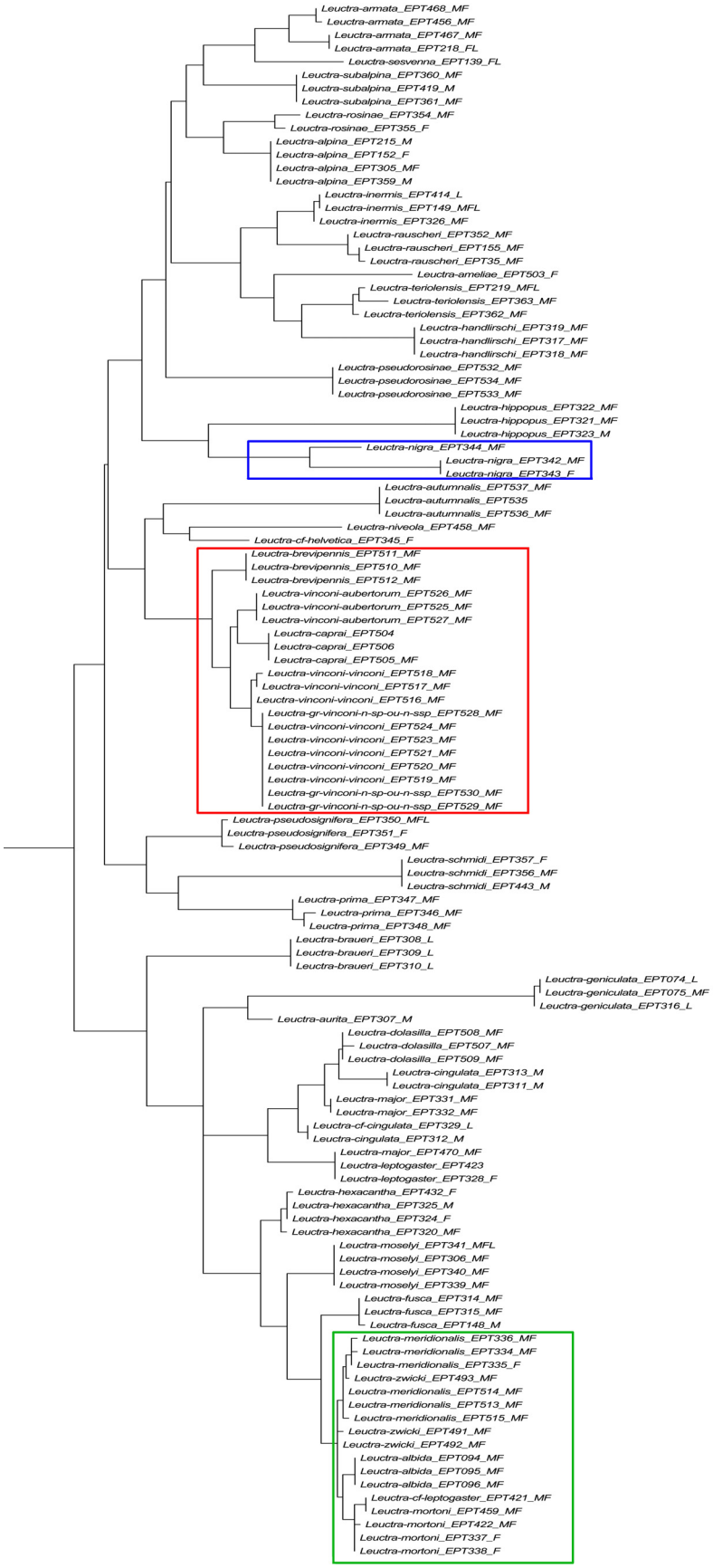

FIGURE 1. COI gene tree for all the specimens belonging to the genus *Leuctra.* Blue frame: *Leuctra nigra* (cf Fig. 2). Red frame: *Leuctra* gr. *brevipennis* (cf Fig. 3). Green frame: *Leuctra* gr. *fusca* (cf Fig. 4). EPTXXX: Specimen code (Number SwissBol), L: larva; M: male imago; F: female imago; MF: both male and female imagos are present in the sample.

TABLE 2. Intra and interspecific distances - Maximum intraspecific and minimum interspecific K2P values found within *Leuctra* (in %). N = number of sequenced specimens.

