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Taxonomic study of the water shrews *Chimarrogale himalayica* and *C. platycephala*

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We investigated karyotypes, mitochondrial cytochrome *b* gene sequences, and cranial morphometrics of the water shrews *Chimarrogale himalayica* (Gray, 1842) and *C. platycephala* (Temminck, 1842) (Insectivora, Soricidae). Karyotypes of *C. himalayica* from Taiwan and *C. platycephala* are 2n = 52 and FNa = 100. Autosomes consisted of 21 large-to-small metacentric or submetacentric pairs, and 4 medium-to-small subtelocentric pairs. The X and Y chromosomes were medium submetacentric and small acrocentric, respectively. The karyotypes of *C. himalayica* and *C. platycephala* were very similar. Secondary constrictions were observed in the largest metacentric pair in both species. In the 930 base-pairs of the cytochrome *b* gene, *C. himalayica* from Taiwan and *C. platycephala* diverged with 9.46% sequence difference; each species diverged from *C. phaeura* with more than 14% sequence difference. The two species *C. himalayica* and *C. platycephala* were well distinguished by morphometric characters, but three subspecies of *C. himalayica* were not clearly separated. We suggest that *C. platycephala* be treated as a valid species and separated from *C. himalayica* in Taiwan.

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Introduction

Chimarrogale Anderson, 1877, is a genus of water shrew belonging to the tribe Neomyini in the subfamily Soricinae of the family Soricidae. This genus consists of 6 species distributed in southeast and east Asia (Hutterer 1993, Wolsan and Hutterer 1998): *C. himalayica* (Gray, 1842), *C. platycephala* (Temminck, 1842), *C. hantu* Harrison, 1958, *C. phaeura* Thomas, 1898, *C. styani* de Winton, 1899, and *C. sumatrana*

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(Thomas, 1921). *Chimarrogale sumatrana* and *C. hantu*, however, were synonymized with *C. phaeura* as allopatric populations by Corbet and Hill (1992), who validated only 4 species. Within the *Chimarrogale* species, *C. himalayica* has the widest distribution from Kashmir, through the Himalayas, Laos, Vietnam and eastern China mainland, and Taiwan (Hutterer 1993). Hoffmann (1987) recognized 3 subspecies within *C. himalayica*: *C. h. himalayica* in the Himalayas, *C. h. varennei* Thomas, 1927 in Burma, Yunnan, Laos, and Vietnam, and *C. h. leander* Thomas, 1902 in the southeastern China and Taiwan.

Hoffmann (1987) considered *C. platycephala* from Japan as a separate species and an insular allospecies of the superspecies *himalayica*. Thereafter, *C. platycephala* was considered a valid species by most authors (Corbet and Hill 1992, Hutterer 1993, Abe 1996, 1999, Wolsan and Hutterer 1998, Koyasu 1998), but it has been and still is considered conspecific with *C. himalayica* by others (Obara *et al*. 1996). Abe (1996) suggested that *C. platycephala* be recognized as a valid species due to uncertain priority between Temminck's (1842) *platycephala* and Gray's (1842) *himalayica* (see also Ellerman and Morrison-Scott 1951, Corbet 1978, Koyasu 1998), even though the comparison in systematic status between *C. platycephala* and *C. himalayica* was incomplete. This priority problem is not yet resolved, as both of the publication date of Temminck (1842) and Gray (1842) are adopted as 31 December 1842 according to the International Commission on Zoological Nomenclature (1999) Article 21 and the available date information for both publications (Ellerman and Morrison-Scott 1951, Holthuis and Sakai 1970).

Except for that of Hoffmann (1987), comparative taxonomic studies for the *Chimarrogale* species have been scarce. Karyological studies were made only for *C. platycephala* (Obara and Tada 1985, Satoh and Obara 1995, Obara *et al*. 1996). Therefore, comparative studies for *C. himalayica* and *C. platycephala* can clarify the relationships between these 2 taxa. To make an interspecific comparison, we examined the karyotype of *C. himalayica* for the first time, based on specimens from Taiwan, and that of *C. platycephala* from Japan. We also compared the mitochondrial cytochrome *b* gene sequences and morphometric characters of *C. himalayica* and *C. platycephala*.

