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Which frog's legs do *froggies* eat? The use of DNA barcoding for identification of deep frozen frog legs (Dicroglossidae, Amphibia) commercialized in France

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Abstract. Several millions frogs captured in the wild in Indonesia are sold for food yearly in French supermarkets, as deep frozen frog legs. They are commercialized as *Rana macrodon*, but up to 15 look-alike species might also be concerned by this trade. From December 2012 to May 2013, we bought 209 specimens of deep frozen frog legs, and identified them through a barcoding approach based on the 16S gene. Our results show that 206 out of the 209 specimens belong to *Fejervarya cancrivora*, two to *Limnonectes macrodon* and one to *F. moodiei*. Thus only 0.96 % of the frogs were correctly identified. Unless misclassification was intentional, it seems that Indonesian frog leg exporters are not able to discriminate between the species. The quasi absence of *L. macrodon* in our samples might be an indication of its rarity, confirming that its natural populations are declining rapidly, in agreement with its "vulnerable" status according to the IUCN Red List. Our results show that the genetic and morphological diversity of the frogs in trade is much higher than the genetic and morphological diversity measured so far by scientific studies. These results underline the need for large scale studies to assess the status of wild populations.

Key words. *Fejervarya cancrivora*, *Limnonectes macrodon*, frog leg trade, barcoding, identification, conservation, Red List.

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Introduction

The international traffic of wild animals is considered an important threat to many animal species and is subject to international regulations. From 1998 to 2007 (10 years), 35 million animals and plants registered on the CITES lists have been exported from South-East Asian countries (Nijman 2010). For

the same period, between 180 million and 1 billion specimens of frogs were collected every year in the wild in Indonesia, and one eighth of these frogs were then exported to Europe (Kusrini 2005; Kusrini & Alfold 2006). For the ten-year period from 2000 to 2009, 46 400 tonnes of frog legs were imported into Europe, which corresponds to approximately 928 million to 2.3 billion frogs (Altherr *et al*. 2011).

The main European countries that import frog legs from Indonesia are France and Belgium (Kusrini & Alfold 2006; Altherr *et al*. 2011). The consumption of frogs is a tradition in French cuisine, causing the French to be called "froggies" by their neighbours, and in former days private people and restaurants would collect local frog specimens at certain periods of the year. However, in the 1970s deep freezing technology was developed, which allowed long term storage and large scale transport of frog legs, and large numbers of frogs were collected by commercial enterprises (Dubois 1983; Neveu 2004). This modified the conditions of traditional local sustainable collection and drove some local frog populations to extinction. A wildlife protection law was voted in 1979 to protect the French frog species (Le Serrec 1988). The market turned to tropical countries, mainly Bangladesh, India and Indonesia, but India and Bangladesh quickly stopped frog exports: the main species concerned (*Hoplobatrachus tigerinus* (Daudin, 1802)) was put on CITES lists and very protective laws for the local anuran species were enacted. Thus, in 1987 Indonesia became the main exporter of frogs and more than 80% of European frog leg imports now come from this country (Altherr *et al*. 2011).

From 1973 to 1987, 830 to 2659 tonnes of frog legs were imported to France from Indonesia (Le Serrec 1988). This import was 5600 tonnes in 1992 (Kusrini & Alford 2006), 4600 tonnes per year in 2000–2009 (Altherr *et al*. 2011), and 2906 to 3275 tonnes per year in 2009–2011 (French Customs 2012). Frog legs from Indonesia are sold in supermarkets, particularly in those specialised in deep frozen food, or in Asian food markets.

Kusirini & Alford (2006) showed that three species are predominantly collected in Indonesia for consumption: the giant Javan frog *Limnonectes macrodon* (Duméril & Bibron, 1841), the crab-eating frog *Fejervarya cancrivora* (Gravenhorst, 1829) and the common grass frog *Fejervarya limnocharis* (Gravenhorst, 1829), the latter probably representing a mixture of *F. limnocharis* and *F. iskandari* Veith, Kosuch, Ohler & Dubois, 2001 (Djong 2007). About fifteen look-alike species of large size might also be involved in collection for international trade (Altherr *et al*. 2011; Ohler unpubl. report), and they all belong to the genera *Fejervarya* Bolkay, 1915 and *Limnonectes* Fitzinger, 1843. Some of these species are common in the wild (e.g., *F. cancrivora*) while others are uncommon and listed as vulnerable in the IUCN Red List (e.g., *L. macrodon*, see Iskandar *et al.* 2004). Nonetheless, the European regulation no. 2065/2001 (Anonymous 2001) asks that the labels of fishery products show the common and scientific names on pre-packed, non-transformed fresh products in order to assure traceability of specimens. In France, deep frozen frog legs are usually sold in the supermarkets in plastic bags of 500 g or 1 kg and bear labels with a Latin species name and their country of origin.

Monitoring and management of harvested populations require accurate species identification; yet, high error rates are likely (Warkentin *et al*. 2009). Frog species can often be distinguished on various morphological characters such as body proportions, foot morphology and coloration pattern. Because exported frogs are skinned and the bodies cut off before packing, the characters allowing a morphological identification cannot be observed on deep frozen legs and thus identification is difficult (Warkentin *et al*. 2009). In this paper we explore the use of DNA barcoding as an identification tool to identify deep frozen frog legs commercialized in France. DNA barcoding is a technique that uses a short DNA sequence from a standard locus as a species identification tool (Hebert *et al*. 2003).

From December 2012 to May 2013, we bought 209 frogs in different supermarkets in France that were labeled as *Rana macrodon* (a variant of the valid name *Limnonectes macrodon*). These frogs were sold under three different brands but all come from the same importer. The samples were identified through a barcoding approach based on the 16S gene. The 16S gene was previously recognized as one of the

Table 1. Species identification recovered from a Blast in GenBank (16S gene) of the 209 specimens of frogs bought in French supermarkets from December 2012 to May 2013 and identified on the label as "*Rana macrodon* from Indonesia".

Month	*Fejervarya cancrivora*	*Fejervarya moodiei*	*Limnonectes macrodon*	Total
December 2012	22	–	–	22
January 2013	48	–	–	48
February 2013	26	1	–	27
March 2013	26	–	–	26
April 2013	46	–	1	47
May 2013	38	–	1	39
Total	206	1	2	209

most effective genes for molecular amphibian species identification (Vences *et al.* 2005; Grosjean *et al.* 2015). Moreover, 16S reference sequences were available in the GenBank database for the 15 species potentially collected in Indonesia for commercialization.

A diminution in size of adult frogs due to overharvesting has been suggested (Iskandar, cited in Anonymous 2007). As snout-vent length and tibia length are highly correlated in frogs (Emerson 1978; Vidal-García *et al.* 2014), we used tibia length to obtain an estimate for the body size of commercialized frogs.

Material and methods

Biological samples, DNA extractions, PCR conditions, DNA sequencing

In this study, a total of 209 biological samples of commercialized frogs were bought in different French supermarkets from December 2012 to May 2013 (Table 1, Appendix). All these samples were sold as frozen frog legs in bags of 500 or 1000 g. They came from Indonesia and were sold as *Rana macrodon*. All these samples are stored in the collections of the National Museum of Natural History, Paris, France (Appendix).

