

Diverse molecular data demonstrate that commercially available medicinal leeches are not *Hirudo medicinalis*

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The European medicinal leech is one of vanishingly few animal species with direct application in modern medicine. In addition to the therapeutic potential held by many protease inhibitors purified from leech saliva, and notwithstanding the historical association with quackery, *Hirudo medicinalis* has been approved by the United States Food and Drug Administration as a prescription medical device. Accurate annotation of bioactive compounds relies on precise species determination. Interpretations of developmental and neurophysiological characteristics also presuppose uniformity within a model species used in laboratory settings. Here, we show, with mitochondrial sequences and nuclear microsatellites, that there are at least three species of European medicinal leech, and that leeches marketed as *H. medicinalis* are actually *Hirudo verbana*. Beyond the obvious need for reconsideration of decades of biomedical research on this widely used model organism, these findings impact regulatory statutes and raise concerns for the conservation status of European medicinal leeches.

Keywords: Annelida; anticoagulants; biodiversity; conservation biology; DNA barcoding; neurobiology

1. INTRODUCTION

Medicinal leeches have been facilitators of phlebotomy since the time of Galen with their use for balancing humours peaking during the post-Napoleonic era when tens of millions were employed across Europe (Moquin-Tandon 1846; Harding & Moore 1927; Elliott & Tullett 1992). At that time, the annual leech harvest was sufficiently intense to stimulate some of the earliest legislative efforts at biological conservation (Elliott & Tullett 1992). Long since repudiated as suitable devices for the treatment of conditions ranging from obesity to pneumonitis (Louis 1828), these much maligned invertebrates are again seeing wide use as biomedical tools. European medicinal leeches returned to the modern clinical toolbox in the context of flap and replantation surgery (Derganc & Zdravic 1960; Foucher *et al.* 1981). Specifically, with artery-only replantation (e.g. of digits), free tissue transfer surgery (e.g. skin flaps) and cases of venous obstruction, the post-operative use of leeches to ameliorate congestion improves prognosis and tissue survival in a cost-effective and reliable manner that has yet to be supplanted by mechanical or pharmaceutical alternatives (Foucher *et al.* 1981; Chepeha *et al.* 2002; Graf *et al.* 2006). Their potential as living apothecaries has stimulated the isolation of scores of bioactive compounds including important anticoagulants, antistasins and other protease inhibitors (Sollner *et al.* 1994; Baskova & Zavalova 2001; Salzet 2002). Leeches also have been favoured model organisms in developmental genetics and in neurobiology in

light of their large, easily manipulated neurons, complex behaviours and integrated locomotion (Kristan *et al.* 2005). More recently, they have become a compelling research model for enteric symbioses (Graf *et al.* 2006). In addition to stimulating lucrative leech farming industries, an enviable status was achieved in 2004 with United States Food and Drug Administration (US FDA) approval to market the European medicinal leech, *Hirudo medicinalis*, as a prescription medical device (Rados 2004)—provided that accurate labelling and branding regulations were followed.

Hirudo medicinalis, described by Linnaeus in 1758, has long been considered the sole European medicinal leech (Sawyer 1986). Yet, by 1827, at least five additional species were recognized (Moquin-Tandon 1827), the varieties of which even Darwin (1859) noted ‘cannot be kept together’. The lack of clear internal anatomical differences has been an impediment to accurately delimiting leech species and taxonomic descriptions often were predicated on subtle variations in colour pattern later considered too variable to be reliable (Moquin-Tandon 1846). In the context of identifying wild and commercially marketed medicinal leeches, we provide DNA barcoding data and, independent of the barcoding locus, nuclear microsatellite data regarding multiple species of *Hirudo* in comparison with commercially available leeches.

