

Mitochondrial diversity of the white-toothed shrews (Mammalia, Eulipotyphla, *Crocidura*) in Vietnam

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Abstract

We explored the patterning of genetic diversity within white-toothed shrews of the genus *Crocidura* from 14 localities across Vietnam. An analysis of cytochrome oxidase *c* subunit I (COI) and cytochrome *b* (*cytb*) gene fragments from 185 specimens of white-toothed shrews of the genus *Crocidura* from 14 localities across Vietnam revealed six deeply divergent (p-distance for both COI and *cytb* >10%) lineages, corresponding to the morphological species *C. sokolovi*, *C. zaitsevi*, *C. phuquocensis*, *C. indochinensis*, *C. tanakae* and, *C. phanluongi*. *Crocidura sokolovi* was distinct from all other Vietnamese white-toothed shrews (~13% on average for both *cytb* and COI). In addition to demonstrating the genetic separation of previously described species, substantial cryptic genetic diversity was revealed. *Crocidura zaitsevi* and *C. tanakae* each included two subgroups that corresponded to geographically remote localities, while *C. indochinensis* contained two distinct subgroups that exhibited co-varying patterns of morphological and ecological differentiation, suggesting that the individuals from Sa Pa represent a separate species (provisionally named *Crocidura* sp. AB1). Mitochondrial data generated for the type specimens of *C. phanluongi* from Yok Don and Bu Gia Map supported the validity of the species while an additional specimen from Binh Chau, South Vietnam originally referred to *C. phanluongi* exhibited a deep genetic split (*cytb*: 8.4%; COI: 8.7%) from a neighbouring population in Yok Don. We propose that the specimen from Binh Chau also represents an undescribed species (provisionally named *Crocidura* sp. AB2). Our *cytb* data were then compared to the sequences of 28 species of *Crocidura* from Southeast Asia available in GenBank, suggesting that three more species occur in northern Vietnam, namely *C. wuchihensis*, *C. attenuata* and *C. fuliginosa/C. dracula*. The discovery of fairly deep genetic divergences among Vietnamese *Crocidura* illustrates that the understudied and largely undescribed diversity of white-toothed shrews in Southeast Asia requires deeper scrutiny. It also shows the useful insights of mitochondrial markers as to the taxonomic resolution of this enigmatic group of mammals.

Key words: DNA barcodes, molecular biodiversity, Soricidae, Southeast Asia, molecular diagnostics, cryptic species

Introduction

The white-toothed shrews (*Crocidura*) have a broad distribution across the Old World tropics and are one of the most speciose genera of mammals (Hutterer 2005). Despite their remarkable alpha-taxonomic diversity, these shrews are morphologically rather uniform, which contributes to their extreme taxonomic complexity. The evolutionary relationships of the species within this genus are the subject of serious debate. Most studies involving protein, chromosomal, and recent molecular data suggest the evolutionary division of *Crocidura* into Afrotropical and Asian lineages (Maddalena 1990; Maddalena & Ruedi 1994; Ruedi 1998; Bannikova *et al.* 2006; Dubey *et al.* 2008; Lavrenchenko *et al.* 2009); however, the monophyly of these clades is still insufficiently supported. Yet both karyological and molecular data strongly suggest that East Asian *Crocidura* comprise a mixture of southern and

northern faunal elements (Motokawa *et al.* 2000, 2005; Dubey *et al.* 2008) and their relationship with Afrotropical species is very complex due to several transcontinental faunal exchanges hypothesized between Eurasia and Africa (Dubey *et al.* 2007).

Vietnam occupies a key geographic position within Southeast Asia, as it spans a majority of the region's ecosystems. However, white-toothed shrews are among the most poorly known mammals in the country and only five species of *Crocidura* were historically documented therein (Heaney & Timm 1983; Huynh *et al.* 1994; Lunde *et al.* 2004; Kuznetsov 2006). More recently, a series of focused collecting trips and in-depth morphological studies have led to the description of several new species and further enhanced the resolution of species' distributions (Lunde *et al.* 2003, 2004; Jenkins *et al.* 2007, 2009, 2010; Abramov *et al.* 2008a). The total number of described *Crocidura* species recorded in Vietnam now stands at eleven: *C. attenuata*, *C. fuliginosa*, *C. indochinensis*, *C. kegoensis*, *C. sokolovi*, *C. phuquocensis*, *C. wuchihensis*, *C. zaitsevi*, *C. guy*, *C. annamitensis* and *C. phanluongi* (Abramov *et al.* 2008a; Can *et al.* 2008; Jenkins *et al.* 2009; Jenkins *et al.* 2010). Although some clarifications regarding the diagnostic morphological features of these species have been provided, the patterning of genetic diversity both among and between them remains largely unexplored.

The purpose of this study represents a first step toward breaching this gap, by employing a combination of two mitochondrial markers. The standardized 657 base pair segment of the 5' "DNA barcode" region of cytochrome *c* oxidase subunit I gene (COI; Hebert *et al.* 2003) was targeted in an effort to document alpha-taxonomic diversity as part of the Barcode of Life initiative. This marker has proven effective in evaluating the genetic diversity of taxonomically complex mammal groups posing difficulties in morphological discrimination (Clare *et al.* 2007; Borisenko *et al.* 2008; Francis *et al.* 2010). Full or partial cytochrome *b* (*cytb*) sequences were also generated from exemplar lineages highlighted by barcoding in order to place our results within an historical context provided by comparing them with the existing *Crocidura cytb* sequences available in GenBank, thus allowing broader comparisons with existing molecular data. Although valuable, GenBank data is known to be error-ridden (Harris 2003) and typically lacks reference to the actual specimens examined (Ruedas *et al.* 2000), making the data problematic for molecular diagnostic applications.

Material and methods

Cytochrome oxidase *c* subunit 1 (COI) and cytochrome *b* (*cytb*). A total of 185 *Crocidura* specimens were analyzed. Pieces of muscle and/or internal organs (liver, heart, kidney) fixed in 96% ethanol were used as sources for DNA extraction. The DNA barcode region of COI was sequenced from 180 specimens and partial or complete *cytb* gene sequences were recovered from an additional 32 specimens. All the specimens were collected between 2002–2009 from among 14 localities surveyed across Vietnam and one locality in Laos (Fig. 1). Included were the holotypes and/or parts of the type series of four recently described species (*C. zaitsevi*, *C. sokolovi*, *C. phuquocensis*, and *C. phanluongi*). Morphological voucher specimens are deposited in the Zoological Institute of the Russian Academy of Sciences (ZIN, St. Petersburg), and the Zoological Museum of Moscow University (ZMMU, Moscow).

The list of Vietnamese specimens analysed including collection localities, museum catalogue numbers, GenBank accession numbers and BOLD process ID is provided in Tables 1 and 2. Besides, seven original sequences obtained from *C. suaveolens*, *C. sibirica* and *C. lasiura* for *cytb* (Ac No HM586991-97) and 38 sequences of *C. suaveolens*, *C. sibirica*, *C. shantungensis*, *C. lasiura*, *C. olivieri* and *C. macmillani* for COI (BOLD No SKMZM365-08, SKMZM366-08, SKMZM371-08, SKMZM372-08, SKMZM486-488-08, SKMZM490-08, SKMZM493-496-08, SKMZM498-08, SKMZM500-504-08, SKMZM800-805-09, SKMZM811-814-09, SKMZM816-09, SKMZM817-09) were used to assess genetic diversity. We also included *cytb* sequence data from several published sources (Ruedi *et al.* 1998; Bannikova *et al.* 2006, 2009; Ohdachi *et al.* 2004, 2006; Dubey *et al.* 2008; Esselstyn *et al.* 2009; Lavrenchenko *et al.* 2009) to place the shrews within Vietnam into a regional phylogeographic context.

DNA isolation, PCR amplification and sequencing. COI. Tissues were submitted to the Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario, University of Guelph (Guelph, Canada) pre-arrayed into 96-well microplates following standard sampling instructions (Borisenko *et al.* 2008; Borisenko *et al.* 2009). Approximately 5 mg or 2 mm³ of tissue was sampled from each specimen. Prior to DNA extraction, each plate well was

filled with 50 μ l of lysis buffer with Proteinase K and the plates were incubated for 12–18 h at 56°C, followed by a robotic DNA extraction protocol as described in Ivanova *et al.* (2006). Standard barcoding protocols for PCR amplification and sequencing were employed, following PCR and sequencing with regular and M13-tailed universal vertebrate primer cocktails (Clare *et al.* 2007; Ivanova *et al.* 2006). In cases where a full length barcode was not recoverable using standard primers, the internal primer RonM (Pfunder *et al.* 2004) and its M13-tailed modification (Borisenko *et al.* 2008) were used to amplify a shorter COI fragment (421 bp). The standard CCDB protocol with 1/24 BigDye dilution (Ivanova & Grainger 2007) was used for sequencing. PCR products were visualized on a 2% agarose gel using an E-Gel96 Pre-cast Agarose Electrophoresis System (Invitrogen) as described in DeWaard *et al.* (2008). Products were labelled by using the BigDye© Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.) as described in (Hajibabaei *et al.* 2005) using a standard 1/24 BigDye dilution (Ivanova & Grainger 2007) and sequenced bidirectionally using an ABI 3730XL capillary sequencer following manufacturer's instructions. Sequences were assembled from raw sequencer trace files using SeqScape v 2.1.1 (Applied Biosystems) and verified by eye. Specimen provenance and barcode sequence data were stored and analyzed using the Barcode of Life Data System — BOLD (Ratnasingham and P.D.N. Hebert 2007) in the publicly accessible project title/code through the online interface at <http://www.barcodinglife.org>.