DNA extracts were prepared from muscle tissue using the NucleoSpin Tissue Core kit (Macherey Nagel). 16S amplification and sequencing were obtained using the primer pair 16SA-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16SB-H (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi *et al.* 1991). The thermal profile consisted of 38 cycles at 94°C for 45 s, 55°C for 45 s and 72°C for 1 min. The amplicons obtained were about 550 bp long. PCR products were purified and sequenced using Abi technology at the Genoscope (Evry, France). Sequences were checked by eye in CodonCode v. 5.1. The sequences obtained were deposited in the GenBank database under accession numbers KX055940−KX055957.

Molecular species identification of samples

To identify all our samples we first determined the number of haplotypes in our dataset using TCS (Clement *et al.* 2000). Each haplotype was then assigned to a species through a nucleotide BLAST approach conducted in GenBank (http://blast.ncbi.nlm.nih.gov) and through a phylogenetic method (Bayesian tree).

BLAST species identification was accomplished following three different criteria (Meier *et al.* 2006): Best Match (BM), Best Close Match (BCM) and All Species Barcode (ASB). The BM criterion assigns

identifications to the closest match regardless of genetic distance. The BCM criterion is similar to BM, but the query is identified by the closest match with a distance below a defined threshold. Finally, the ASB criterion is similar to the BCM by applying a threshold but it returns all the sequences within it. A query is identified when all the matching sequences below the threshold are conspecific. For BCM and ASB, a query may provide ambiguous results if sequence divergences of different species are below the threshold (ASB) or sequences from different species are the closest match below the threshold (BCM).

A tree-based approach of species delimitation was also used. The tree-based criterion of reciprocal monophyly was used to define species boundaries. In our analysis we included: 1) all the haplotypes recovered in our deep frozen frog legs, 2) all species of the genera *Fejervarya* and *Limnonectes* known from Indonesia (except *Limnonectes dammermani* (Mertens, 1929), *L. kenepaiensis* (Inger, 1966), *L. rhacodus* (Inger, Boedi & Taufik, 1996), *L. sinuatodorsalis* Matsui, 2015, *L. timorensis* (Smith, 1927) and *Fejervarya schlueteri* (Werner, 1893) for which no 16S gene sequences are currently available in GenBank), and 3) one sequence of *Hoplobatrachus rugulosus* (Wiegmann, 1834) and one sequence of *Occidozyga laevis* (Günther, 1858) included as outgroups. Depending on 16S gene sequence availability in GenBank, we included one to four individuals per species in our analysis. Sequences were aligned with ClustalW (Thompson *et al*. 1994). In order to minimize the number of missing data we kept 553 sites in our final analyses. Evolutionary relationships among sequences were estimated by conducting Bayesian Markov chain Monte Carlo phylogenetic analyses (MCMC) with MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001). MrModeltest v. 3.04 (Nylander 2004) was used to evaluate the fit of 24 nested models of nucleotide substitution to the data. According to the Akaike information criterion, MrModeltest recommended the GTR + I + G model. This model was used in the Bayesian analysis. Three heated chains and one single cold chain were employed, and runs were initiated with random trees. Two independent MCMC runs were conducted with five million generations per run; trees (and parameters) were sampled every 100 generations. Stationarity was assessed by examining the average standard deviation of split frequencies and the Potential Scale Reduction Factor (Ronquist *et al.* 2005). For each run, the first 25% of sampled trees were discarded as burn-in.

Geographical origin of commercialized frogs

For the species *Fejervarya cancrivora*, 16S sequences of specimens from different geographical regions are available in GenBank (Table 2). We conducted a phylogeographic analysis to see if there is any phylogeographic signal within this species and whether the geographical origin of the commercialized frogs could thus be determined based on 16S gene sequences. To this aim, 16S gene sequences of *F. cancrivora* for which the geographical origin was known were retrieved from GenBank. To minimize the number of missing data, 257 sequences of 494 bp were retained in final analyses (206 sequences of commercialized frogs + 51 specimens from GenBank). Relationships among haplotypes were inferred by constructing a network using the median-joining method available in Network v. 5.0.0.0 (Bandelt *et al.* 1999).

Morphometry

Snout vent length (SVL) and tibia length (TL) of *Fejervarya cancrivora* were obtained from Boulenger (1920) and from measurements of adult specimens from Borneo, Java and Sumatra stored at the Field Museum of Natural History, Chicago (FMNH 256671, 256688, 256690, 256692–97, 256709–14). Tibia length of frog legs was measured with a slide caliper (Appendix). The Pearson correlation coefficient showed a highly significant correlation between snout vent length and tibia length ($r = 0.973$; $p < 0.001$***). For all specimens measured we calculated the ratio $r = TL/SVL$. We used R = mean of all r to estimate body size depending on TL. We obtained the value $R = 0.48177$; $n = 28$; standard deviation = 0.197. Thus, we could use the following formula to calculate an estimate for snout vent length for the frog legs: $SVL = TL / 0.48177$.

Table 2. List of specimens of *Fejervarya cancrivora* used in our phylogeographic analysis.

GenBank no.	Source	Country	Island	Locality	No. of individuals
KX055940	this study	Indonesia	–	–	1
KX055941	this study	Indonesia	–	–	54
KX055942	this study	Indonesia	–	–	7
KX055943	this study	Indonesia	–	–	66
KX055944	this study	Indonesia	–	–	1
KX055945	this study	Indonesia	–	–	6
KX055946	this study	Indonesia	–	–	1
KX055947	this study	Indonesia	–	–	5
KX055948	this study	Indonesia	–	–	1
KX055949	this study	Indonesia	–	–	8
KX055950	this study	Indonesia	–	–	49
KX055951	this study	Indonesia	–	–	1
KX055952	this study	Indonesia	–	–	1
KX055953	this study	Indonesia	–	–	1
KX055954	this study	Indonesia	–	–	1
KX055955	this study	Indonesia	–	–	3
AB570273	Kurniawan *et al.* 2014	Indonesia	Bali	Denpasar	6
AB570274	Kurniawan *et al.* 2014	Indonesia	Bali	Denpasar	1
AB570275	Kurniawan *et al.* 2014	Indonesia	Bali	Denpasar	1
AB570276	Kurniawan *et al.* 2014	Indonesia	Bali	Denpasar	1
AB570277	Kurniawan *et al.* 2014	Indonesia	Bali	Denpasar	1
AB444684	Kurniawan *et al.* 2010	Indonesia	Bangka	Tempilang	1
AB570273	Kurniawan *et al.* 2014	Indonesia	Java	Banyumas	1
AB570273	Kurniawan *et al.* 2014	Indonesia	Java	Banyuwangi	1
AB444684	Kurniawan *et al.* 2010	Indonesia	Java	Bogor	1
AB570273	Kurniawan *et al.* 2014	Indonesia	Java	Cilacap	9
AB570273	Kurniawan *et al.* 2014	Indonesia	Java	Kediri	2
AB570273	Kurniawan *et al.* 2014	Indonesia	Java	Malang	2
AB444684	Kurniawan *et al.* 2010	Indonesia	Sumatra	Padang	1
AB570273	Kurniawan *et al.* 2010	Indonesia	Java	Pelabuhan ratu	1
AB570273	Kurniawan *et al.* 2014	Indonesia	Kalimantan	Pontianak	6
AB570273	Kurniawan *et al.* 2014	Indonesia	Sumatra	Jamb	1
AB444684	Kurniawan *et al.* 2014	Indonesia	Sumatra	Jamb	1
AB570273	Kurniawan *et al.* 2014	Indonesia	Sumatra	Lampung	1
AB444684	Kurniawan *et al.* 2010	Indonesia	Sumatra	Langkat	3
AB444685	Kurniawan *et al.* 2010	Indonesia	Sumatra	Padang	1
AB570273	Kurniawan *et al.* 2014	Indonesia	Sumatra	Palembang	1
AB444685	Kurniawan *et al.* 2010	Indonesia	Sumatra	Panti	1
AB444685	Kurniawan *et al.* 2010	Indonesia	Sumatra	Payakumbuh	1
AB444684	Kurniawan *et al.* 2010	Malaysia	Selangor	Selangor	1
EU435279	Kung *et al.* unpubl.	Taiwan	Donggang	Donggang	1
EU435280	Kung *et al.* unpubl.	Taiwan	Fangliao	Fangliao	1
EU365387	Hsu *et al.* unpubl.	Taiwan	Jiadong	Jiadong	3
EU365389	Hsu *et al.* unpubl.	Taiwan	Sinpi	Sinpi	1