Species	N	Maximum intraspecific distance	Nearest neighbor	Nearest neighbor distance
L. albida	3	0.3	*L. mortoni*	2.0
L. alpina	4	0.0	*L. rosinae*	9.0
L. ameliae	1	0.0	*L. teriolensis*	10.3
L. armata	4	3.6	*L. sesvenna*	11.7
L. aurita	1	0.0	*L. moselyi*	9.6
L. autumnalis	3	0.2	*L. vinconi*	17.3
L. braueri	3	0.0	*L. helvetica*	17.5
L. brevipennis	3	0.0	*L. vinconi*	5.0
L. caprai	3	0.3	*L. vinconi*	5.0
L. cingulata	3	5.8	*L. major*	4.9
L. dolasilla	3	0.3	*L. major*	1.8
L. fusca	3	0.8	*L. albida*	3.8
L. geniculata	3	0.2	*L. major*	16.7
L. handlirschi	3	0.0	*L. inermis*	13.4
L. hexacantha	4	1.3	*L. moselyi*	6.4
L. helvetica	1	0.0	*L. niveola*	12.9
L. hippopus	3	0.3	*L. pseudosignifera*	16.2
L. inermis	3	0.3	*L. rauscheri*	6.5
L. leptogaster	2	0.2	*L. cingulata*	5.9
L. major	2	0.0	*L. dolasilla*	1.8
L. meridionalis	6	0.8	*L. zwicki*	0.8
L. mortoni	4	1.3	*L. albida*	2.0
L. moselyi	4	0.2	*L. hexacantha*	6.3
L. nigra	3	10.6	*L. pseudosignifera*	11.4
L. niveola	1	0.0	*L. helvetica*	12.9
L. prima	3	0.7	*L. pseudosignifera*	11.4
L. pseudorosinae	3	0.0	*L. alpina*	12.0
L. pseudosignifera	3	0.6	*L. prima*	11.4
L. rauscheri	3	0.8	*L. inermis*	6.5
L. rosinae	2	1.9	*L. alpina*	9.0
L. schmidi	3	0.0	*L. prima*	17.5
L. sesvenna	1	0.0	*L. armata*	11.7
L. subalpina	3	0.2	*L. alpina*	12.0
L. teriolensis	3	1.1	*L. inermis*	9.9
L. vinconi aubertorum	3	0.0	*L. vinconi vinconi*	3.2
			L. caprai	4.9
L. vinconi vinconi	8	1.0	*L. vinconi aubertorum*	3.2
			L. caprai	5.0
L. zwicki	3	0.8	*L. meridionalis*	1.4

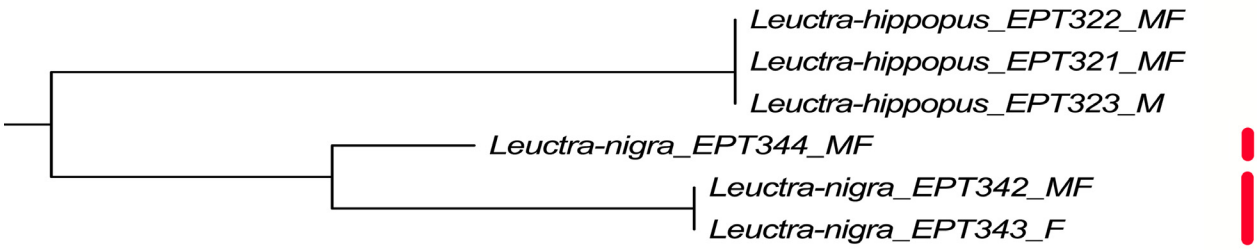

FIGURE 2. CO1 gene tree for *Leuctra nigra.* For abbreviations see Fig. 1.