2. MATERIAL AND METHODS

Wild European medicinal leeches were collected from France, Italy, Slovenia, Croatia, Ukraine and Macedonia (table 1 and

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Table 1. Allelic profiles for European medicinal leeches.

sample	locality	morphospecies	HvA10		HvH07		Hm8		Hm10		Hm12		Hv351		HvT379		Hm1		Hm2	
			allele2	allele1	allele2	allele1	allele2	allele1	allele2	allele1	allele2	allele1	allele2	allele1	allele2	allele1	allele2	allele1	allele2	allele1
HM04	Paimpont, France	<i>H. medicinalis</i>	—	—	—	—	234	—	329	—	209	—	148	—	133	—	104	—	158	—
HR20	Hrast, SE Slovenia	<i>H. medicinalis</i>	—	—	—	—	231	—	329	—	209	—	150	—	133	—	104	—	158	—
HR21	Hrast, SE Slovenia	<i>H. medicinalis</i>	—	—	—	—	231	—	329	205	209	—	150	—	133	—	104	—	158	—
HR22	Hrast, SE Slovenia	<i>H. medicinalis</i>	—	—	—	—	231	—	329	209	—	150	—	133	104	107	—	—	—	—
X2	Kharkiv, E Ukraine	<i>H. medicinalis</i>	—	—	—	—	231	—	329	209	—	148	150	—	133	—	104	—	158	—
KO66	Kocevje, S Slovenia	<i>H. medicinalis</i>	—	—	—	—	231	—	329	209	—	150	168	—	133	—	104	—	158	—
KO67	Kocevje, S Slovenia	<i>H. medicinalis</i>	—	—	—	—	231	—	329	209	—	150	168	—	133	—	104	—	158	—
X3	Leipzig, E Germany	<i>H. medicinalis</i>	—	—	—	—	237	—	329	209	197	136	148	—	133	—	104	—	158	—
PO56	Podvinci, NE Slovenia	<i>H. medicinalis</i>	—	—	—	—	231	—	329	205	217	146	148	—	133	—	104	—	155	—
PO58	Podvinci, NE Slovenia	<i>H. medicinalis</i>	—	—	—	—	231	—	329	205	205	148	150	—	133	—	104	—	158	—
PO59	Podvinci, NE Slovenia	<i>H. medicinalis</i>	—	—	—	—	231	234	329	205	209	146	168	—	133	—	104	—	158	—
PO60	Podvinci, NE Slovenia	<i>H. medicinalis</i>	—	—	—	—	231	—	329	209	148	150	—	133	—	104	—	158	—	—
PO61	Podvinci, NE Slovenia	<i>H. medicinalis</i>	—	—	—	—	231	—	329	209	150	168	—	133	—	104	—	158	—	—
HM 97	Paimpont, France	<i>H. medicinalis</i>	—	—	—	—	234	—	329	209	146	168	—	133	—	110	—	158	—	—
C1	S Italy	<i>H. verbana</i>	321	348	174	177	—	316	319	—	—	140	136	138	92	95	—	158	—	—
KR40	Gabrovica, SW Slovenia	<i>H. verbana</i>	—	309	—	171	—	316	319	—	—	153	—	136	92	—	155	—	—	—
KR41	Gabrovica, SW Slovenia	<i>H. verbana</i>	—	309	—	171	—	316	319	—	—	188	—	136	92	—	155	—	—	—
PA54	Island Pag, Croatia	<i>H. verbana</i>	306	348	174	177	—	—	323	—	—	162	172	—	136	95	—	158	—	—
PA55	Island Pag, Croatia	<i>H. verbana</i>	—	306	—	174	—	—	323	—	—	162	—	136	95	—	155	—	—	—
OH31	Lake Ohrid, Macedonia	<i>H. verbana</i>	—	309	—	174	—	—	319	—	—	166	—	136	92	101	—	155	—	—
OH32	Lake Ohrid, Macedonia	<i>H. verbana</i>	—	306	—	174	—	—	319	—	—	155	190	133	136	92	—	155	—	—
OH33	Lake Ohrid, Macedonia	<i>H. verbana</i>	—	309	174	177	—	—	313	—	—	166	133	136	92	101	—	161	—	—
OH34	Lake Ohrid, Macedonia	<i>H. verbana</i>	—	309	174	177	—	—	316	—	—	142	166	133	136	92	—	155	—	—
X1	Lecce, S Italy	<i>H. verbana</i>	—	306	—	174	—	—	313	—	—	140	166	—	136	92	95	—	158	—
KA46	Povir, SW Slovenia	<i>H. verbana</i>	—	309	171	180	—	—	319	—	—	180	—	136	92	—	158	—	—	—
KA47	Povir, SW Slovenia	<i>H. verbana</i>	—	306	—	180	—	—	319	—	—	146	—	136	92	—	158	—	—	—
KA49	Povir, SW Slovenia	<i>H. verbana</i>	—	309	171	180	—	316	319	—	—	180	—	136	92	98	—	155	—	—
Graf	Commercial Graf Lab		—	306	—	177	—	316	329	—	—	170	172	—	136	92	—	161	—	—
Carolina	Commercial C. Biological		—	306	—	183	—	—	316	—	—	170	—	138	92	—	161	—	—	—
HM LM 2	Commercial Mehaffey Lab		—	306	171	177	—	—	316	—	—	172	176	136	92	98	—	164	—	—
HM UK	Commercial Biopharm UK		—	306	—	183	—	—	313	—	—	170	—	138	92	—	164	—	—	—
HM LM 1	Commercial Mehaffey Lab		—	306	171	177	—	—	313	—	—	170	180	136	92	—	155	—	—	—
Leeches USA	Commercial		—	309	—	177	—	265	316	252	283	180	194	136	92	—	—	—	—	—
Leeches USA	Commercial		—	306	—	183	—	274	316	223	248	172	184	139	92	—	161	—	—	—
Leeches USA	Commercial		—	306	—	183	274	282	316	248	252	172	136	139	92	95	—	—	—	—
Leeches USA	Commercial		—	306	177	183	262	271	316	248	252	186	192	139	95	—	161	—	—	—