Results

Species identification

After alignment, our dataset contained 585 sites. Eighteen haplotypes were recovered (Appendix), and they clustered in three groups: (1) haplotypes h01 to h16 (206 individuals) which differ from one another by less than 6 mutations (i.e., 1% sequence divergence), (2) haplotype h17 (one individual) and (3) haplotype h18 (2 individuals). Haplotype h17 differs from haplotypes h01 to h16 by 50 to 56 mutations (8.5 to 9.5% of absolute sequence divergence) and from haplotype h18 by 104 mutations (17.8% of absolute sequence divergence). Haplotype h18 differs from haplotypes h01 to h16 by 108 to 111 mutations (18.7 to 19.0% of absolute sequence divergence).

Our nucleotide BLAST analysis showed that haplotypes h01 to h16 correspond to the species *Fejervarya cancrivora* (best close match: 99 to 100% of sequence identity; Table 1). Three distinct species have been recognized in "*Fejervarya cancrivora*" (designated as large, mangrove and Sulawesi types; Hasan *et al.* 2012). The large type of *F. cancrivora* was designated as the nominal *F. cancrivora* (Kotaki *et al*. 2010), while the mangrove and Sulawesi types were designated as *F. moodiei* (Taylor, 1920) and an undescribed species, respectively (Kurniawan *et al*. 2011). Our haplotypes h01 to h16 cluster with the large *F. cancrivora* (99% sequence identity). The percentage of sequence identity with the mangrove type (represented in GenBank by 10 sequences: EU652694, DQ458252, AF206473, AB543602, AB070738, AB530508, AB372018, AY841754, AB444692, AB444691) is 91%, and it is 93% with the Sulawesi type (represented in GenBank by 13 sequences: EU979849, AB444693, AB570278, AB570283, AB570287, AB570286, AB570290, AB570288, AB570289, AB570284, AB570282, AB570280, AB570281). Based on the percentage of sequence identity, the other closest species are several species of *Fejervarya* (88–89% of identity with *F. vittigera* (Wiegmann, 1834), *F. triora* Stuart, Chuaynker, Chan-ard & Inger, 2006, *F. iskandari*, *F. multistriata* (Hallowell, 1861), *F. limnocharis*, *F. sakishimensis* Matsui, Toda & Ota, 2008) and *Hoplobatachus rugulosus*. A threshold of 5% is often considered to correspond to distinct species of *Fejervarya* (Kurabayashi *et al*. 2005). Based on this threshold, the results of the BM, BCM and ASB methods are congruent and show that haplotypes h01 to h16 can be attributed to the species *F. cancrivora*.

Haplotype h17 is highly similar (99–100% identity) to the mangrove type (*F. moodiei*). This haplotype is also close (90–92%) to the large type and the Sulawesi type of *F. cancrivora*. Based on the percentage of sequence identity, the other closest species are several species of *Fejervarya* (88–90% of identity with *F. vittigera*, *F. triora*, *F. iskandari, F. multistriata*, *F. limnocharis* and *F. sakishimensis*) and *Hoplobatachus rugulosus* (88–89%).

Haplotype h18 corresponds to the species *Limnonectes macrodon* (99% of sequence identity). Based on the percentage of sequence identity, the other closest species are *Limnonectes shompenorum* Das, 1996 (94%), *L. leporinus* Anderson, 1923 (91%), *L. leytensis* (Boettger, 1893) (91%) and *L magnus* (Stejneger, 1910) (90%).

In our phylogenetic tree (Fig. 1), all Indonesian species of the genera *Fejervarya* and *Limnonectes* are reciprocally monophyletic and Bayesian posterior probabilities are high ($pp > 0.98$) for all species. This tree clearly shows that haplotypes h01 to h16 correspond to the species *Fejervarya cancrivora*, haplotype 17 corresponds to the species *F. moodiei* and haplotype h18 corresponds to the species *Limnonectes macrodon.*

Phylogeography of *Fejervarya cancrivora*

The results of our network analysis show that there is some genetic variability within *F. cancrivora* (Fig. 2): 21 haplotypes differing by 1 to 5 mutations were found. Thirteen out of the 16 haplotypes

identified in the commercialized frogs (representing 95 individuals) have not been recorded in previous analyses. Haplotype h02, found in 54 commercialized frogs, was recovered in Taiwan and several Indonesian islands (Kalimantan, Sumatra, Bali and Java). Haplotype h11 (49 commercialized frogs) was recovered in Malaysia and several Indonesian islands (Bangka, Sumatra, Java). Haplotype h10