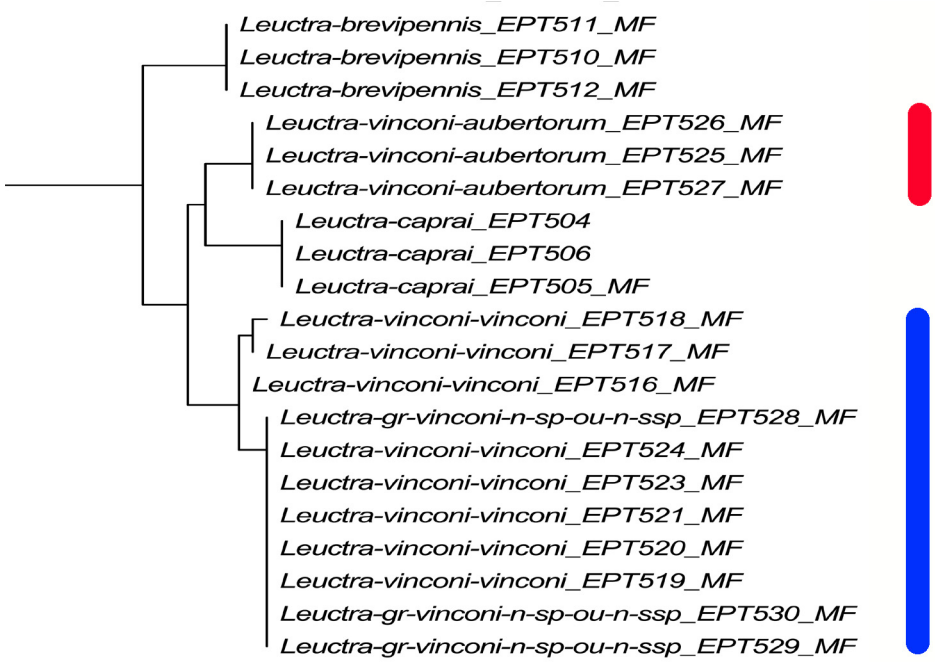

FIGURE 3. CO1 gene tree for *Leuctra* gr. *brevipennis.* Red dash: *Leuctra vinconi aubertorum.* Blue dash: *Leuctra v. vinconi.* For abbreviations see Fig. 1.

Discussion

With the present database, we could authoritatively identify 90% of the Swiss species and almost 99% of the specimens sequenced for this project. Twelve of the 112 species have not been collected in Switzerland since 1970 and may be extirpated from the country (Lubini *et al.* 2012b). Most of these species (*Brachyptera braueri* (Klapálek, 1900), *B. monilicornis* (Pictet, 1841), *Xanthoperla apicalis* (Newmann, 1836), *Isoperla obscura* (Zetterstedt, 1840), *Isogenus nubecula* (Newmann, 1833) and *Besdolus ventralis* (Pictet, 1841)) inhabited lowland rivers that have been modified due to channel regulation or water pollution. The other missing species are rare in Switzerland, having either extremely restricted distributions (*Leuctra ravizzai* Ravizza-Dematteis & Vinçon, 1994, *Nemoura undulata* Ris, 1902, *Isoperla oxylepis* Despax, 1936 and *Perlodes dispar* (Rambur, 1842)), or being at the northern limit of their distribution in southern Switzerland (*Nemoura pesarinii* Ravizza & Ravizza Dematteis, 1979, *N. palliventris*, Aubert, 1953, *Leuctra elisabethae* Ravizza, 1985*, L. festai* Aubert, 1954*, L. insubrica,* Aubert, 1949, *L. muranyii* Vinçon & Graf, 2011, *Isoperla carbonaria* Aubert, 1953*, I. lugens* (Klapalek, 1923) and *I. orobica* Ravizza, 1975).

The Swiss Plecoptera DNA barcode library consists of a relative low number of specimens per species (generally three specimens from different areas: Jura, Plateau and Alps). Although high levels of intraspecific divergence have been reported for Plecoptera (Gill *et al.* 2015), we detected intraspecific distances higher than the established threshold of 3.5 % in only a few cases.

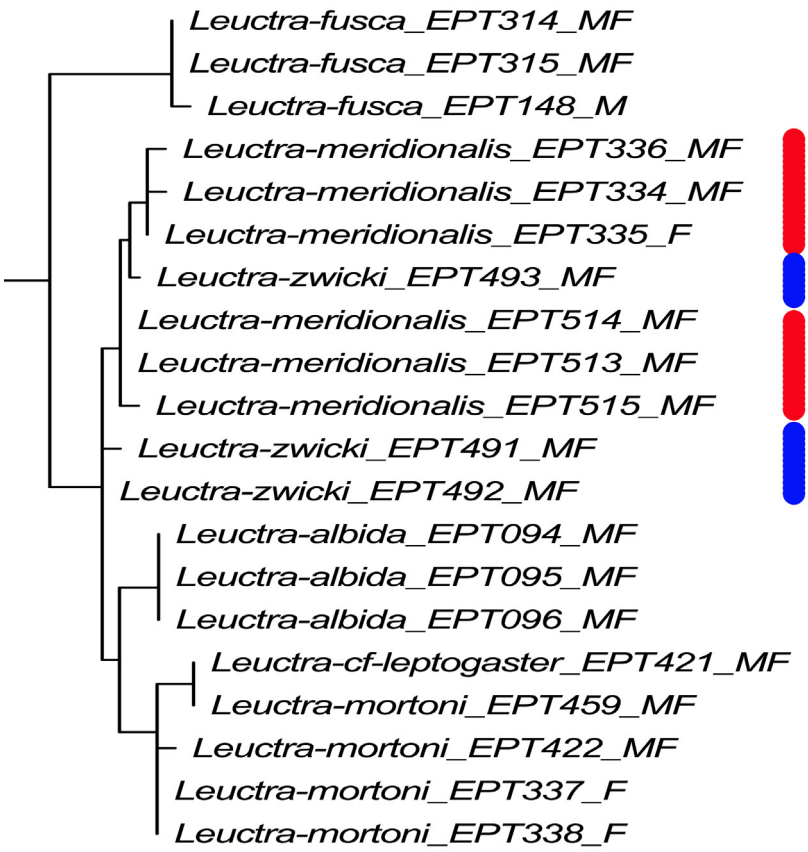

FIGURE 4. CO1 gene tree for *Leuctra* gr. *fusca.* Red dash: *Leuctra meridionalis.* Blue dash: *Leuctra zwicki.* For abbreviations see Fig. 1.