see Trontelj *et al.* 2004). These included 13 wild *H. medicinalis* and 13 wild *Hirudo verbana* distinguished on the basis of colour patterns (figure 1). Commercially available leeches were obtained from four suppliers (Leeches USA Ltd, Westbury, NY, USA; Biopharm Leeches supplied through Carolina Biological Supply Company, Burlington, NC, USA; Ward's Natural Science, Rochester, NY, USA; and Biopharm Leeches (UK), Hendy, Carmarthenshire, UK) as well as from the laboratories of Joerg Graf (University of Connecticut, Storrs, CT, USA) and Leathem Mehaffey (Vassar College, Poughkeepsie, NY, USA) wherein leeches are used as model organisms. Microsatellite libraries were enriched and screened using a protocol (Budinoff *et al.* 2004) modified from Dr Travis Glenn's group (Savannah River Ecology Laboratory, University of Georgia, Aiken, SC, USA). Twenty-nine clones contained microsatellite regions with flanking sequences appropriate for primer design. Primers were designed using MACVECTOR v. 7.0 (Accelrys, San Diego, CA, USA). An M13 forward primer sequence was added to the 5' end of each forward primer, following the protocol of Boutin-Ganache *et al.* (2001). Amplification reactions for microsatellite analyses were performed with genomic DNA from 13 specimens of *H. medicinalis* and 13 specimens of wild *H. verbana* using equal quantities of each microsatellite primer pair and a 6-FAM fluorescently labelled M13 forward primer. Reactions consisted of 1 µl genomic DNA, 0.5 µl of each 10 µM primer, 2.5 µl 10× PCR Buffer II (Amersham Pharmacia Biotech, Piscataway, NJ, USA), 2 µl of 10 µM dNTP mixture, 0.13 µl AmpliTaq Gold (Amersham Biosciences) and 16.5 µl H₂O. Reactions were run at 94°C for 10 min, followed by 40 cycles of 94°C for 30 s, primer-specific annealing temperature (table 2) for 30 s and 72°C for 45 s. Reactions ended with a 72°C extension for 7 min.