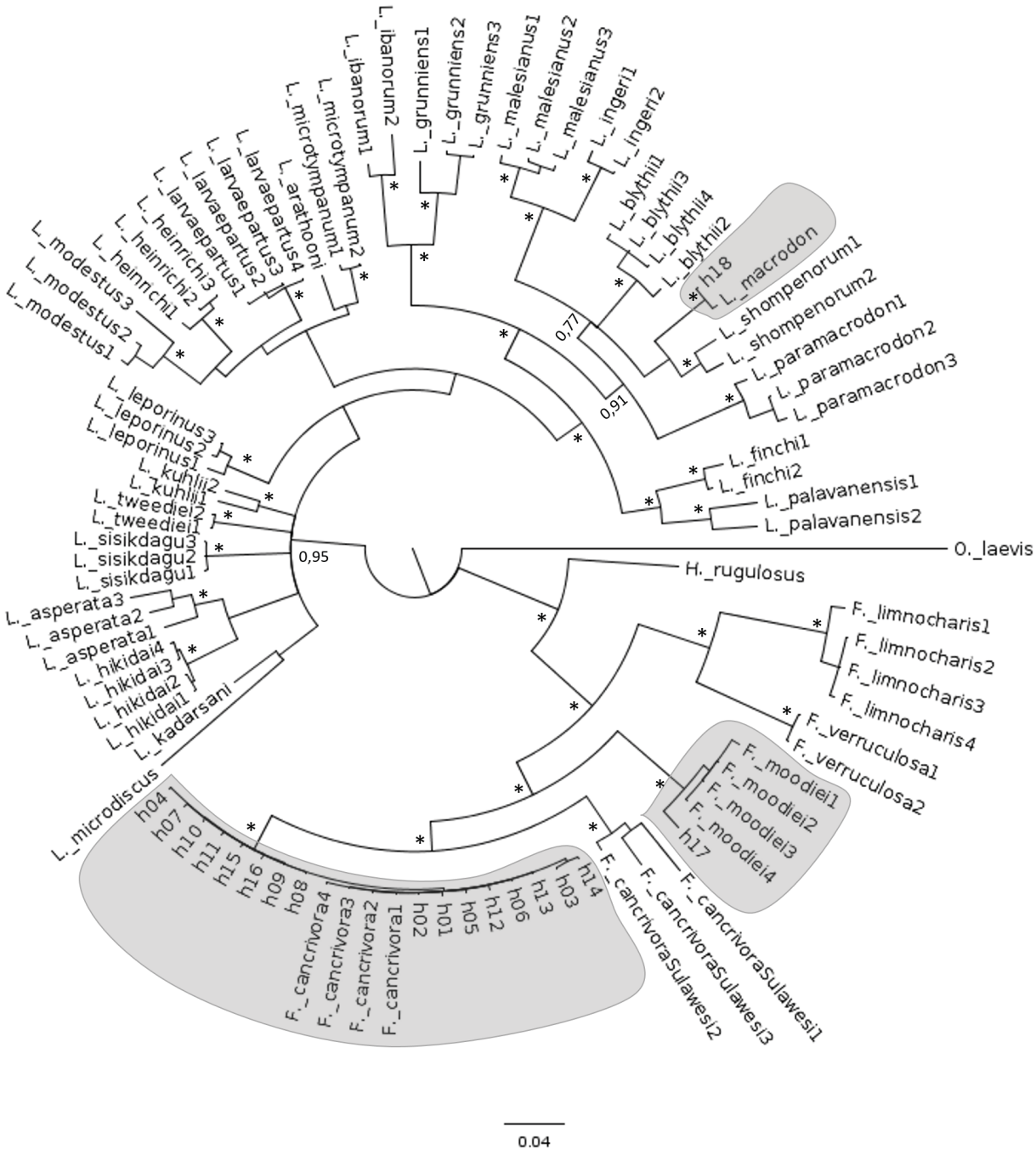

Fig. 1. Phylogeny of Indonesian species of *Fejervarya* and *Limnonectes* recovered by the Bayesian analysis (GTR + I + G model). *Hoplobatrachus rugulosus* (Wiegmann, 1834) and *Occidozyga laevis* (Günther, 1858) were used as outgroups. Numbers on nodes represent Bayesian posterior probabilities, * indicates a value higher than 0.98. Only values higher than 0.75 are represented. h01 to h18 indicate the 18 haplotypes from frozen frog legs recovered in this study.

(8 commercialized frogs) is present in Taiwan. Due to the lack of phylogeographic structure within previously sequenced *F. cancrivora* specimens and to the high number of new haplotypes detected in commercialized frogs, it is not possible to infer the geographical origin of the frogs sold in France.

Estimation of snout-vent length of frog legs in trade in France

For 192 frog legs, the estimated snout-vent length ranged from 59.4 to 111.7 mm (mean value: 79.0; standard deviation: 11.37). The histogram (Fig. 3, Appendix) of these measurements shows a bimodal distribution which does not correspond to the size distribution of *Fejervarya cancrivora* specimens collected in natural populations without sampling bias. This is also reflected by the relatively low standard deviation. Measurements of specimens from Borneo, Java and Sumatra published in Boulenger (1920) vary from 54 to 88 mm (mean value: 72.8; standard deviation: 13.34), whereas specimens collected in the early 1990s range from 49.7 to 101.6 mm (mean value: 78.9; standard deviation: 14.98).

Discussion

Misidentification of frogs commercialized in France

Results based on the phylogentic tree and the BLAST approach (BM, BCM and ASB) are congruent and show (threshold of 5%; Kurabayashi *et al*. 2005) that 206 out of the 209 analyzed specimens belong

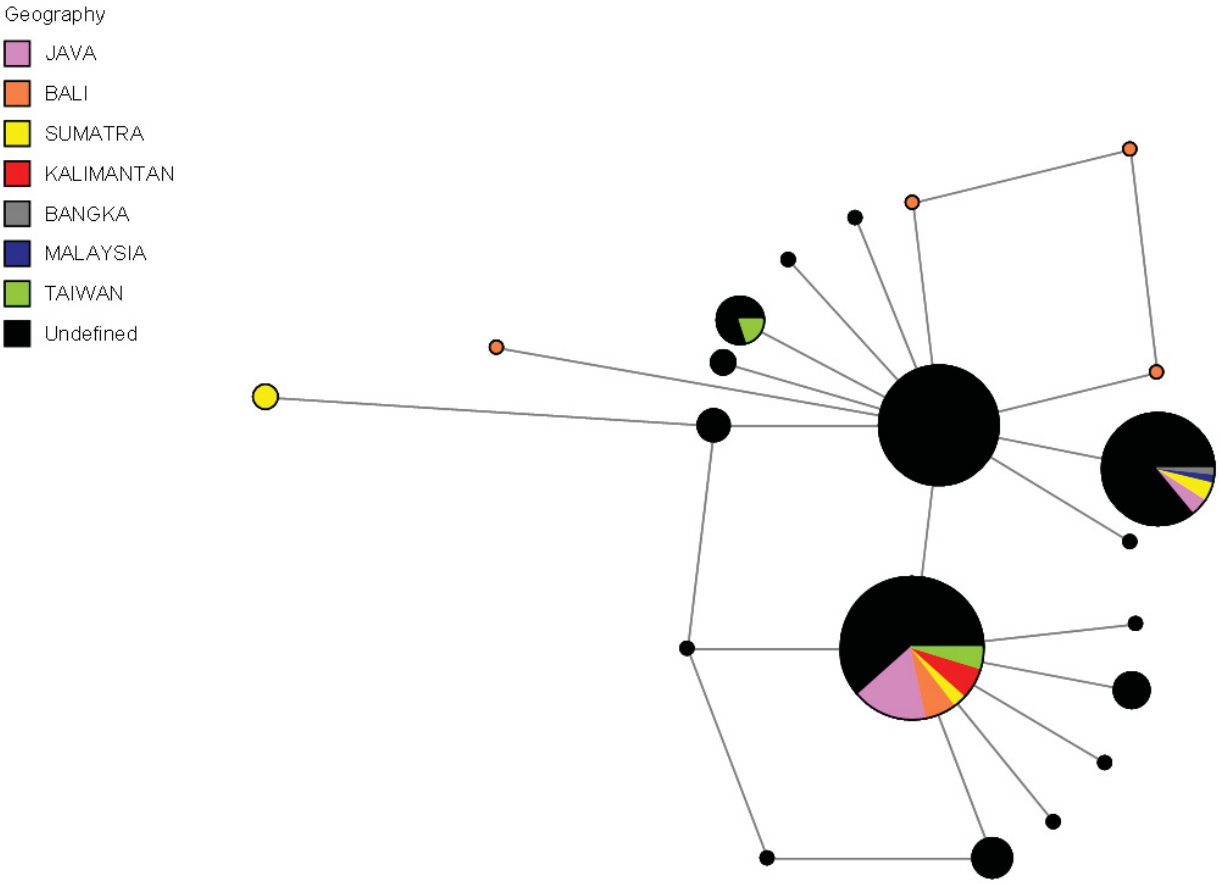

Fig. 2. Minimum spanning network depicting relationships among 16S haplotypes of *Fejervarya cancrivora* (Gravenhorst, 1829). The size of each circle is proportional to the haplotype frequency and the lengths of the connecting lines are proportional to the number of mutations. Colors refer to distinct regions (Indonesia: Java, Sumatra, Bali, Kalimantan, Bangka; Malaysia; Taiwan) and commercialized frogs of unknown origin are in black.

to the species *Fejervarya cancrivora*, two to the species *Limnonectes macrodon* and one to the species *F. moodiei*. Thus only 0.96% of the frogs were correctly identified.