Material and methods

Twenty specimens of *C. himalayica* and 8 specimens of *C. platycephala* were examined. They are deposited in the Natural History Museum, London (BMNH), Department of Biology, Tunghai University, Taichung (THU), Osaka City University Graduate School of Medicine, Osaka (MH), and Kyoto University Museum, Kyoto (KUZ). We used most specimens for morphometric analysis; 2 specimens of *C. himalayica* from Taiwan and 2 of *C. platycephala* for karyological analysis; and 4 *C. himalayica* from Taiwan and 1 *C. platycephala* for the mitochondrial cytochrome *b* gene sequence analysis. We followed the subspecies classification of Hoffmann (1987) in morphometric analysis to investigate intraspecific variation within *C. himalayica*. A list of specimens used follows (unless otherwise stated examined only for morphometric analysis): *C. himalayica leander*: THU Lala-1 (morphometric and DNA analyses), Lala-2 (morphometric, karyological, and DNA analyses), Lala-3 (morphometric and DNA analyses), Lala-4 (morphometric, karyological, and DNA analyses) from Lala River, Fusheng Township, Taoyuan County, Taiwan (24 41'47.9"N and 121°23'53.8"E, 800 m in altitude); KUZ M3409, M3410 from Wulien, Houpen Township, Taichung County, Taiwan; BMNH 2.6.10.3 (holotype of *C. h. leander*) from Kuatun, Fokien (= Fujian), China; *C. h. varennei*: BMNH 26.10.4.44 (holotype of *C. h. varennei*) from Dak-to, Annan, Vietnam; BMNH 33.4.1.167 from Chapa, Vietnam; BMNH 32.11.1.14, 50.494, -495 from Nam Tamai, Kachin District, Burma; BMNH 50.496 from Waurayang, Kachin District, Burma; BMNH 76.1232 from Hkinlum Triangle, Kachin District, Burma; *C. h. himalayica*: BMNH 42.2.18.1 (holotype of *C. h. himalayica*) from Himalayas; BMNH 8.7.6.17 from Liddar River, Kashmir; BMNH 35.10.10.2 from Dhodi Tal, Gharwal; BMNH 90.1.1.18 from Darjeeling; BMNH 90.1.1.4 from Sikkim; BMNH 20.6.6.5 from Pangth, Naga Hills, Assam; *C. platycephala* from Japan: BMNH 5.1.4.36 from Nikko; BMNH 98.1.2.10 from Tokyo; BMNH 6.1.4.46 from Tajima, east coast of Izu Peninsula; KUZ M3408 from Miyazu City, Kyoto Prefecture; KUZ M3413 from Kawakami Village, Yoshino County, Nara Prefecture; MH8493 from Yamasaki Town, Shisou County, Hyogo Prefecture; MH5699 (karyological analysis only) from Kozagawa Town, Higashimuro County, Wakayama Prefecture; MH8201 (karyological and DNA analyses) from Hashimoto City, Wakayama Prefecture.

The cytological preparations were made from tail tissue culture cells as described in Harada and Yosida (1978) by using the standard air drying method. Differential staining of G-band and C-band techniques followed Seabright (1971) and Sumner (1972), respectively.

Total DNA was purified from liver and muscle tissues according to the method of Sambrook *et al.* (1989). Based on the complete mitochondrial DNA sequence of *Soriculus fumidus* published by Lin *et al.* (2002), two primers were newly

designed for amplification of mitochondrial cytochrome *b* gene, L14182: 5'-CATCGTTGTTATTCAACTATAGGAAC-3' and H15445: 5'-GAATATCAGCTTGGGTGTTGATA-3'. Polymerase chain reaction (PCR) mixture of 50 µl contained approximately 50 ng of genomic DNA, 7.5 pmol of each primer and 0.2 mM of dNTP mix in reaction buffer including 2.0 mM Tris-HCl (pH 8.0), 0.01 mM EDTA, 0.1 mM DDT, 0.1% Triton X-100, 5% glycerol, and 1U of Taq DNA polymerase (Viogene). Amplifications were performed in a GeneAmp PCR System 2400 thermal cycler employing 35 cycles as follows: denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute, and extension at 72°C for 2 minutes. The final extension was 72°C for 10 minutes. Approximately 100 ng of purified PCR product was used for sequencing with PCR primer L14182 and another internal primer:
5'-GTAATAGCCACCGCCTTTATAGG-3'. Additionally, two internal primers were also designed to get complete sequence of cytochrome *b* gene:
5'-CTAGTCCTGTTCTCCCCAGACC-3' and
5'-CCTATAAAGGCGGGTGGCTATTAC-3'. Sequences were determined by automated sequencer (ABI 3730 Genetic Analyzer).