TABLE 1. List of the specimens analysed for the cytochrome oxidase *c* subunit 1 (COI) gene. Holotypes are marked in bold.

| species | BOLD Accession No | Catalogue number and field specimen code | Specimen code on Fig. 2–3 | Collecting locality |
|----------------------|-------------------|--|--|---|
| <i>C. sokolovi</i> | ABMIV152 08 | ZIN 91232, CVN179 | 1 | Vietnam, Kon Tum Province, Ngoc Linh Mt.; 15°05'N, 107°57'E |
| | ABMIV083 08 | ZIN 96404, CVN12 | 2 | |
| | ABMIV082 08 | ZIN 96396, CVN4 | 3 | |
| | ABIOW052 08 | ZIN 91233, CVN180 | 4 | |
| | ABMIV081 08 | ZIN 96394, CVN2 | 5 | |
| <i>C. zaitsevi</i> A | ABMIV091 08 | ZIN 96343, CVN43 | 1 | Vietnam, Kon Tum Province, Ngoc Linh Mt.; 15°05'N, 107°57'E |
| | ABMIV093 08 | ZIN 96348, CVN48 | 2 | |
| | ABMIV149 08 | ZIN 91219, CVN172 | 3 | |
| | ABMIV146 08 | ZIN 91214, CVN167 | 4 | |
| | ABMIV097 08 | ZIN 96379, CVN79 | 5 | |
| | ABMIV095 08 | ZIN 96359, CVN59 | 6 | |
| | ABIOW119 08 | ZIN 96345, CVN45 | 7 | |
| | ABIOW108 08 | ZIN 96358, CVN58 | 8 | |
| | ABIOW115 08 | ZIN 96340, CVN40 | 9 | |
| | ABIOW114 08 | ZIN 96339, CVN39 | 10 | |
| | ABIOW100 08 | ZIN 96364, CVN64 | 11 | |
| | ABIOW092 08 | ZIN 96372, CVN72 | 12 | |
| | ABIOW120 08 | ZIN 96346, CVN46 | 13 | |
| | ABIOW117 08 | ZIN 96342, CVN42 | 14 | |
| | ABMIV151 08 | ZIN 91225, CVN178 | 15 | |
| | ABMIV094 08 | ZIN96354, CVN54 | 16 | |
| | ABIOW088 08 | ZIN 96377, CVN77 | 17 | |
| | ABMIV088 08 | ZIN 96331, CVN31 | 18 | |
| | ABMIV150 08 | ZIN 91224, CVN177 | 19 | |
| | ABMIV085 08 | ZIN 96319, CVN19 | 20 | |
| | ABMIV098 08 | ZIN 96380, CVN80 | 21 | |
| ABIOW041 08 | ZIN 97511, CVN213 | 22 | Vietnam, Quang Binh Province, Phong Nha - Ke Bang National Park; 17°38'N, 106°06'E | |

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TABLE 1. (continued)

| species | BOLD Accession No | Catalogue number and field specimen code | Specimen code on Fig. 2–3 | Collecting locality |
|-------------------------|-------------------|--|---------------------------|--|
| <i>C. zaitsevi</i> B | ABMIV120 08 | ZIN 96413, CVN121 | 1 | Vietnam, Khanh Hoa Province, Hon Ba Mt.; 12°07' N, 108°57' E |
| | ABMIV121 08 | ZIN 96414, CVN122 | 2 | |
| | ABMIV159 08 | ZMMU S-175185, CVN187 | 3 | |
| | ABIOW149 09 | ZIN 97629, CVN215 | 4 | Vietnam, Lam Dong Province, Bi Doup Mt.; 12°11' N, 108°41' E |
| | ABIOW166 09 | ZIN 97643, CVN232 | 5 | |
| | ABIOW172 09 | ZIN 97635, CVN238 | 6 | |
| | ABIOW173 09 | ZIN 97648, CVN239 | 7 | |
| | ABIOW203 09 | ZIN 97659, CVN 270 | 8 | |
| | ABIOW195 09 | ZIN 97636, CVN262 | 9 | |
| | ABIOW198 09 | ZIN 97639, CVN265 | 10 | |
| | ABIOW133 09 | ZIN 98949, CVN338 | 11 | |
| | ABIOW165 09 | ZIN 97642, CVN231 | 12 | |
| | ABIOW130 09 | ZIN 98946, CVN335 | 13 | |
| | ABIOW153 09 | ZIN 97630, CVN219 | 14 | |
| | ABIOW171 09 | ZIN 97634, CVN237 | 15 | |
| | ABIOW169 09 | ZIN 97646, CVN235 | 16 | |
| | ABIOW170 09 | ZIN 97647, CVN236 | 17 | |
| <i>C. phuquocensis</i> | ABMIV158 08 | ZIN 96662, CVN186 | 1 | Vietnam, Kien Giang Province, Phu Quoc Isl.; 10°23' N, 104°00' E |
| | ABMIV155 08 | ZIN 96659, CVN183 | 2 | |
| | ABMIV157 08 | ZIN 96661, CVN185 | 3 | |
| | ABMIV156 08 | ZIN96660, CVN184 | 4 | |
| <i>Crocidura</i> sp.AB1 | ABMIV111 08 | ZIN 96276, CVN104 | 1 | Vietnam, Lao Cai Province, Sa Pa District, 22°20' N, 103°50' E |
| | ABMIV106 08 | ZIN 96269, CVN99 | 2 | |
| | ABMIV109 08 | ZIN 96274, CVN102 | 3 | |
| | ABIOW222 09 | ZIN 97797, CVN289 | 4 | |
| | ABIOW051 08 | ZIN 98250, CVN58 27 | 5 | |
| | ABIOW217 09 | ZIN 97792, CVN284 | 6 | |
| | ABIOW075 08 | ZIN 96434, CVN109 | 7 | |
| | ABIOW072 08 | ZIN 96499, CVN120 | 8 | |
| | ABIOW216 09 | ZIN 97791, CVN283 | 9 | |
| | ABIOW240 09 | ZIN 97815, CVN307 | 10 | |
| | ABIOW230 09 | ZIN 97805, CVN297 | 11 | |
| | ABIOW014 08 | ZIN 98248, CVN31 60 | 12 | |
| | ABIOW220 09 | ZIN 97795, CVN287 | 13 | |
| | ABIOW049 08 | ZIN 98260, CVN74 124 | 14 | |
| | ABIOW050 08 | ZIN 98267, CVN93 142 | 15 | |
| | ABIOW233 09 | ZIN 97808, CVN300 | 16 | |
| | ABIOW221 09 | ZIN 97796, CVN288 | 17 | |
| | ABIOW223 09 | ZIN 97798, CVN290 | 18 | |
| | ABIOW015 08 | ZIN 98249, CVN32 85 | 19 | |
| | ABIOW069 08 | ZIN 96442, CVN117 | 20 | |
| | ABMIV108 08 | ZIN 96271, CVN101 | 21 | |