Twenty primer sets were eliminated from consideration in light of mismatches between observed and expected amplification product sizes, inconsistent or weak amplification, hypervariability and lack of variability. Nine primers sets (table 2) were employed for this study for which all 26 wild leeches and 10 commercially obtained leeches were scored with GENEMAPPER (Chatterji & Pachter 2006). Microsatellite profiles were subjected to analyses of population structure with STRUCTURE (Pritchard *et al.* 2000) and analysis of molecular variance (AMOVA) with ARLEQUIN (Schneider *et al.* 1996).

Each leech included in the microsatellite analyses was also subjected to DNA Barcoding protocols. The universal primers (Folmer *et al.* 1994), LCO1490, 5' GGTCACA-CAAATCATAAAGATATTGG 3' and HCO2198, 5' TAAACTTCAGGGTGACCAAAAATCA 3', were used to amplify *cox1*. All amplifications used Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ), 0.5 µl of each 10 µM primer, 1 µl DNA template and 23 µl RNase-free H₂O run for 35 cycles of 94°C (45 s), 46°C (30 s) and 68°C (45 s). Amplification products were purified with the AMPure system PCR Cleaning protocol (Agencourt, Beverly, MA, USA) and were sequenced in both directions with the same primers. Each sequencing reaction, including 1 µl BigDye (Applied Biosystems, Perkin-Elmer Corporation), 1 µl of 1 µM primer (single primer for each direction) and 3 µl of DNA template, ran for 35 cycles of 96°C (30 s), 50°C (30 s) and 60°C (4 min). Sequences were purified by following the CleanSEQ protocol (Agencourt, Beverly, MA, USA). Products were re-suspended in 6 µl of formamide and electrophoresed in an ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA). Sequences of complimentary

strands were edited and reconciled using CodonCode Aligner (CodonCode, Dedham, MA, USA). Aligned *cox1* sequences (GenBank Accession Members—AF116029, AY425449–52, AY763148–55, AY786458, EF446680–EF446713) were subjected to parsimony analysis, 100 bootstrapping replicates as well as Kishino-Hasegawa (KH) and Templeton tests in PAUP* (Swofford 2003). Branch lengths were calculated using a general time-reversible model and gamma-distributed rate parameter as specified by MODELTEST (Posada & Crandall 1998).

3. RESULTS

Microsatellite profiles (table 1) clearly distinguished between wild *H. medicinalis* and *H. verbana*. Four microsatellite loci showed clear evidence of species-level segregation: two loci, Hm8 and Hm12, would not amplify from wild *H. verbana*. Two other loci, HvA10 and HvH07, would not amplify from wild *H. medicinalis*. Three of the five loci that amplified from all wild European medicinal leeches, Hm2, Hm10, and HvT397, showed no allelic variation in wild *H. medicinalis*, yet were variable for wild *H. verbana*. The remaining two amplifiable loci with variability for all wild leech isolates, Hm1 and Hv351, nonetheless had no alleles in common between the two species. In light of these clear differences, it is not surprising that analyses of population structure assigned wild-caught individuals to either *H. medicinalis* or *H. verbana* with more than 98.5% confidence in a manner precisely concordant with dorsal colour patterns evident in figure 1. AMOVA demonstrated that differences between these two species accounted for nearly half of all of the genetic variability evidenced in the microsatellite loci ($F_{CT}=0.481$; $p<0.004$). Inverted nesting of the AMOVA (i.e., presuming phenotypic plasticity in two colour variants of but a single widespread species) implied that more than 90% of allelic variation was accounted for by this obvious morphological difference and with no remaining variation explainable by geography ($F_{CT}=-0.37$; $p<0.97$).