Within the genus *Leuctra*, the genetic distance between conspecific specimens is generally low (Table 2). Except for *Leuctra cingulata* Kempny, 1899, the maximum intraspecific variability (Table 2) is lower than the distance to the nearest neighbour corroborating the presence of the so-called DNA barcode gap (Zhou *et al.* 2010). The case of *Leuctra nigra* is an exception with a distance between the two populations much higher than what is generally considered as an intraspecific value (K2P distance = 0.106) (Table 2, Fig. 2). Therefore, as 10% of divergence most probably indicates the presence of putative cryptic species, additional sequences are highly desirable. In this particular case, morphological differences allow discrimination of two groups of *L. nigra*, one occurring on the northern slopes of the Alps and the other restricted to the southern slopes of the Alps. These results will be investigated and discussed in more detail in a complementary research study with morphological analysis at population level (Vinçon & Gattolliat, unpublished data).

Conversely, low interspecific differences were also detected between some *Leuctra* species (Table 2; Fig. 4). For instance, the genetic distance between *Leuctra albida*, *L. meridionalis*, *L. mortoni* and *L. zwicki* is lower than 3%. Furthermore, the very low genetic distance between *L. albida* and *L. meridionalis* confirms their sister species status. On the basis of COI results, these species could be considered as two geographic sister species or subspecies, as they respectively occur on the northern and southern slopes of the Alps. The last glaciations certainly had an important impact on the speciation process within different stonefly genera and within *Leuctra* in particular (Vinçon 2012). As this process is rather recent, the time to accumulate mutations has been rather short. We also need to consider that mitochondrial introgression may introduce an error into the DNA barcode-based species database (Dussex *et al.* 2015). For example, COI cannot reliably distinguish between *L. fusca* and *L. digitata* in Northern Europe due to the introgression of a *L. fusca* mitochondrion into a *L. digitata* population (Boumans & Tierno de Figueroa 2016). Introgression may partially explain the complex relationships observed for *L. zwicki* and *L. meridionalis* (Fig. 4).

The COI reconstruction also has important implications for the status of some taxa considered as subspecies. For instance, two subspecies within the species *Leuctra vinconi* Ravizza & Ravizza-Dematteis,

1993 (Fig. 3) were described. The subspecies *Leuctra vinconi aubertorum* Ravizza & Ravizza-Dematteis, 1994 appears closer to *Leuctra caprai* Festa, 1939 than to the nominotypical subspecies *L. v. vinconi*. The genetic distance between the two subspecies is slightly higher than the intraspecific distance. According to these preliminary results, *L. vinconi aubertorum* should be revaluated to the species rank rather than subspecies.

Our study contributed much to the completion of the DNA barcode library for Swiss stoneflies and it already constitutes a reliable tool for identification. A few specimens collected from nearby countries (France and Italy) have been crucial to complete our dataset and we believe that we need to continue this effort by collecting and sequencing additional specimens that belong to very rare species or are no longer reported from Switzerland. Moreover, we should pay attention to species not yet reported from Switzerland but occurring close to the Swiss borders. These should be added to the library, especially those species increasingly widely distributed (related, for example, to climate change).

Our study highlighted some cases that deserve particular attention, especially within *Leuctra.* A careful and complete analysis of these cases should be undertaken before taking any systematic decision. We defend the fact that revaluation of species status should be based on both morphological characters and molecular data. Examination of morphology of male terminalia and of female vaginal sclerites and sternal plates (such as in Grubbs 2015) will be necessary to reach conclusions on distinctness of species and the usefulness of current subspecies designations in closely related groups.

Acknowledgments

This study was sponsored by a grant from the Swiss Federal Office for the Environment to SwissBOL, with the help of the Museum of Zoology, Lausanne (MZL). We wish to thank Sandra Knispel, Jean-Paul Reding and Verena Lubini for the gift of material and identification, or re-identification, of some specimens. Many thanks also to Albertine Roulet, Marion Podolak and Nicolas Hazi for searching and preparing the specimens stored in the MZL collection. Our appreciations go to Louis Boumans (Oslo, Norway) and an anonymous reviewer for useful comments on an early version of the manuscript.

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