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TABLE 1. (continued)

| species | BOLD Accession No | Catalogue number and field specimen code | Specimen code on Fig. 2–3 | Collecting locality |
|-----------------------------|-------------------|--|---------------------------|--|
| | ABMIV101 08 | ZIN 96264, CVN94 | 22 | |
| | ABMIV117 08 | ZIN 96438, CVN113 | 23 | |
| | ABMIV110 08 | ZIN 96275, CVN103 | 24 | |
| | ABMIV100 08 | ZIN 96262, CVN93 | 25 | |
| | ABMIV118 08 | ZIN 96439, CVN114 | 26 | |
| | ABMIV116 08 | ZIN 96436, CVN111 | 27 | |
| | ABMIV114 08 | ZIN 96433, CVN108 | 28 | |
| | ABIOW225 09 | ZIN 97800, CVN292 | 29 | |
| | ABIOW074 08 | ZIN 96432, CVN107 | 30 | |
| | ABIOW232 09 | ZIN 97807, CVN299 | 31 | |
| | ABIOW219 09 | ZIN 97794, CVN286 | 32 | |
| <i>Crocidura</i> sp. AB2 | ABMIV171 08 | ZIN 97089, CVN201 | | Vietnam, Ba Ria – Vung Tau Province, Binh Chau - Phuoc Buu Nature Reserve; 10°32'N, 107°29'E |
| <i>C. indochinensis</i> | ABIOW202 09 | ZIN 97672, CVN269 | 1 | Vietnam, Lam Dong Province, Bi Doup Mt.; 12°11'N, 108°41'E |
| | ABIOW199 09 | ZIN 97670, CVN266 | 2 | |
| | ABIOW200 09 | ZIN 97671, CVN267 | 3 | |
| | ABIOW145 09 | ZIN 98962, CVN350 | 4 | |
| | ABIOW177 09 | ZIN 97668, CVN243 | 5 | |
| | ABIOW206 09 | ZIN 97673, CVN273 | 6 | |
| | ABIOW207 09 | ZIN 97674, CVN274 | 7 | |
| <i>C. tanakae</i> A | ABMIV145 08 | ZIN 91230, CVN165 | 1 | Vietnam, Kon Tum Province, Ngoc Linh Mt.; 15°05'N, 107°57'E |
| | ABMIV142 08 | ZIN 91226, CVN161 | 2 | |
| | ABMIV143 08 | ZIN 91227, CVN162 | 3 | |
| | ABMIV144 08 | ZIN 91229, CVN164 | 4 | |
| | ABIOW063 08 | ZIN 91228, CVN163 | 5 | |
| | ABIOW062 08 | ZIN 91231, CVN166 | 6 | |
| | ABMIV129 08 | ZIN 96412, CVN138 | 7 | |
| | ABIOW040 08 | ZIN 97510, CVN212 | 8 | Vietnam, Quang Binh Province, Phong Nha - Ke Bang National Park; 17°38'N, 106°06'E |
| | ABIOW128 09 | ZIN 98933, CVN333 | 9 | Vietnam, Lam Dong Province, Bi Doup Mt.; 12°11'N, 108°41'E |
| | ABIOW143 09 | ZIN 98960, CVN348 | 10 | |
| | ABIOW150 09 | ZIN 97606, CVN216 | 11 | |
| | ABIOW179 09 | ZIN 97615, CVN245 | 12 | |
| | ABIOW144 09 | ZIN 98691, CVN349 | 13 | |
| | ABIOW161 09 | ZIN 97613, CVN227 | 14 | |
| | ABIOW158 09 | ZIN 97610, CVN224 | 15 | |
| | ABIOW160 09 | ZIN 94612, CVN226 | 16 | |
| | ABIOW183 09 | ZIN 97625, CVN249 | 17 | |
| | ABIOW174 09 | ZIN 97623, CVN240 | 18 | |
| | ABIOW164 09 | ZIN 97622, CVN230 | 19 | |

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TABLE 1. (continued)

| species | BOLD Accession No | Catalogue number and field specimen code | Specimen code on Fig. 2–3 | Collecting locality |
|----------------------|-------------------|--|---------------------------|---|
| | ABIOW175 09 | ZIN 97624, CVN241 | 20 | |
| | ABIOW155 09 | ZIN 97608, CVN221 | 21 | |
| | ABMIV164 08 | ZMMU S-173391, CVN192 | 22 | |
| | ABMIV168 08 | ZMMU S-173397, CVN198 | 23 | |
| | ABMIV165 08 | ZMMU S-173392, CVN193 | 24 | |
| | ABIOW138 09 | ZIN 98940, CVN343 | 25 | |
| | ABIOW135 09 | ZIN 98937, CVN340 | 26 | |
| | ABIOW136 09 | ZIN 98938, CVN341 | 27 | |
| | ABIOW140 09 | ZIN 98942, CVN345 | 28 | |
| | ABIOW137 09 | ZIN 98939, CVN342 | 29 | |
| | ABIOW139 09 | ZIN 98941, CVN344 | 30 | |
| | ABIOW154 09 | ZIN 97607, CVN220 | 31 | |
| | ABIOW181 09 | ZIN 97651, CVN247 | 32 | |
| | ABIOW201 09 | ZIN 97620, CVN268 | 33 | |
| | ABIOW178 09 | ZIN 97614, CVN244 | 34 | |
| | ABIOW193 09 | ZIN 97619, CVN260 | 35 | |
| | ABIOW021 08 | ZIN 97504, CVN207 | 36 | Vietnam, Quang Tri Province, Huong Hoa Nature Reserve; 16°56'N, 106°35'E |
| | ABIOW022 08 | ZIN 97505, CVN208 | 37 | |
| | ABIOW024 08 | ZIN 97507, CVN210 | 38 | |
| | ABIOW023 08 | ZIN 97506, CVN209 | 39 | |
| | ABIOW039 08 | ZIN 97508, CVN211 | 40 | |
| | ABIOW126 09 | ZIN 98935, CVN331 | 41 | Vietnam, Binh Phuoc Province, Bu Gia Map National Park; 12°11'N, 107°12'E |
| | ABIOW127 09 | ZIN 98936, CVN 332 | 42 | |
| <i>C. tanakae</i> B | ABIOW033 08 | ZIN 91195, CVN145 | 43 | Vietnam, Lao Cai Province, Van Ban District; 21°58'N, 104°02'E |
| | ABIOW064 08 | ZIN 91209, CVN159 | 44 | |
| | ABMIV131 08 | ZIN 91190, CVN140 | 45 | |
| | ABMIV140 08 | ZIN 91208, CVN158 | 46 | |
| | ABMIV137 08 | ZIN 91204, CVN154 | 47 | |
| | ABIOW036 08 | ZIN91198, CVN148 | 48 | |
| | ABIOW032 08 | ZIN91193, CVN143 | 49 | |
| | ABMIV135 08 | ZIN 91201 CVN151 | 50 | |
| | ABMIV141 08 | ZIN 91210, CVN160 | 51 | |
| | ABMIV138 08 | ZIN 91205, CVN155 | 52 | |
| | ABMIV136 08 | ZIN 91202, CVN152 | 53 | |
| | ABMIV139 08 | ZIN 91207, CVN157 | 54 | |
| | ABMIV133 08 | ZIN 91194, CVN144 | 55 | |
| <i>C. phanluongi</i> | ABMIV172 08 | ZIN 97090, CVN202 | 1 | Vietnam, Dak Lak Province, Yok Don National Park; 12°58'N, 107°49'E |
| | ABIOW019 08 | ZIN 97092, CVN204 | 2 | |
| | ABIOW018 08 | ZIN 97091, CVN203 | 3 | |
| | ABIOW020 08 | ZIN 97093, CVN205 | 4 | |
| | ABIOW124 09 | ZIN 98930, CVN329 | 5 | |
| | | | | Vietnam, Binh Phuoc Province, Bu Gia Map National Park; 12°11'N, 107°12'E |

TABLE 2. List of specimens analysed for the cytochrome *b* (*cytb*) gene. Holotypes are marked in bold. The sequences retrieved from GenBank are given with asterisks.