In addition to definitively distinguishing the two wild species of *Hirudo*, our microsatellite loci also inferred, for all commercially obtained leeches, an unambiguous ancestry ($p<0.006$) with *H. verbana* notwithstanding their having been marketed as *H. medicinalis*. Though the commercially available leeches, unlike wild *H. verbana*, amplified for loci Hm8 and Hm12, none of the alleles found were shared with wild *H. medicinalis*. Lest this slightly different microsatellite profile be perceived as ambiguous, forcing an analysis of population structure to consider the possibility of three distinct groups split the wild *H. verbana* roughly into northwestern and southeastern groups and confidently ($p<0.01$) placed the commercially marketed leeches among these two. DNA barcoding was consistent with all of the foregoing in depicting distinct monophyletic groups for *H. medicinalis* and *H. verbana* each with independent histories and averaging 8.5% sequence divergence between the two species relative to less than 2% sequence variation within either species (figure 2). Commercially labelled *H. medicinalis* leeches were monophyletic (non-parametric bootstrap=100) with wild *H. verbana* (uncorrected p distance averaged 1.81%) and distinct from wild *H. medicinalis* (uncorrected p distance averaged 9.03%).

Table 2. Microsatellite loci, primers and annealing temperatures.

locus	forward primer ^a	reverse primer	annealing T (°C)
HvA10	CACGACGTTGTAAAACGACGAAAAGGT- CAAAGGTCACAACGAC	TTGCCCTGGATGCCATACAG	50
HvH07	CACGACGTTGTAAAACGACCTCGTAAGAAATG- GAAACTG	GATGTTGAAACCCGAAGC	55
Hm8	CACGACGTTGTAAAACGACTGGATGA- TAATGCGTCGTCAGTC	TCTTTGGTTCACAGGGCTTAGTTG	55
Hm10	CACGACGTTGTAAAACGACCTCTCCACAGCCT- TATGGTGTG	AGGATGAGGTGAAGCGGGGATG	55
Hm12	CACGACGTTGTAAAACGACGCACCAAGCCA- GAATAAGGTTAG	TCTCCACTTTCCTCCCGCTC	50
Hv351	CACGACGTTGTAAAACGACCGCAATCTGCCT- GAAAAAGTAAAC	CGAATGTGTTGGATGCCACTAAC	50
HvT379	CACGACGTTGTAAAACGACATTGGGAACAA- GAACAAGCC	CAGAAGGTGGAGAAGTGGTTG	50
Hm1	CACGACGTTGTAAAACGACTCAGGCGA- CATCCTCTTCATCG	ATGGCTACCACTGCGTTGTTG	50
Hm2	CACGACGTTGTAAAACGACAGGAACTCAT- CATTTATCTCCAGCC	ACGGAAACCAGGTCTCCAAGAG	50
HvD7	CACGACGTTGTAAAACGACTCTACTTCCA- GAACTCGTG	GCAGCCTTTACGGAGCGG	50

^a The first 19 nt of each forward primer corresponds to a fluorescently labelled M13 primer used in the reaction.



Figure 1. Distinctive colour patterns for two species of European medicinal leech. (a) *Hirudo medicinalis* Linnaeus 1758. (b) *Hirudo verbana* Carena 1820.

Trees constraining commercially available leeches to be monophyletic with wild *H. medicinalis* were significantly suboptimal in KH and Templeton tests ($p=0.0019$ and 0.0020 , respectively).

4. DISCUSSION

The foregoing confirms recent work pointing to more than one distinct species of European medicinal leech (Nesemann & Neubert 1999; Trontelj *et al.* 2004; Trontelj & Utevsy 2005) and a suspicion that widely used laboratory model organisms are incorrectly identified (Kutschera 2006). Whether the erroneous marketing of *H. verbana* as *H. medicinalis* might be relevant to efficacy is not thoroughly determined but seems doubtful. Nevertheless, US FDA regulations currently do not expressly permit the use of *H. verbana*, and specifically proscribe the mislabelling of a device. The implications of our findings are more far reaching. Already invertebrate neurobiological and developmental biology communities are grappling with the recognition that apparent phenotypic plasticity in some leeches actually belies species heterogeneity (Bely & Weisblat 2006). A detailed EST project, supposedly for *H. medicinalis*, is well underway at the French National Sequencing Centre, Genoscope.