In a previous study on frog legs imported to Belgium as *F. cancrivora*, *L. macrodon*, *L. limnocharis* and *Rana catesbeiana* Shaw, 1802, all samples proved to be *Fejervarya cancrivora*. In this case, 34.5% of the identifications were correct (Veith *et al*. 2000). In that study 36.8% of the frog legs were sold as *Limnonectes macrodon*. Although all our samples were labelled as *Rana macrodon*, only 2 frog legs (0.96%) could be identified as this species. As proposed by several authors (Kusrini 2005; Kusrini & Alford 2006; Veith *et al.* 2000), it seems likely that misclassification is not intentional but due to the fact that frog leg exporters are not able to discriminate between the species in trade. Managers of export companies stipulate that they should be supplied only with frog legs of *L. macrodon* (Kusrini & Alford 2006). On the other hand, in the local markets *L. macrodon* and *F. cancrivora* are correctly identified and sold at different prices, because the meat of *L. macrodon* is considered of better taste (Kusrini & Alford 2006; A. Ohler, pers. obs.).

Sustainability and conservation

The species concerned by international trade are of similar, large size but show differences in breeding biology and habitat, although relevant data are scarce. *Fejervarya cancrivora* inhabits marshes and paddy fields, not avoiding habitat modified by man and thus a large area of potential habitat is available (Inger 1966; Iskandar 1998; Yuan *et al*. 2004). Virtually nothing is known of the habitat and ecology of *F. moodiei* (Ohler 2004). *Fejervarya cancrivora* lays a relatively large number (up to more than 2500) of small-sized eggs in successive clutches (Inger 1956; Alcala 1962), which develop in lotic habitats. *Limnonectes macrodon* is present in riparian secondary forests (Inger 1966). It can be observed in clearings and secondary growth, and it is very rare in primary forests. A single clutch of about 1000 eggs is laid in side pools of rivers (Iskandar 1998). Overharvesting should thus have a higher impact on *L. macrodon,* as breeding capacity is smaller in *L. macrodon* than in *F. cancrivora* and riverside habitats are scarcer than ponds and paddy fields. The conservation status of *F. cancrivora* was evaluated as "*Least Concern*" "in view of its wide distribution, tolerance of a broad range of habitats, presumed large

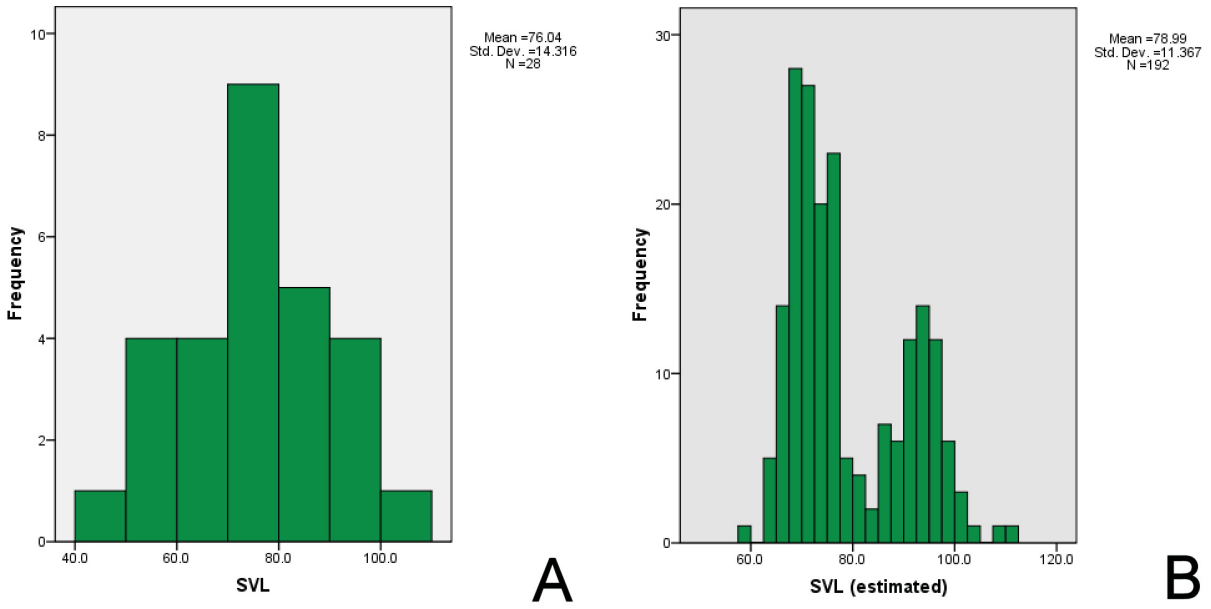

Fig. 3. Histograms. **A**. Snout vent length (in mm) in adult *Fejervarya cancrivora* (Gravenhorst, 1829) from samples collected for scientific purposes (Boulenger 1920) and collection specimens as mentioned in Material and methods. **B**. Snout vent length estimated from tibia length of genetically identified frog legs from French supermarkets (specimen list, see Appendix).

population, and because it is unlikely to be declining to qualify for listing in a more threatened category" (Yuan *et al*. 2004). *Fejervarya moodiei* is listed as "*Data deficient* in view of continuing doubts as to its extent of occurrence, status and ecological requirements" (Ohler 2004). *Limnonectes macrodon* is listed as "*Vulnerable* because it depends on streams in lowland forest, and so its Area of Occupancy is probably less than 2000 km^2, its distribution is severely fragmented, and there is continuing decline in the extent and quality of its habitat, the number of locations, and the number of mature individuals" (Iskandar *et al*. 2004). These evaluations are not based on population studies that might measure the impact of the heavy harvesting ongoing for decades. However, the quasi absence of *L. macrodon* in our samples might be an indication of its rarity in the field and the fact that its natural populations are declining rapidly.

In the 1990s, researchers already considered that large-sized frogs had completely disappeared in many parts of Java and Sumatra (Manthey & Grossmann 1997; Inger & Stuebing 1997). In 2006, 18.8% of the frogs captured by harvesters in West Java and East Java belonged to the species *L. macrodon* (Kuzrini & Alford 2006). In 2012–2013, only 0.96% of the samples from trade sold in France belonged to this species.