| species | GenBank Accession No | Catalogue and/or field code | number specimen | Specimen code (on Fig. 3 if present) | Collecting locality |
|--------------------------|-------------------------|-----------------------------------|--------------------|--|---|
| <i>C. sokolovi</i> | HM586998 | ZIN 96393, CVN1 | 1 | | Vietnam, Kon Tum Province, Ngoc Linh Mt.; 15°05'N, 107°57'E |
| | HM586999 | ZIN 96394, CVN2 | 2 | | |
| | HM587000 | ZIN 96396, CVN4 | - | | |
| <i>C. zaitsevi</i> A | HM587002 | ZIN 96319, CVN19 | 1 | | Vietnam, Kon Tum Province, Ngoc Linh Mt.; 15°05'N, 107°57'E |
| | HM587004 | ZIN 96321, CVN21 | 2 | | |
| | HM587003 | ZIN 96320, CVN20 | - | | |
| <i>C. zaitsevi</i> B | HM587021 | ZIN 97628, CVN217 | 1 | | Vietnam, Lam Dong Province, Hon Giao Mt.; 12°11'N, 108°48'E |
| | HM587025 | ZIN 97642, CVN231 | 2 | | Vietnam, Lam Dong Province, Bi Doup Mt.; 12°11'N, 108°41'E |
| | HM587026 | ZIN 97643, CVN232 | - | | |
| | HM587013 | ZIN 96413, CVN121 | - | | Vietnam, Khanh Hoa Province, Hon Ba Mt., 12°07'N, 108°57'E |
| | HM587014 | ZMMU S-175185, CVN187 | - | | |
| <i>C. phuquocensis</i> | HM587011 | ZIN 96660, CVN184 | ZIN 96660 | | Vietnam, Kien Giang Province, Phu Quoc Isl.; 10°23'N, 104°00'E |
| <i>Crociodura</i> AB1 | sp. HM587006 | ZIN 96264, CVN94 | ZIN 96264 | | Vietnam, Lao Cai Province, Sa Pa District, 22°20'N, 103°50'E |
| | HM587005 | ZIN 96262, CVN93 | - | | |
| | HM587007 | ZIN 96265, CVN95 | - | | |
| <i>C. indochinensis</i> | HM587023 | ZIN 97669, CVN261 | 1 | | Vietnam, Lam Dong Province, Bi Doup Mt.; 12°11'N, 108°41'E |
| | HM587024 | ZIN 97670, CVN266 | 2 | | |
| <i>C. tanakae</i> | AB175081* | | 1 | | Taiwan, Nantou Co. |
| | AB175080* | | 2 | | |
| | HM587017 | ZMMU S-173392, CVN193 | 3 | | Vietnam, Lam Dong Province, Bi Doup Mt.; 12°11'N, 108°41'E |
| | HM587022 | ZIN 97627, CVN218 | 4 | | Vietnam, Lam Dong Province, Hon Giao Mt.; 12°11'N, 108°48'E |
| | HM587012 | ZIN 91190, CVN140 | - | | Vietnam, Lao Cai Province, Van Ban District, 21°58'N, 104°02'E |
| | HM587001 | ZIN 96409, CVN17 | - | | Vietnam, Kon Tum Province, Ngoc Linh Mt.; 15°05'N, 107°57'E |
| | HM587010 | ZIN 96411, CVN139 | - | | |
| | | ZIN 96412, CVN138 | - | | Vietnam, Khanh Hoa Province, Hon Ba Mt.; 12°07'N, 108°57'E |
| | FJ814038* | ROM115021 | 5 | | China, Hunan |
| | FJ814037* | ROM11505 | 6 | | |
| | FJ814036* | ROM11504 | 7 | | |
| | FJ814035* | ROM114960 | 8 | | |
| | FJ814033* | ROM111317 | 9 | | Vietnam |
| | FJ814032* | ROM111293 | 10 | | |
| HM587027 | ZIN 98935, CVN331 | 11 | | Vietnam, Binh Phuoc Province, Bu Gia Map National Park; 12°11'N, 107°12'E | |
| HM587028 | ZIN 98936, CVN 332 | 12 | | | |
| HM587031 | ZIN 99026, Laos317 | 13 | | Laos, Khammouane Province, Ban Doy Area; 17°33'N, 104°49'E | |
| HM587032 | ZIN 99028, Laos319 | 14 | | | |

continued next page

TABLE 2. (continued)

| species | GenBank Accession No | Catalogue and/or field code | number specimen | Specimen code (on Fig. 3 if present) | Collecting locality |
|-------------------------|-------------------------|-----------------------------------|--------------------|--|--|
| | EU122211 | MVZ185237 | 15 | | Vietnam, Vinh Phu Province, Vin Yen District, Tam Dao; 21°27' N, 105°38'E |
| | FJ814031* | ROM107661 | 16 | | Vietnam |
| | FJ814047* | ROM116443 | 17 | | China, Hunan |
| | FJ814042* | ROM116114 | 18 | | |
| | FJ814044* | ROM116366 | 19 | | |
| | FJ814046* | ROM116432 | 20 | | |
| | FJ814045* | ROM116426 | 21 | | |
| | HM587029 | ZIN 98937, CVN340 | 22 | | Vietnam, Lam Dong Province, Bi Doup Mt.; 12°11' N, 108°41' E |
| | HM587030 | ZIN 98938, CVN341 | 23 | | |
| <i>Crocidura</i> sp.AB2 | HM587018 | ZIN 97089, CVN201 | ZIN 97089 | | Vietnam, Ba Ria – Vung Tau Province, Binh Chau - Phuoc Buu Nature Reserve; 10°32' N, 107°29' E |
| <i>C. phanluongi</i> | HM587019 | ZIN 97091, CVN203 | - | | Vietnam, Dak Lak Province, Yok Don National Park; 12°58' N, 107°49' E |
| | HM587020 | ZIN 97092, CVN204 | ZIN 97092 | | |
| <i>C. attenuata</i> | AB175083* | AMNH101493 | 1 | | Vietnam, Ha Giang Province, Mt. Tay Con Linh II; 22°45' N, 104°50' E |
| | AB175082* | AMNH101492 | 2 | | |
| | FJ814039* | ROM116033 | 3 | | China, Guangxi, |
| | FJ814034* | ROM114916 | 4 | | China, Hunan, |
| <i>C. wuchihensis</i> | AB175084* | AMNH101499 | 1 | | Vietnam, Ha Giang Province; Mt. Tay Con Linh II; 22°45' N, 104°50' E |
| | AB175085* | AMNH101508 | 2 | | |
| | FJ814043* | ROM116129 | 3 | | China, Guangxi |
| | FJ814041* | ROM116095 | 4 | | |
| | FJ814040* | ROM116090 | 5 | | |
| | EU122212* | MVZ186404 | 6 | | Vietnam, Vinh Phu Province, Vin Yen District, Tam Dao; 21°27' N, 105°38' E |
| <i>C. dracula</i> | AB175079* | AMNH101526 | 1 | | Vietnam, Ha Giang Province, Mt. Tay Con Linh II; 22°45' N, 104°50' E |
| <i>C. fuliginosa</i> | FJ813925* | IZEA3753 | 2 | | Peninsular Malaysia, Cameron Highland |
| | FJ813924* | IZEA3553 | 3 | | |

Cytb. Genomic DNA was isolated from ethanol-fixed liver, kidney or muscles by proteinase K digestion, phenol-chloroform deproteinization and isopropanol precipitation (Sambrook *et al.* 1989). The mitochondrial H-strand region containing the complete *cytb* gene (1140 bp) was amplified by PCR with the forward/reverse primer combination L14728_Cr/H1310_Cr or L14723_Cr/H1310_Cr. In cases when DNA was degraded, fragments of *cytb* were amplified in two PCR reactions using L363A/ Cro_481b which was also used for sequencing. All primers were designed specifically for this study (Table 3). The PCR reaction conditions for *cytb* amplification included the initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 s, annealing at 57–60°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min, and indefinite storage at 4°C. PCR products were visualized on 1% agarose gel and then purified using DEAE Watman or NH₄EtOH. Approximately 10–40 ng of the purified PCR product was used for sequencing with each primer by the ABI 3100-Avant autosequencing system using ABI PRISM®BigDye™ Terminator v. 3.1.



FIGURE 1. Distribution of sampling localities in Vietnam: 1—Lao Cai Province, Sa Pa District; 2—Lao Cai Province, Van Ban District; 3—Ha Giang Province, Mt. Tay Con Linh II (data from GenBank); 4—Vinh Phu Province, Vin Yen District, Tam Dao (data from GenBank); 5—Quang Binh Province, Phong Nha - Ke Bang National Park; 6—Quang Tri Province, Huong Hoa Nature Reserve; 7—Kon Tum Province, Ngoc Linh Mt.; 8—Dak Lak Province, Yok Don National Park; 9—Binh Phuoc Province, Bu Gia Map National Park; 10—Lam Dong Province, Bi Doup Mt.; 11—Lam Dong Province, Hon Giao Mt.; 12—Khanh Hoa Province, Hon Ba Mt; 13—Ba Ria-Vung Tau Province, Binh Chau - Phuoc Buu Nature Reserve; 14—Kien Giang Province, Phu Quoc Isl.; and in Laos: 15—Khammouane Province, Ban Doy Area.

Analyses of sequences. *Cytb* and COI gene sequences were aligned by eye using BioEdit v.7.0.5.3 (Hall 1999). The final alignment of the mitochondrial regions included 1140 bp for *cytb* and 657 bp for COI. Patterns of evolutionary sequence diversification for both COI and *cytb* were assessed using neighbour joining methods (NJ) implemented on PAUP* version 4.0b10 (Swofford 1998), based on pairwise p-distance matrix. To assess nodal support, 1000 bootstrap pseudoreplicates were generated. In order to cluster sequences likely belonging to different species we used the approach of Francis *et al.* (2010). To assess the genetic independence of species, we first

defined genetically distinct haplotype clusters on the NJ tree, using a combination of genetic distances and bootstrap support. We then calculated the mean genetic distance from each cluster to other clusters both within and among species. We defined the minimum interspecific distance as the minimum distance between specimens belonging to different species.

TABLE 3. Primers designed for amplification and sequencing of cytochrome b (*cytb*) gene in the Vietnamese species of *Crocidura*.

| primer | sequence (5'-3') |
|-----------|-------------------------------|
| L14728_Cr | GACATGAAAAATCATCGTTGTTCTTCAAC |
| L14723_Cr | CCTATGACATGAAAAATCATCGTTGTT |
| H1310_Cr | GAATATCAGCTTTGGGTGYTGATGGTGG |
| Cro_481b | ACGGAAAAGCCTCCTCAGATTCATTCTAC |
| L363A | CGCAGTTATAGCCACCGCCTTTATAGG |

Results

The results of the NJ analysis of the COI and *cytb* p-distance matrices are presented on Figs. 2–3, and in Tables 4–5. The different interpretations from COI and *cytb* are due to the differences in the comparative context available for these two markers: COI includes significantly more specimens analyzed in this study, while there is a greater taxonomic sampling of *cytb* in GenBank.