There are more than 300 entries in public genome databases and nearly 700 entries in PubMed with *H. medicinalis* as the primary taxonomic key for work in neurobiology, developmental genetics and even enteric symbiosis models (Graf *et al.* 2006). Nearly all of these concern research using commercially available leeches as model organisms. Indeed, more than 300 scholarly articles concerning leeches indicate just such a commercial source. Given our results, molecular characterization of laboratory strains of apparent *H. medicinalis* is now warranted. To our knowledge, there are no isogenic strains of *H. medicinalis*, though many undoubtedly exist for *H. verbana* which is undoubtedly the object of genomic and peptidome studies (Baskova *et al.* 2004; Wang *et al.* 2005; Yanes *et al.* 2005).

At least 115 bioactive compounds have been isolated from medicinal leeches mostly being putative anticoagulants, antistasis and other protease inhibitors (Sollner *et al.* 1994; Baskova & Zavalova 2001; Salzet 2002). Antistasis, like other coagulation factor Xa inhibitors, have antimetastatic properties (Tuszinsky *et al.* 1987). Amino acid sequences from these short peptides are substantially different between closely related species (Kim & Kang 1998) and even among paralogues of this serine protease family within a species (Moser *et al.* 1998). Hirudin and calin, heretofore presumed to be from *H. medicinalis*, inhibit coagulation by interfering with thrombin or platelet aggregation, respectively (Markwardt 1955; Deckmyn *et al.* 1995). A thorough investigation of the various species of European medicinal leech should reveal yet more of these powerful peptides and could further enrich the suite of available therapeutic agents and biomedical research tools.

Medicinal leeches, once abundant across Europe, suffered a precipitous decline in the nineteenth century. A century of harvesting tens of millions of leeches each year, the advent of intensive farming and widespread