A diminution in size of adult frogs due to overharvesting has been suggested (Iskandar, cited in Anonymous 2007). The estimated size of the frogs from deep frozen specimens of *F. cancrivora* is not smaller than specimens collected 20 or a hundred years ago. The frogs measured in Boulenger (1920) are slightly smaller in size than the frogs collected in the early 1990s and in 2012–2013. However, the body size of the frogs in trade is clearly biased. After collection, frogs are separated into size categories and only large specimens are chosen for export (Kusrini & Alford 2006). In samples studied by Church (1960) and Kusrini & Alford (2006) that had been collected for trade, very large-sized frogs were present (maximum size of 132 mm for 1325 frogs, and of 162 mm for 555 frogs, respectively). In the samples bought in the supermarkets in France imported by the same company, two size classes of frogs are present, but very large frogs have not been observed in our sample. The absence of small-sized frogs is due to the sorting out of larger specimens for export (Kusrini & Alford 2006). Nevertheless, the presence of numerous large frogs means that such large specimens can be found in the field. Based on our genetic results, we could not allocate our samples to precise geographic origins. The absence of an effect of overharvesting on body size can only be studied when the precise geographic origin of specimens is known, because body size varies geographically (Kurniawan *et al*. 2011). The specimens studied in the present work may well come from newly harvested populations. In fact, the presence of *F. moodiei* in our samples, a species which has a known range outside Java and Sumatra, a range that includes the Philippines, the northern coasts of the Gulf of Thailand, Bangladesh and Orissa (Kurniawan *et al*. 2010), indicates that the specimens studied here may have come from other islands than Java and south-eastern Sumatra.

"At present, nothing is known about the impact of extensive frog harvesting on Indonesian frog populations and agricultures." This phrase of Veith *et al*. (2000) can be cited as such, as there has been no change in the last 15 years. There are still no studies on the effects of frog harvesting on natural populations. Our results show that the genetic and morphological diversity of the frogs in trade is much higher than the genetic and morphological diversity measured so far by scientific studies (Kurniawan *et al*. 2010, 2011). These results underline the need for large-scale studies on the taxonomy, population structure, reproductive data and ecology of the species concerned in international trade. In 2000, Veith *et al*. concluded that the development of a "quick and cheap test for management authorities" was necessary to monitor the international trade in frog legs. Such a test is already available through DNA sequencing, and the price is decreasing rapidly with the development of next generation sequencing techniques. The lack of any progress in the development of identification tools and assessing the status of wild populations is not due to an absence of convenient methodology but it reflects the fact that conservation is focused on flagship species, such as tigers and pandas, or species that qualify for rarity.

The edible frogs of Indonesia, although billions of individuals are killed annually, are the scarcely visible part of the bulk of species that are disappearing.

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Appendix

List of samples of deep frozen frog legs with purchase date, collection number, haplotype number, taxonomic identification, tibia length (TL) and estimated snout vent length (SVL).

Date	Museum number	Haplotype number	Species identification (Blast)	TL	SVL (estimated)
April 2013	2013.0317	h01	*Fejervarya cancrivora*	34.2	71.0
April 2013	2013.0318	h02	*Fejervarya cancrivora*	34.9	72.4
April 2013	2013.0319	h03	*Fejervarya cancrivora*	32.5	67.5
April 2013	2013.0320	h04	*Fejervarya cancrivora*	32.2	66.8
April 2013	2013.0321	h03	*Fejervarya cancrivora*	36.7	76.2
April 2013	2013.0322	h02	*Fejervarya cancrivora*	32.9	68.3
April 2013	2013.0323	h02	*Fejervarya cancrivora*	36.8	76.4
April 2013	2013.0324	h02	*Fejervarya cancrivora*	37.0	76.8
April 2013	2013.0325	h02	*Fejervarya cancrivora*	33.9	70.4
April 2013	2013.0326	h04	*Fejervarya cancrivora*	33.8	70.2
April 2013	2013.0327	h02	*Fejervarya cancrivora*	38.5	79.9
April 2013	2013.0328	h02	*Fejervarya cancrivora*	34.1	70.8
April 2013	2013.0329	h02	*Fejervarya cancrivora*	32.0	66.4
April 2013	2013.0330	h02	*Fejervarya cancrivora*	36.8	76.4
April 2013	2013.0331	h02	*Fejervarya cancrivora*	36.3	75.3
April 2013	2013.0332	h04	*Fejervarya cancrivora*	33.6	69.7
April 2013	2013.0333	h02	*Fejervarya cancrivora*	32.9	68.3
April 2013	2013.0334	h05	*Fejervarya cancrivora*	35.5	73.7
April 2013	2013.0335	h04	*Fejervarya cancrivora*	38.4	79.7
April 2013	2013.0336	h06	*Fejervarya cancrivora*	32.8	68.1
April 2013	2013.0337	h04	*Fejervarya cancrivora*	35.1	72.9
April 2013	2013.0338	h04	*Fejervarya cancrivora*	33.3	69.1
April 2013	2013.0339	h04	*Fejervarya cancrivora*	35.4	73.5
April 2013	2013.0340	h07	*Fejervarya cancrivora*	34.5	71.6
April 2013	2013.0341	h02	*Fejervarya cancrivora*	33.2	68.9
March 2013	2013.0342	h02	*Fejervarya cancrivora*	33.3	69.1
March 2013	2013.0343	h02	*Fejervarya cancrivora*	37.2	77.2
March 2013	2013.0344	h02	*Fejervarya cancrivora*	36.2	75.1
March 2013	2013.0345	h02	*Fejervarya cancrivora*	39.7	82.4
March 2013	2013.0346	h02	*Fejervarya cancrivora*	39.2	81.4
March 2013	2013.0347	h04	*Fejervarya cancrivora*	34.6	71.8
March 2013	2013.0348	h02	*Fejervarya cancrivora*	34.0	70.6
March 2013	2013.0349	h08	*Fejervarya cancrivora*	36.6	76.0
March 2013	2013.0350	h08	*Fejervarya cancrivora*	33.1	68.7
March 2013	2013.0351	h02	*Fejervarya cancrivora*	34.7	72.0
March 2013	2013.0352	h09	*Fejervarya cancrivora*	37.8	78.5
March 2013	2013.0353	h10	*Fejervarya cancrivora*	35.6	73.9
March 2013	2013.0354	h02	*Fejervarya cancrivora*	33.3	69.1

Appendix (cont.)