The NJ tree for COI (Fig. 2) contains 180 specimens of shrews from Vietnam which split into six highly divergent (p-dist > 10%) lineages. These haplogroups correspond to the morphological species *C. sokolovi*, *C. zaitsevi*, *C. phuquocensis*, *Crocidura* ex. gr. *indochinensis*, *C. cf. tanakae*, and *C. phanluongi*. Four of these clusters are further subdivided into subgroups with varying levels of genetic differentiation. The haplogroups containing *C. zaitsevi* and *C. tanakae* each include two subgroups: *C. zaitsevi* A and B and *C. cf. tanakae* A and B separated by a p-distance of 1.5% and 2.3%, accordingly. *C. phanluongi* is represented by two similar, but strongly divergent (8.7%) lineages. The cluster identified here as *C. ex gr. indochinensis* contains two subgroups with fairly deep genetic divergence (p-dist = 4.0%). Each haplogroup and subgroup is supported with high bootstrap values; however, their relationships remain unclear.

The *cytb* dendrogram (Fig. 3) contains fewer specimens of Vietnamese shrews but a broader set of sequences of *Crocidura* species from extralimital regions of Southeast Asia. All groups seen on the COI dendrogram are present and supported by high bootstrap values in the *cytb* dendrogram as well. *C. zaitsevi* is the nearest neighbour to the *C. indochinensis/C. wuchihensis* group and, together with it, clusters with the *C. dsinezumi/C. attenuata/C. kurodae/C. lasiura* branch. The similarity of two lineages referred to *C. phanluongi* sensu lato (p-dist = 7.9%) is more obvious, compared to COI, with a bootstrap support of 94%. Mean p-distances between the Vietnamese species range between 7.75% (*C. wuchihensis/C. indochinensis* s.l.) and 15.5% (*C. sokolovi/C. phuquocensis*).

Discussion

The patterning of genetic diversity within and among Vietnamese *Crocidura* obtained using mitochondrial markers enabled us to discriminate all described morphospecies and to highlight hidden genetic divergences that may taxonomic refinements. Specific details are discussed below.

Crocidura sokolovi and *C. zaitsevi*

Both COI and *cytb* mitochondrial sequence data confirmed the genetic authenticity of *C. sokolovi* and *C. zaitsevi* recently described from central Vietnam (Jenkins *et al.* 2007). The relative amount of separation from the respective closest groups (COI/*cytb* of 11.5/11.1% and 9.4/9.9%, correspondingly) falls within the range of other species-level divergences observed from our data and the literature. Interestingly, the mitochondrial DNA data further highlight the fact that *C. sokolovi* from high altitudes of Ngoc Linh Mountain is not only distinct, but also

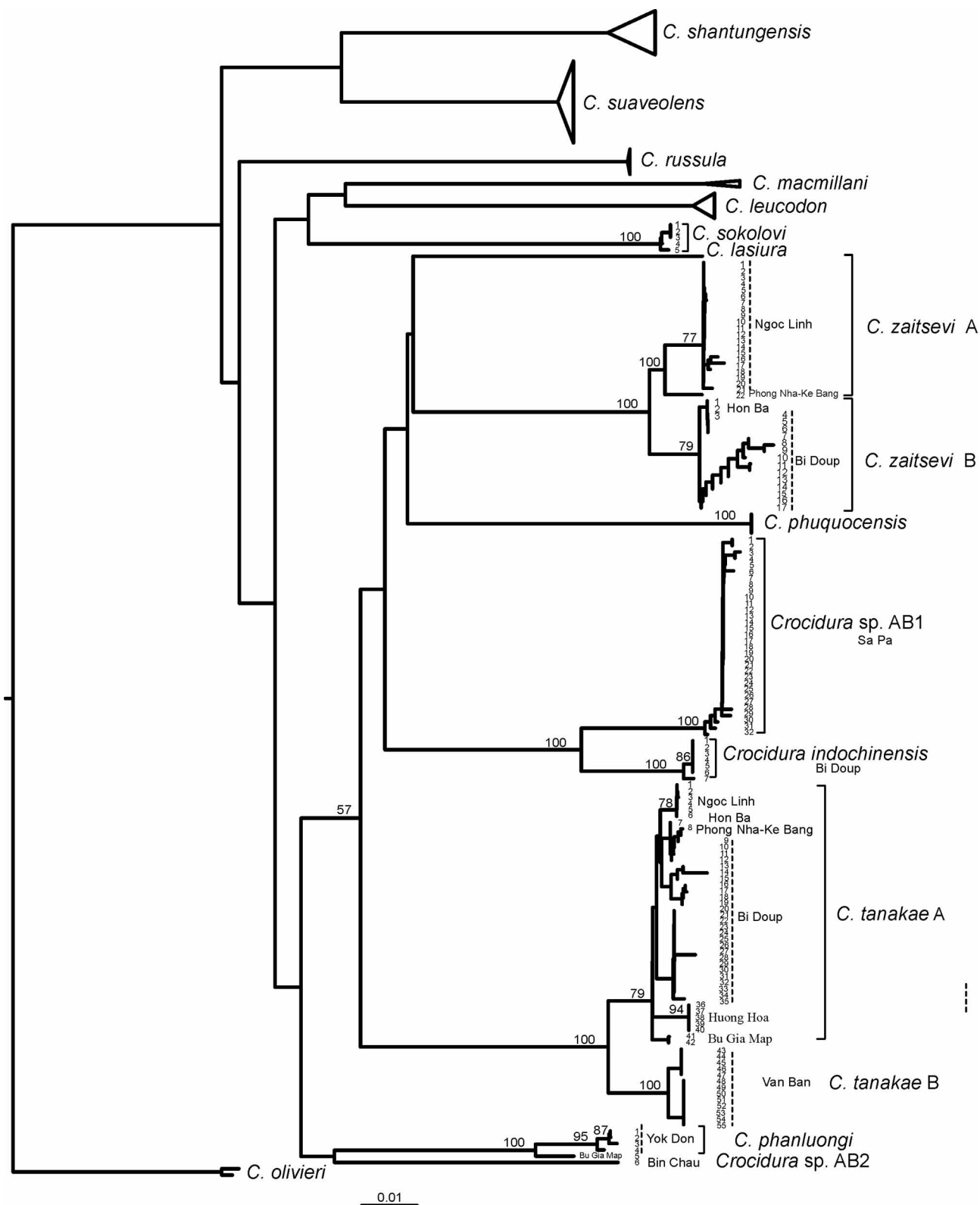


FIGURE 2. The Neighbor-Joining tree for the cytochrome oxidase *c* subunit 1 (*COI*) gene fragment. The bootstrap values ($\geq 50\%$) obtained from 1000 pseudoreplications are presented above the branches. *Crocidura olivieri* is used as outgroup.