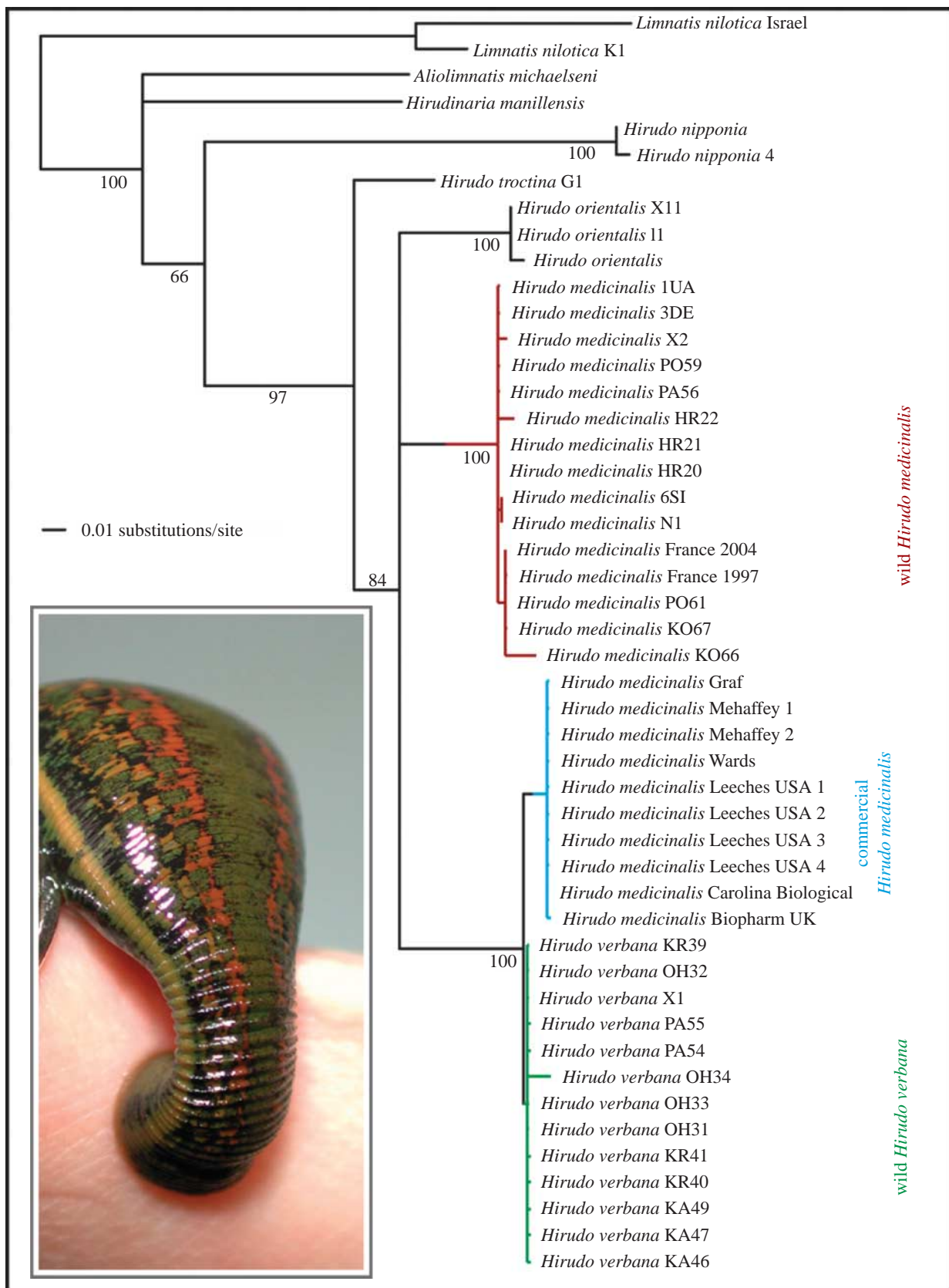


Figure 2. DNA barcoding distinguishes European medicinal leech species. Multiple equally parsimonious trees varied on resolution within each of the wild or commercial medicinal leech clades, and on whether *Hirudo orientalis* is sister to *H. medicinalis* or to the other European medicinal leeches. Clade support values are non-parametric bootstrap proportions. Mean genetic distances: 8.55% between wild *H. medicinalis* and *H. verbana*; 1.81% between leeches commercially marketed *Hirudo medicinalis* and wild *H. verbana*; 9.03% between leeches commercially marketed *Hirudo medicinalis* and wild *H. medicinalis*; 8.60% between *H. orientalis* and the other wild *Hirudo* species. Branch lengths are based on a GTR + G model of nucleotide substitution. A commercially obtained *Hirudo medicinalis* (i.e. *H. verbana*) is shown feeding (inset).

draining of European wetlands together led to dramatic shortages, unregulated smuggling and some of the first European legislative efforts in biological conservation in the early 1900s (Harding & Moore 1927; Elliott & Tullett 1992). In UK, it is still an offence to injure, possess and sell medicinal leeches or damage their natural habitat (Elliott & Tullett 1992). *Hirudo medicinalis* is afforded threatened species status under IUCN, is regulated by CITES, the Berne Convention and the European Union Habitat Directive (Elliott & Tullett 1992; Trontelj & Utevsky 2005). Such regulations necessarily are species specific and *H. verbana* has no legal protection whatsoever. Pending a more detailed evaluation of the conservation status of these historically compelling and medically valuable invertebrate animals, we urge the extension of all proscriptions equally to *H. verbana* and to the newly discovered *H. orientalis* (Utevsky & Trontelj 2005).

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