Date	Museum number	Haplotype number	Species identification (Blast)	TL	SVL (estimated)
March 2013	2013.0355	h04	*Fejervarya cancrivora*	33.5	69.5
March 2013	2013.0356	h02	*Fejervarya cancrivora*	36.0	74.7
March 2013	2013.0357	h04	*Fejervarya cancrivora*	35.7	74.1
March 2013	2013.0358	h04	*Fejervarya cancrivora*	36.5	75.8
March 2013	2013.0359	h02	*Fejervarya cancrivora*	35.3	73.3
March 2013	2013.0360	h02	*Fejervarya cancrivora*	33.8	70.2
March 2013	2013.0361	h08	*Fejervarya cancrivora*	36.6	76.0
March 2013	2013.0363	h02	*Fejervarya cancrivora*	33.8	70.2
March 2013	2013.0364	h04	*Fejervarya cancrivora*	32.9	68.3
March 2013	2013.0365	h04	*Fejervarya cancrivora*	31.1	64.6
March 2013	2013.0366	h04	*Fejervarya cancrivora*	38.9	80.7
March 2013	2013.0367	h02	*Fejervarya cancrivora*	34.9	72.4
March 2013	2013.0368	h02	*Fejervarya cancrivora*	37.3	77.4
February 2013	2013.0369	h11	*Fejervarya cancrivora*	33.3	69.1
February 2013	2013.0371	h11	*Fejervarya cancrivora*	34.4	71.4
February 2013	2013.0372	h11	*Fejervarya cancrivora*	32.6	67.7
February 2013	2013.0373	h11	*Fejervarya cancrivora*	31.3	65.0
February 2013	2013.0374	h11	*Fejervarya cancrivora*	33.3	69.1
February 2013	2013.0375	h11	*Fejervarya cancrivora*	36.2	75.1
February 2013	2013.0376	h11	*Fejervarya cancrivora*	35.3	73.3
February 2013	2013.0377	h11	*Fejervarya cancrivora*	32.4	67.3
February 2013	2013.0378	h11	*Fejervarya cancrivora*	28.6	59.4
February 2013	2013.0379	h11	*Fejervarya cancrivora*	31.0	64.3
February 2013	2013.0380	h11	*Fejervarya cancrivora*	32.1	66.6
February 2013	2013.0381	h11	*Fejervarya cancrivora*	32.7	67.9
February 2013	2013.0382	h11	*Fejervarya cancrivora*	31.8	66.0
February 2013	2013.0383	h11	*Fejervarya cancrivora*	31.5	65.4
February 2013	2013.0384	h11	*Fejervarya cancrivora*	33.6	69.7
February 2013	2013.0385	h11	*Fejervarya cancrivora*	32.0	66.4
February 2013	2013.0386	h17	*Fejervarya moodiei*	31.2	–
February 2013	2013.0387	h11	*Fejervarya cancrivora*	33.1	68.7
February 2013	2013.0388	h11	*Fejervarya cancrivora*	32.7	67.9
February 2013	2013.0389	h11	*Fejervarya cancrivora*	33.5	69.5
February 2013	2013.0390	h11	*Fejervarya cancrivora*	30.4	63.1
February 2013	2013.0391	h11	*Fejervarya cancrivora*	31.2	64.8
February 2013	2013.0392	h11	*Fejervarya cancrivora*	30.9	64.1
February 2013	2013.0393	h11	*Fejervarya cancrivora*	34.6	71.8
February 2013	2013.0394	h11	*Fejervarya cancrivora*	31.6	65.6
February 2013	2013.0395	h11	*Fejervarya cancrivora*	32.6	67.7
February 2013	2013.0396	h11	*Fejervarya cancrivora*	35.4	73.5

Appendix (cont.)

Date	Museum number	Haplotype number	Species identification (Blast)	TL	SVL (estimated)
January 2013	2013.0397	h02	*Fejervarya cancrivora*	42.6	88.4
January 2013	2013.0398	h02	*Fejervarya cancrivora*	41.9	87.0
January 2013	2013.0399	h04	*Fejervarya cancrivora*	43.4	90.1
January 2013	2013.0400	h04	*Fejervarya cancrivora*	45.8	95.1
January 2013	2013.0401	h02	*Fejervarya cancrivora*	43.5	90.3
January 2013	2013.0402	h04	*Fejervarya cancrivora*	46.6	96.7
January 2013	2013.0403	h02	*Fejervarya cancrivora*	42.3	87.8
January 2013	2013.0404	h08	*Fejervarya cancrivora*	44.7	92.8
January 2013	2013.0405	h04	*Fejervarya cancrivora*	43.4	90.1
January 2013	2013.0406	h10	*Fejervarya cancrivora*	47.4	98.4
January 2013	2013.0407	h06	*Fejervarya cancrivora*	40.0	83.0
January 2013	2013.0408	h04	*Fejervarya cancrivora*	43.0	89.3
January 2013	2013.0409	h02	*Fejervarya cancrivora*	44.3	92.0
January 2013	2013.0410	h02	*Fejervarya cancrivora*	46.0	95.5
January 2013	2013.0411	h04	*Fejervarya cancrivora*	43.5	90.3
January 2013	2013.0412	h04	*Fejervarya cancrivora*	39.9	82.8
January 2013	2013.0413	h06	*Fejervarya cancrivora*	43.5	90.3
January 2013	2013.0414	h02	*Fejervarya cancrivora*	50.2	104.2
January 2013	2013.0415	h02	*Fejervarya cancrivora*	47.1	97.8
January 2013	2013.0416	h02	*Fejervarya cancrivora*	41.0	85.1
January 2013	2013.0417	h04	*Fejervarya cancrivora*	48.0	99.6
January 2013	2013.0418	h02	*Fejervarya cancrivora*	46.7	96.9
April 2013	2013.0419	h04	*Fejervarya cancrivora*	45.4	94.2
April 2013	2013.0420	h04	*Fejervarya cancrivora*	43.4	90.1
April 2013	2013.0421	h04	*Fejervarya cancrivora*	45.2	93.8
April 2013	2013.0422	h04	*Fejervarya cancrivora*	53.8	111.7
April 2013	2013.0423	h04	*Fejervarya cancrivora*	44.7	92.8
April 2013	2013.0424	h04	*Fejervarya cancrivora*	45.2	93.8
April 2013	2013.0425	h04	*Fejervarya cancrivora*	42.5	88.2
April 2013	2013.0426	h04	*Fejervarya cancrivora*	44.1	91.5
April 2013	2013.0427	h04	*Fejervarya cancrivora*	45.9	95.3
April 2013	2013.0428	h04	*Fejervarya cancrivora*	46.7	96.9
April 2013	2013.0429	h04	*Fejervarya cancrivora*	45.2	93.8
April 2013	2013.0430	h18	*Limnonectes macrodon*	43.3	–
April 2013	2013.0431	h04	*Fejervarya cancrivora*	51.8	107.5
April 2013	2013.0432	h04	*Fejervarya cancrivora*	44.2	91.7
April 2013	2013.0433	h10	*Fejervarya cancrivora*	45.8	95.1
April 2013	2013.0434	h04	*Fejervarya cancrivora*	44.7	92.8
April 2013	2013.0435	h10	*Fejervarya cancrivora*	46.4	96.3
April 2013	2013.0436	h04	*Fejervarya cancrivora*	42.0	87.2

Appendix (cont.)