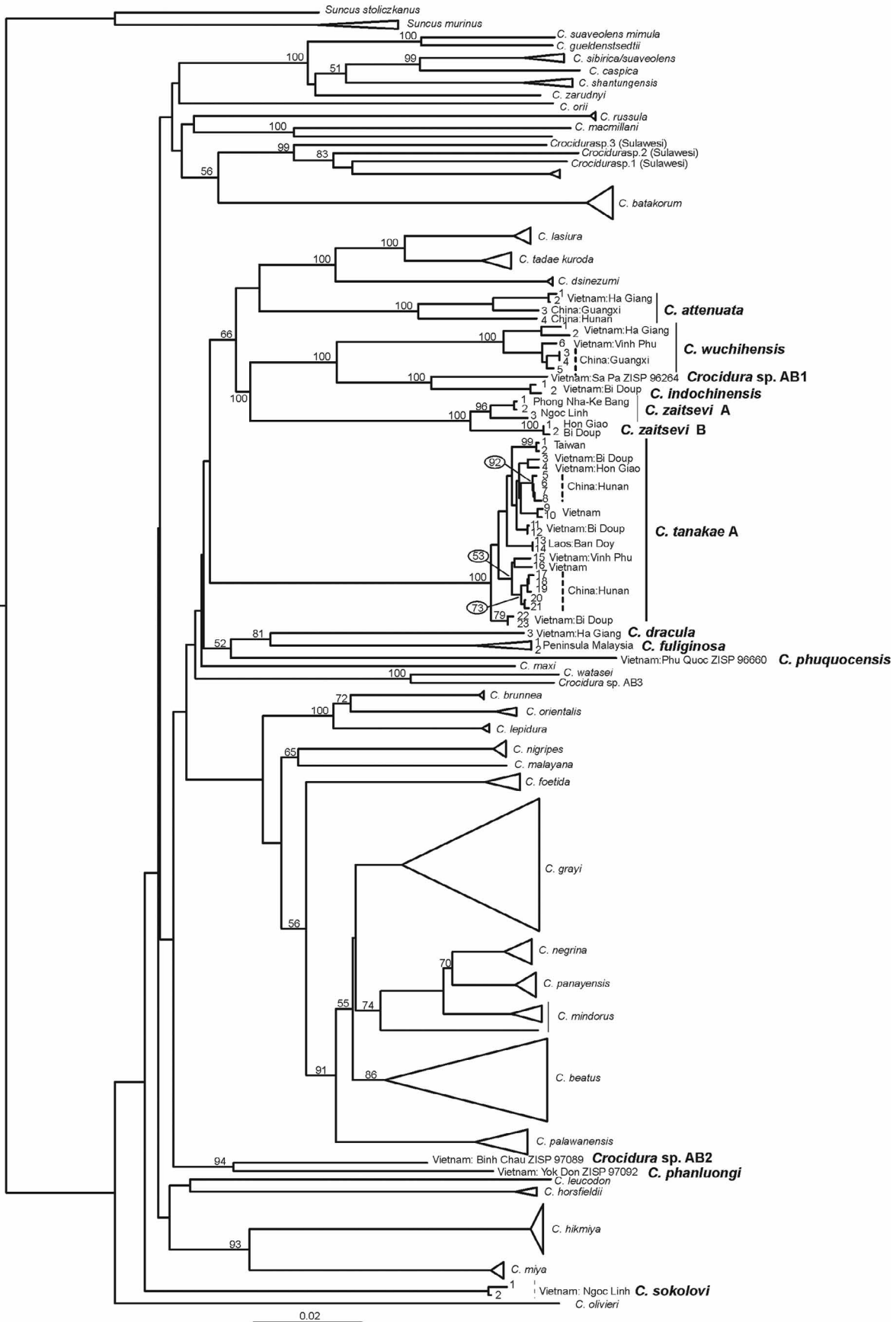


FIGURE 3. The Neighbor-Joining tree for the cytochrome *b* (*cytb*) gene fragment. Designations as on the Fig. 2. *Suncus murinus* and *S. stoliczkanus* are used as outgroup.

TABLE 4. Comparison of intraspecific vs. interspecific p-distances (%) using the cytochrome oxidase *c* subunit 1 (COI) gene. Average /maximum intragroup distances are given on the diagonal; average/minimum distances between groups or species are shown under the diagonal.

| | <i>C. lasiura</i> | <i>C. sokolovi</i> | <i>C. zaitsevi</i> | <i>C. zaitsevi</i> | <i>C. zaitsevi</i> | <i>Crociodura</i> sp. Sapa | <i>Crociodura</i> sp. Sapa + <i>C. indochinensis</i> | <i>Crociodura</i> sp. <i>C. tanakae</i> | <i>C. tanakae</i> | <i>C. tanakae</i> | <i>C. tanakae</i> | <i>Crociodura</i> sp. Bihn Chau | <i>Crociodura</i> sp. <i>phanluongi</i> | <i>Crociodura</i> sp. Bihn Chau + <i>Crociodura</i> sp. Bihn Chau |
|--|-------------------|--------------------|--------------------|--------------------|--------------------|----------------------------|--|---|-------------------|-------------------|-------------------|---------------------------------|---|---|
| <i>C. sokolovi</i> | 12.81/12.71 | 0.15/0.30 | | | | | | | | | | | | |
| <i>C. zaitsevi</i> A | 9.21/8.71 | 13.82/13.46 | 0.18/1.50 | | | | | | | | | | | |
| <i>C. zaitsevi</i> B | 9.59/9.37 | 14.36/13.70 | 1.50/1.00 | 0.17/0.71 | | | | | | | | | | |
| <i>C. zaitsevi</i> combined | 9.37/8.71 | 14.06/13.46 | - | 0.84/2.20 | | | | | | | | | | |
| <i>Crociodura</i> sp. Sapa | 10.79/10.57 | 12.51/12.23 | 10.94/8.49 | 10.80/8.81 | 10.88/8.49 | 0.10/0.50 | | | | | | | | |
| <i>C. indochinensis</i> | 10.60/10.51 | 13.87/13.67 | 11.27/10.19 | 11.12/9.19 | 11.2/9.2 | 4.00/3.83 | 0.90/0.30 | | | | | | | |
| <i>Crociodura</i> sp. Sapa + <i>C.</i> | 10.75/10.51 | 12.75/12.23 | 11/8.49 | 10.86/8.81 | 10.94/8.49 | - | 1.28/4.28 | | | | | | | |
| <i>indochinensis</i> | | | | | | | | | | | | | | |
| <i>C. tanakae</i> A | 10.72/9.71 | 12.83/12.16 | 10.95/10.25 | 11.43/10.24 | 11.16/10.25 | 11.32/9.38 | 10.27/8.09 | 11.13/8.09 | 0.61/1.65 | | | | | |
| <i>C. tanakae</i> B | 10.64/10.57 | 13.30/13.15 | 10.18/9.94 | 10.82/9.94 | 10.46/9.94 | 11.69/11.169 | 9.63 | 11.38/9.63 | 2.35/1.98 | 0.23/0.46 | | | | |
| <i>C. tanakae</i> combined | 10.79/9.71 | 12.94/12.16 | 10.77/9.94 | 11.28/9.94 | 10.99/9.94 | 11.41/9.39 | 10.19/8.09 | 11.19/8.09 | - | 1.23/3.63 | | | | |
| <i>C. phanluongi</i> | 11.96/10.86 | 11.53/10.86 | 12.15/11.77 | 12.61/11.62 | 12.35/11.62 | 12.88/10.78 | 12.63/10.86 | 12.84/10.78 | 10.08/10.01 | 11.20/10.70 | 10.9/10.13 | 0.80/1.97 | | |
| <i>Crociodura</i> sp. Bihn Chau | 11.49/11.49 | 12.01/11.93 | 11.59/11.04 | 12.07/11.77 | 11.8/11.04 | 12.4/12.21 | 12.53/12.39 | 12.42/12.21 | 11.31/10.68 | 11.19/11.11 | 11.28/10.68 | 8.73/8.50 | - | |
| <i>C. phanluongi</i> + <i>Crociodura</i> sp. Bihn Chau | 11.88/10.86 | 11.61/10.86 | 12.06/11.04 | 12.52/11.62 | 12.26/11.04 | 12.8/10.78 | 12.62/10.86 | 12.77/10.78 | 10.89/10.13 | 11.2/10.7 | 10.96/10.13 | - | 3.45/8.89 | |
| <i>C. phanluocensis</i> | 10.41/10.41 | 12.74/12.63 | 10.35/9.55 | 10.12/8.43 | 10.25/8.43 | 11.02/10.47 | 10.21/9.94 | 10.87/9.94 | 11.47/10.87 | 11.79/11.72 | 11.55/10.87 | 11.37/11.16 | 11.11/11.11 | 11.33/11.11 |

TABLE 5. Average/maximum intragroup distances and average/minimum distances between closest haplogroups of Vietnamese *Crocidura* using the cytochrome *b* (*cytb*) gene.

| | Within group variation | Closest group in the NJ dendrogram | Average distance to the closest group (according to the NJ topology) | Minimum distance between haplotypes belonging to different groups |
|--------------------------------|------------------------|---|--|---|
| <i>C. zaitsevi</i> A | 1.60/2.44 | <i>C. zaitsevi</i> B | 2.28 | 2.03 |
| <i>C. zaitsevi</i> B | 0 | <i>C. zaitsevi</i> A | | |
| <i>C. zaitsevi</i> combined | - | <i>C. wuchichensis</i> + <i>C. indochinensis</i> + <i>Crocidura</i> sp. Sapa | 10.91 | 9.30 |
| <i>C. wuchichensis</i> | 1.31/2.54 | <i>C. indochinensis</i> + <i>Crocidura</i> sp. Sapa | 7.75 | 7.36 |
| <i>C. indochinensis</i> | 0.28 | <i>Crocidura</i> sp. Sapa | 4.09 | 4.03 |
| <i>Crocidura</i> sp. Sapa | - | <i>C. indochinensis</i> | | |
| <i>C. tanakae</i> A | 0.88/1.58 | <i>C. lasiura</i> + <i>C. dsinezumi</i> + <i>C. tadae</i> + <i>C. zaitsevi</i> + <i>C. attenuate</i> + <i>C. wuchichensis</i> + <i>C. indochinensis</i> + <i>Crocidura</i> sp. Sapa | 11.7 | 9.91 |
| <i>C. phanluongi</i> | - | <i>Crocidura</i> sp. Bihn Chau | 7.93 | 7.93 |
| <i>Crocidura</i> sp. Bihn Chau | - | <i>C. phanluongi</i> | | |
| <i>C. phuquocensis</i> | - | <i>C. dracula</i> + <i>C. fuliginosa</i> | 12.61 | 12.02 |
| <i>C. fuliginosa</i> | 1.74 | <i>C. dracula</i> | 9.28 | 9.27 |
| <i>C. dracula</i> | - | <i>C. fuliginosa</i> | | |
| <i>C. sokolovi</i> | 0.38 | all studied species except <i>C. olivieri</i> | 13.34 | 10.90 |

deeply divergent from all other Vietnamese white-toothed shrews: ~13% on average (for both *cytb* and COI). While it has been supposed that *C. zaitsevi* is endemic to Ngoc Linh Mountain (Jenkins *et al.* 2007, 2009), our data suggest this species to be more widely distributed across South Vietnam. Two haplogroups (p-dist = 2.3%, *cytb* and 1.5%, COI) were found within *C. zaitsevi*, which correspond to two geographically remote localities: haplogroup A containing specimens from the type locality (Ngoc Linh Mt., Central Vietnam), and haplogroup B found in Hon Ba Mt. and Bi Doup Mt., South Vietnam (Fig. 2–3). The haplogroup from the Phong Nha—Ke Bang area in central Vietnam is associated with haplogroup A; however, it is distinct (p-dist = 1.3%, COI) and may represent a separate subgroup. Thus, the intraspecific differentiation of *C. zaitsevi* is relatively high and comparable with that of *C. wuchihensis* and *C. tanakae*.

Crocidura indochinensis and *C. wuchihensis*

The group containing *C. indochinensis* and *C. wuchihensis* appears on the *cytb* tree with 100% bootstrap support.

Originally described from Langbian Mountain, Dalat Plateau (South Vietnam), *C. indochinensis* occurs in Myanmar, Yunnan China and Vietnam (Hutterer 2005). Our dataset includes a small series of *C. indochinensis* from the vicinity of Bi Doup Mountain, about 25 km from its type locality. These are medium-sized shrews with a relatively long tail. This species is poorly represented in collections, which led Jenkins *et al.* (2009) to suggest that it has a disjunct distribution. Aside from the Da Lat Plateau in southern Vietnam, *C. indochinensis* has also been recorded from the north of the country (Can *et al.* 2008; Jenkins *et al.* 2009), particularly in the vicinity of Sa Pa (specimen FMNH 39029 at the Field Museum of Natural History, Chicago – see Jenkins *et al.* 2009). Nonetheless, we did not find any northern members of this haplogroup, either in our collection or among GenBank sequences. On both the *cytb* and COI trees specimens from Sa Pa are separated from *C. indochinensis* by p-distances of 4.1 % (*cytb*) and 4.0% (COI). While the shrews from Bi Doup and Sa Pa may be treated as genetically divergent populations of the same species because they are nearest neighbours on mtDNA trees, morphological data support their separation as different species. In our sample, specimens from Sa Pa differ from the Bi Doup of *C. indochinensis* in

smaller size and shorter tail and thus resemble *C. wuchihensis* (sensu Jenkins *et al.* 2009, 2010). Furthermore, there are significant discrepancies in habitat preferences and altitudinal distribution of these two haplogroups (Abramov *et al.* 2008b, 2010). Thus, we suggest that specimens from Sa Pa could be specifically distinct and propose a temporary designation under *Crocidura* sp. AB1, pending a formal species description.

The haplogroup on Fig. 3 assigned to *C. wuchihensis* includes shrews from Tay Con Linh II Mountain, Ha Giang Province, northern Vietnam (see Lunde *et al.* 2003) and those from China, Guangxi, sequenced by Esselstyn & Brown (2009). At this point, no genomic data are available for *C. wuchihensis* from the type locality (Hainan Is., China); however, specimens from the most proximate mainland area (Guangxi Province) have been referred to as *C. wuchihensis* (Esselstyn & Brown 2009), which makes this name the best available for this haplogroup. The specimen MVZ186404 from Vinh Phu Province, northern Vietnam (code 6 on Fig.3) was deposited in GenBank as *C. fuliginosa* (EU122212, Meegaskumbura *et al.* 2007); however, its position within the *C. wuchihensis* haplogroup suggests an identification error, a problem not uncommon with GenBank sequence submissions (Harris, 200X). Jenkins *et al.* (2009) state that *C. wuchihensis* is widely distributed across Vietnam, including the provinces Lao Cai, Ha Giang and Lang Son in the north and Ha Tinh and Quang Nam in Central Vietnam. Our mtDNA data contradict this assertion, suggesting that the distribution of *C. wuchihensis* is restricted to areas east and north of the Red River (Fig.1). Furthermore, no genetically similar haplotypes were detected among specimens from Lao Cai Province (Van Ban and Sa Pa areas). Within northern Vietnam, *C. wuchihensis* splits into two lineages (p-distance of 2.1% for *cytb*) which probably represent distinct geographic populations.

Crocidura fuliginosa and *C. dracula* group

Crocidura fuliginosa Blyth, 1855 was among the first species of *Crocidura* documented from Vietnam (Heaney & Timm 1983). GenBank holds two complete *cytb* sequences assigned to this species from Cameron Highland, Peninsular Malaysia (codes 1, 2 on Fig. 3) and one from northern Vietnam, Ha Giang Province (code 3 on Fig. 3). Specimens from these two localities are separated by a p-distance of 9.3% for *cytb*, suggesting that they are specifically distinct. GenBank also holds several short sequences that were not included in our dendrograms. Three of them (AF003759, EF524752, EF524708) are part of the Malayan cluster while sequences from Yunnan (EF524792, EF524775) are neighbours with the specimen from northern Vietnam. Moreover, the analysis of nuclear BRCA1 and ApoB genes (Dubey *et al.* 2008) also supports the distinction between *C. fuliginosa* of Peninsular Malaysia and the specimens from Yunnan which are named there as *C. dracula*. The name *C. dracula* was used to define shrews from South China (Ellerman & Morrison-Scott 1951), until Jenkins (1976) proposed it to be a subspecies of *C. fuliginosa*—a notion later followed by others (Heaney & Timm 1983; Jiang & Hoffmann 2001; Hutterer 2005). Comparison of mtDNA data suggests that specimens from northern Vietnam and southern China (close to the type locality of *C. dracula*) belong to the same haplogroup, which is deeply divergent from Malayan specimens. *Crocidura fuliginosa* was described from Shwegyin (=Schwegyin), central Burma (Corbet and Hill 1992), so biogeographically it is unclear whether this name can be applied to the Malayan haplogroup. Recognising that this question requires resolution, we provisionally apply the name “*fuliginosa*” to shrews from the southern part of Southeast Asia, including Malaysia and South Myanmar. Following the above interpretation, the occurrence of *C. fuliginosa* s. str. in Vietnam, to this end, has not been confirmed genetically. In their latest review of Southeast Asian white-toothed shrews, Jenkins *et al.* (2009) provide records of *C. fuliginosa* only from northern Vietnam and southern China. The record of *C. fuliginosa* (*s.lato*) from Kon Tum and Lam Dong Provinces in Central Vietnam (Can *et al.* 2008) is not supported by collection material. It is still possible that *C. fuliginosa* s.str extends its range into southern Vietnam. A ZMMU specimen S-144368 from Bai Canh Is. off the coast of southern Vietnam was identified as *C. fuliginosa* (Paulina Jenkins, pers. comm., 2009). Furthermore, Van Peenen *et al.* (1970) recorded a specimen of large shrew from the neighbouring island of Con Son (Con Dao Archipelago), identified as *C. fuliginosa* (USNM 357348, Smithsonian Institution).

Comparison of *cytb* and COI sequences obtained from *C. phuquocensis* with available reference data corroborate the specific status of this taxon recently described from Phu Quoc Island (Abramov *et al.* 2008a). On the *cytb* tree, this haplogroup is the nearest neighbour to the *C. fuliginosa*–*C. dracula* group, although with low bootstrap support.

Crocidura phanluongi

C. phanluongi is a species recently described from the lowlands of southern Vietnam and north-eastern Cambodia (Jenkins *et al.* 2010). Our *cytb* and COI data for the type series of *C. phanluongi* from Yok Don and Bu Gia

Map provide genetic support for its species status. The specimen ZIN 97089 from Binh Chau, Ba Ria—Vung Tau Province, southern Vietnam, which was also referred to *C. phanluongi* by Jenkins *et al.* (2010), is deeply divergent from its nearest neighbours collected in Yok Don (p-distance=8.4% for *cytb* and 8.7% for COI), suggesting that it may represent a separate, undescribed species, provisionally defined here as *Crocidura* sp. AB2. The corresponding voucher specimen from Binh Chau also has a slightly shorter tail (61% of head and body length), compared to *C. phanluongi* from other localities (68–83%; see Jenkins *et al.* 2010).