Date	Museum number	Haplotype number	Species identification (Blast)	TL	SVL (estimated)
April 2013	2013.0437	h04	*Fejervarya cancrivora*	45.3	94.0
April 2013	2013.0438	h04	*Fejervarya cancrivora*	45.7	94.9
April 2013	2013.0439	h04	*Fejervarya cancrivora*	47.4	98.4
April 2013	2013.0440	h04	*Fejervarya cancrivora*	48.5	100.7
January 2013	2013.0441	h04	*Fejervarya cancrivora*	35.4	73.5
January 2013	2013.0442	h12	*Fejervarya cancrivora*	35.2	73.1
January 2013	2013.0443	h02	*Fejervarya cancrivora*	34.1	70.8
January 2013	2013.0444	h11	*Fejervarya cancrivora*	37.3	77.4
January 2013	2013.0445	h04	*Fejervarya cancrivora*	33.5	69.5
January 2013	2013.0446	h03	*Fejervarya cancrivora*	32.3	67.0
January 2013	2013.0447	h13	*Fejervarya cancrivora*	32.0	66.4
January 2013	2013.0448	h04	*Fejervarya cancrivora*	37.1	77.0
January 2013	2013.0449	h03	*Fejervarya cancrivora*	34.9	72.4
January 2013	2013.0450	h02	*Fejervarya cancrivora*	31.8	66.0
January 2013	2013.0451	h03	*Fejervarya cancrivora*	37.9	78.7
January 2013	2013.0452	h14	*Fejervarya cancrivora*	34.4	71.4
January 2013	2013.0453	h02	*Fejervarya cancrivora*	34.1	70.8
January 2013	2013.0454	h04	*Fejervarya cancrivora*	36.0	74.7
January 2013	2013.0455	h04	*Fejervarya cancrivora*	36.5	75.8
January 2013	2013.0456	h04	*Fejervarya cancrivora*	34.9	72.4
January 2013	2013.0457	h11	*Fejervarya cancrivora*	33.5	69.5
January 2013	2013.0458	h03	*Fejervarya cancrivora*	35.8	74.3
January 2013	2013.0459	h02	*Fejervarya cancrivora*	36.4	75.6
January 2013	2013.0460	h02	*Fejervarya cancrivora*	36.5	75.8
January 2013	2013.0461	h02	*Fejervarya cancrivora*	34.2	71.0
January 2013	2013.0462	h04	*Fejervarya cancrivora*	35.7	74.1
January 2013	2013.0463	h04	*Fejervarya cancrivora*	37.3	77.4
January 2013	2013.0464	h04	*Fejervarya cancrivora*	33.0	68.5
January 2013	2013.0465	h11	*Fejervarya cancrivora*	39.2	81.4
January 2013	2013.0466	h03	*Fejervarya cancrivora*	32.7	67.9
December 2012	2013.0467	h02	*Fejervarya cancrivora*	41.0	85.1
December 2012	2013.0468	h02	*Fejervarya cancrivora*	48.3	100.3
December 2012	2013.0469	h11	*Fejervarya cancrivora*	44.8	93.0
December 2012	2013.0470	h11	*Fejervarya cancrivora*	43.6	90.5
December 2012	2013.0471	h11	*Fejervarya cancrivora*	43.2	89.7
December 2012	2013.0472	h11	*Fejervarya cancrivora*	43.5	90.3
December 2012	2013.0473	h11	*Fejervarya cancrivora*	46.8	97.1
December 2012	2013.0474	h11	*Fejervarya cancrivora*	45.2	93.8
December 2012	2013.0475	h11	*Fejervarya cancrivora*	45.5	94.4
December 2012	2013.0476	h11	*Fejervarya cancrivora*	47.8	99.2

Appendix (cont.)

Date	Museum number	Haplotype number	Species identification (Blast)	TL	SVL (estimated)
December 2012	2013.0477	h11	*Fejervarya cancrivora*	47.7	99.0
December 2012	2013.0478	h11	*Fejervarya cancrivora*	42.5	88.2
December 2012	2013.0479	h11	*Fejervarya cancrivora*	42.0	87.2
December 2012	2013.0480	h11	*Fejervarya cancrivora*	41.5	86.1
December 2012	2013.0481	h11	*Fejervarya cancrivora*	46.4	96.3
December 2012	2013.0482	h11	*Fejervarya cancrivora*	46.1	95.7
December 2012	2013.0483	h11	*Fejervarya cancrivora*	46.2	95.9
December 2012	2013.0484	h11	*Fejervarya cancrivora*	45.6	94.7
December 2012	2013.0485	h11	*Fejervarya cancrivora*	48.8	101.3
December 2012	2013.0486	h11	*Fejervarya cancrivora*	41.0	85.1
December 2012	2013.0487	h11	*Fejervarya cancrivora*	44.6	92.6
December 2012	2013.0488	h11	*Fejervarya cancrivora*	43.8	90.9
May 2013	2013.0489	h04	*Fejervarya cancrivora*	37.6	78.0
May 2013	2013.0490	h04	*Fejervarya cancrivora*	34.2	71.0
May 2013	2013.0491	h06	*Fejervarya cancrivora*	35.3	73.3
May 2013	2013.0492	h02	*Fejervarya cancrivora*	34.1	70.8
May 2013	2013.0493	h04	*Fejervarya cancrivora*	32.8	68.1
May 2013	2013.0494	h10	*Fejervarya cancrivora*	35.2	73.1
May 2013	2013.0495	h02	*Fejervarya cancrivora*	34.1	70.8
May 2013	2013.0496	h02	*Fejervarya cancrivora*	35.0	72.6
May 2013	2013.0497	h10	*Fejervarya cancrivora*	31.7	65.8
May 2013	2013.0498	h10	*Fejervarya cancrivora*	35.4	73.5
May 2013	2013.0499	h04	*Fejervarya cancrivora*	36.0	74.7
May 2013	2013.0500	h15	*Fejervarya cancrivora*	35.6	73.9
May 2013	2013.0501	h10	*Fejervarya cancrivora*	36.2	75.1
May 2013	2013.0502	h04	*Fejervarya cancrivora*	31.4	65.2
May 2013	2013.0503	h04	*Fejervarya cancrivora*	36.3	75.3
May 2013	2013.0504	h06	*Fejervarya cancrivora*	36.5	75.8
May 2013	2013.0505	h02	*Fejervarya cancrivora*	33.8	70.2
May 2013	2013.0506	h02	*Fejervarya cancrivora*	34.0	70.6
May 2013	2013.0507	h02	*Fejervarya cancrivora*	34.4	71.4
May 2013	2013.0508	h04	*Fejervarya cancrivora*	36.9	76.6
May 2013	2013.0509	h02	*Fejervarya cancrivora*	36.9	76.6
May 2013	2013.0510	h06	*Fejervarya cancrivora*	34.3	71.2
May 2013	2013.0511	h02	*Fejervarya cancrivora*	32.7	67.9
May 2013	2013.0512	h04	*Fejervarya cancrivora*	33.0	68.5
May 2013	2013.0515	h02	*Fejervarya cancrivora*	–	–
May 2013	2013.0516	h16	*Fejervarya cancrivora*	–	–
May 2013	2013.0517	h08	*Fejervarya cancrivora*	–	–
May 2013	2013.0518	h04	*Fejervarya cancrivora*	–	–

Appendix (cont.)

Date	Museum number	Haplotype number	Species identification (Blast)	TL	SVL (estimated)
May 2013	2013.0519	h04	*Fejervarya cancrivora*	–	–
May 2013	2013.0520	h04	*Fejervarya cancrivora*	–	–
May 2013	2013.0521	h16	*Fejervarya cancrivora*	–	–
May 2013	2013.0522	h16	*Fejervarya cancrivora*	–	–
May 2013	2013.0523	h18	*Limnonectes macrodon*	–	–
May 2013	2013.0524	h04	*Fejervarya cancrivora*	–	–
May 2013	2013.0525	h04	*Fejervarya cancrivora*	–	–
May 2013	2013.0526	h04	*Fejervarya cancrivora*	–	–
May 2013	2013.0527	h04	*Fejervarya cancrivora*	–	–
May 2013	2013.0528	h02	*Fejervarya cancrivora*	–	–
May 2013	2013.0529	h04	*Fejervarya cancrivora*	–	–