Crocidura attenuata and *Crocidura tanakae*

The genetic analysis presented here of the most common and widely distributed Vietnamese shrew *C. attenuata* revealed an unexpected and previously undocumented pattern. Comparison of original *cytb* sequence information with GenBank data suggests that specimens from Vietnam are genetically similar to *C. tanakae* described from Taiwan. Because *C. tanakae* is currently recognized as a separate species (Motokawa *et al.* 1997, 2001; Hutterer 2005), our results call for the reappraisal of the taxonomic status of Vietnamese shrews previously referred to as *C. attenuata*. Until this is done, we propose to apply the name *C. tanakae* to the specimens that are genetically similar to the Taiwanese haplogroup. Such specimens have been collected across Vietnam, suggesting broad geographic distribution of this species on the mainland. In contrast, *C. attenuata* proper appears to be found only in the northernmost part of Vietnam (Ha Giang Province; Tay Con Linh II Mountain). Similar to *C. wuchihensis* (in its present interpretation), it is restricted to areas to the north and east of the Red River (Fig. 1) and is replaced by *C. tanakae* elsewhere.

Our relatively limited *cytb* sample shows little structuring among Chinese and Southeast Asian *C. tanakae*; however, the broader COI data demonstrates two clearly defined haplogroups within Vietnam (p-dist = 2.3%): one of them (A, Fig. 1) occurs in Central and South Vietnam (Huong Hoa, Phong Nha—Ke Bang, Ngoc Linh, Hon Ba, Bi Doup), while the other (B, Fig. 1) is restricted to the northern part of the country (Hoang Lien Mountains, Van Ban District).

Other *Crocidura* species

Three available GenBank sequences of *C. horsfieldii* included in the present analysis suggest that the sequence AB175078 of a specimen from Thailand forms a highly supported cluster with *C. watasei* but not with specimens of *C. horsfieldii* from Sri Lanka and India. Since the name *C. horsfieldii* Tomes, 1856 was described from Sri Lanka (Corbet & Hill 1992), we find it hardly applicable to the Thai specimen referred to *C. horsfieldii* in Dubey *et al.* (2008), Esselstyn *et al.* (2009), and Esselstyn & Brown (2009). We propose that it should be regarded as a yet unidentified species, provisionally named *Crocidura* sp. AB3.

The inclusion of available *Crocidura cytb* sequences from GenBank in our study revealed a disturbing trend, colloquially referred to as the “taxonomic impediment” (Carvalho *et al.* 2007). Over time, more and more DNA sequences are submitted to GenBank that are attributed to species names that appear to be assigned without proper taxonomic verification. At the other extreme, a growing pool of publications highlights the significant increase in the number of species of *Crocidura* described from Southeast Asia since Ruedi’s (1996) work—both within the region on the whole (Dubey *et al.* 2008; Esselstyn *et al.* 2009) and within Vietnam in particular (Lunde *et al.* 2003, 2004; Jenkins *et al.* 2007, 2009, 2010; Abramov *et al.* 2008a). The discovery of more new species in this area is very likely. Several recently described species of *Crocidura* reported from Vietnam based on morphological data are known only from type localities (*C. rapax*, *C. kegoensis*, *C. annamitensis*, and *C. guy*) with no published sequence data available for them, causing further confusion. The data standards associated with barcoding (Hanner 2009) represent a significant improvement to the status quo, particularly if new species descriptions include barcodes, a move that could help to disambiguate the application of names going forward.

As an example, several specimens from Gia Lai and Lam Dong provinces in southern Vietnam formerly reported as *C. indochinensis* (Heaney & Timm 1983) were recently re-identified as *C. rapax* (Jenkins *et al.* 2009). However, a targeted study of a large series (96 specimens) of shrews from the Dalat Plateau, Lam Dong Province (Abramov *et al.* 2010) did not reveal any individuals matching the morphological diagnosis of this species. The question remains open whether this was due to the actual differences in the species diversity sampled or due to the differential interpretation of morphological diagnoses.

While our pilot study of Southeast Asian *Crocidura* is insufficient to draw phylogenetic conclusions or to make a substantial taxonomic revision of this genus, it highlights some key points related to our assessment of the

alpha-taxonomic diversity and species diagnostics of its Vietnamese members. In particular, this study characterized the genetic profiles of several recently described species: *C. sokolovi*, *C. zaitsevi*, *C. phuquocensis*, and *C. phanluongi*. It also suggested the existence of two possibly undescribed species: *Crocidura* sp. AAB1 from Sa Pa and *Crocidura* sp. AB2 from Binh Chau. Finally, it helped formulate working hypotheses related to the taxonomic status and distribution patterns of species groups *C. attenuata/C. tanakae*, *C. fuliginosa/C. dracula*, and *C. indo-chinensis/C. wuchihensis*. These questions and others, such as the taxonomic status of the “northern” and “southern” forms of *C. tanakae* and *C. zaitsevi* are in need of further research.

This study underpins the need for an integrative approach towards the systematics and diagnostics of speciose and morphologically cryptic taxa, such as the genus *Crocidura*. Further progress would be aided by a more in-depth phylogenetic study, one involving not only mitochondrial, but also nuclear markers, a suite of morphological and karyological characters, as well as ecological peculiarities and distributional patterns. We hope that the approach used here and the presented data will contribute towards building a more comprehensive picture of the taxonomic and phylogeographic diversity of Southeast Asian white-toothed shrews.

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APPENDIX

The list of non-Vietnamese specimens used in DNA barcoding and sequences retrieved from GenBank.

COI

Crocidura russula: AY332675, AY332676, AY769263, AY769264, NC006893. *C. shantungensis*: SKMZM500 08-SKMZM503 08, SKMZM494 08-SKMZM496 08, SKMZM498 08. *C. suaveolens*: SKMZM490 08, SKMZM493 08, SKMZM802 09, SKMZM804 09, SKMZM805 09, SKMZM807 09, SKMZM808 09, SKMZM811 09, SKMZM814 09, SKMZM816 09, SKMZM817 09, SKMZM486 08-SKMZM488 08. *C. leucodon*: SKMZM800 09, SKMZM801 09, SKMZM803 09, SKMZM812 09, SKMZM813 09. *C. lasiura*: SKMZM504 08. *C. olivieri*: SKMZM365 08, SKMZM366 08. *C. macmillani*: SKMZM371 08, SKMZM372 08.

Cytb

S. murinus: DQ630386, DQ630390. *Suncus stoliczkanus*: AB175077. *Crocidura orii*: AB175087. *C. russula*: AY769263, AY769264. *C. shantungensis*: AB077082, AB077078, EU742592-EU742594, EU742584-EU742585, EU742589, EU742590, EU742586, EU742587, EU742589. *C. suaveolens*: AB077280, AB077288, EU742613, AY994379. *C. gueldenstaedtii*: AY994376. *C. sibirica*: EU742583, AB077279. *C. caspica*: AY994370. *C. zarudnyi*: AY925211. *C. lasiura*: AB077071, AB077072. *C. leucodon*: DQ065609. *C. olivieri*: EU742597. *C. macmillani*: EU742601. *Crocidura* cf. *lucina*: EU742603. *C. tadar kurodai*: AB057420, AB062686, AB115557. *Crocidura* sp.1: FJ814025. *Crocidura* sp.2: FJ814026. *Crocidura* sp.3: FJ814027. *C. maxi*: FJ814024. *C. dsinezumi*: AB077274, AB077277. *C. batakorum*: FJ813972-FJ813974, FJ813976, FJ813977. *C. musseri*: FJ813927, FJ813929. *C. lasiura*: AB077071-AB077072. *C. maxi*: FJ814024. *C. watasei*: ABO77074. *Crocidura* sp.AB3: AB175078. *C. brunnea*: DQ630385. FJ814030. *C. orientalis*: FJ814029. *C. lepidura*: FJ814023, FJ814022. *C. nigripes*: DQ630384, FJ13926, FJ13928. *C. malayana*: DQ630381. *C. foetida*: FJ814053-FJ814055. *C. grayi*: FJ814051, FJ814049, FJ813900, FJ813967, FJ813942, FJ813830, FJ813865, FJ813867, FJ813880, FJ813930, FJ813872, FJ813839, FJ813898, FJ813895, FJ813856, FJ813848, FJ813857. *C. negrina*: FJ813961, FJ813962, FJ813951, FJ813952. *C. panayensi*: FJ813944-FJ813947. *C. mindorus*: FJ813940-FJ813943. *C. beatus*: FJ813980, FJ813884, FJ813844, FJ813847, FJ813838, FJ813837, FJ813879, FJ813846, FJ814019, FJ814010, FJ814015. *C. palawanensis*: FJ813978, FJ813902, FJ813915, FJ813912. *C. horsfieldii*: EU122213, FJ814028. *C. hik-miya*: EU122219-21, EU122217, EU122218, EU122222, EU122223. *C. miya*: EU122214-EU122216.