The partial sequences (930 base pairs) of central portion of cytochrome *b* gene were aligned with Clustal W in BioEdit 5.0.9 (Hall 1999) and corrected by eye. Sequence data are deposited in GenBank with accession numbers AY526739-AY526743. Phylogenetic analyses were conducted by neighbor-joining (NJ) method and genetic distances were calculated by the Kimura's (1980) two-parameter model using the MEGA program version 2.1 (Kimura *et al.* 2001). Confidence limits in estimated relationships were determined using 1000 bootstrap pseudoreplicates (Felsenstein 1985). We also analyzed one *C. phaeura* from Mahua, Sabah in Borneo (MH 8426, AY526744) and one *Anourosorex yamashinai* Kuroda, 1935 (species taxonomy following Motokawa *et al.* 2004), a member of the tribe Neomyini (see Repenning 1967) from Taiwan (THU H1, AY575067) for outgroup.

Nineteen cranial measurements were taken with digital calipers to the nearest 0.01 mm by the first author: condylo-incisive length (CIL); breadth of braincase (BB); interorbital breadth (IOB); rostral length (RL); postpalatal depth (PPD); braincase depth excluding auditory ring (BD); rostral breadth (RB); palatal length from the anterior surface of the 1st incisor to the most posterior portion of the palate (PL); postpalatal length (PPL); condyle to glenoid length (CG); upper toothrow length (UIM); length of upper molariform teeth (UPM); greatest width between M2s (M2W); mandibular length (ML); mandibular height (MH); lower toothrow length (LIM); lower toothrow length between canine and 3rd molar (LCM); lower molar row length (LM); and greatest length of mandibular condyle (CD). Of these, 11 variables (CIL, BB, IOB, RL, PPD, RB, PPL, CG, UIM, UPM, and M2W) follow the criterion of Ruedi (1995) and 4 (ML, MH, LIM, and LM) follow that of Motokawa *et al.* (1996). The remaining 4 variables (BD, PL, LCM, and CD) were taken as mentioned above. To identify variation, principal component analyses (PCA) was conducted with the PRINCOMP procedure of SAS version 6 (SAS 1990) using the correlation matrix of cranial log-transformed measurements. We made two analyses by using either all 19 variables or 6 variables taken from mandibles (ML, MH, LIM, LCM, LM, and CD). The latter analysis was intended to improve sample size of specimens in this study and to infer the variation pattern of broken or incomplete specimens.

Results

The autosomes of *C. himalayica* from Taiwan (Fig. 1) consisted of 21 large-to-small metacentric or submetacentric pairs gradually decreasing in size (nos. 1–21) and four medium-to-small subtelocentric pairs (nos. 22–25). The X chromosome was medium submetacentric and the Y chromosome was small acrocentric. Therefore, the 2n and FNa were 52 and 100, respectively. In the largest metacentric pair (no. 1), a distinct secondary constriction was observed on the short arm near the centromeric region (indicated with arrowheads in Fig. 1), a portion not stained in G-band and C-band karyotypes. In C-band karyotype, centromeric regions of all chromosome pairs, the distal part of the short arm of no. 1 chromosomes, and whole of Y chromosome were well stained. Conventional, G-band, and C-band karyotypes of *C. himalayica* resembled those for *C. platycephala* from Japan (Fig. 2). We could not find any difference. The size heteromorphism of the short arms of no. 1 chromosome pair (C-band positive regions) was found in 1 of 2 *C. platycephala* (MH 8201), but was not so evident in both individuals of *C. himalayica*.

In the mitochondrial cytochrome *b* gene analysis (930 bp), 2 haplotypes were observed from 4 *C. himalayica* specimens from Taiwan, which differed by 2 base-pairs (0.22% difference). Both of these *C. himalayica* haplotypes differed from the *C. platycephala* haplotype in 88 base-pairs (9.46% difference). They differed from *C. phaeura* in 136 base-pairs (14.6% difference). The *C. platycephala* haplotype differed from that of *C. phaeura* in 137 base-pairs (14.7% difference). In the neighbor-joining tree (Fig. 3), *C. himalayica* haplotypes were more closely related to *C. platycephala* before being clustered with *C. phaeura*. Each node was supported with high bootstrap values.

The external measurements (average and range) of four fresh Taiwan *C. himalayica* speci-

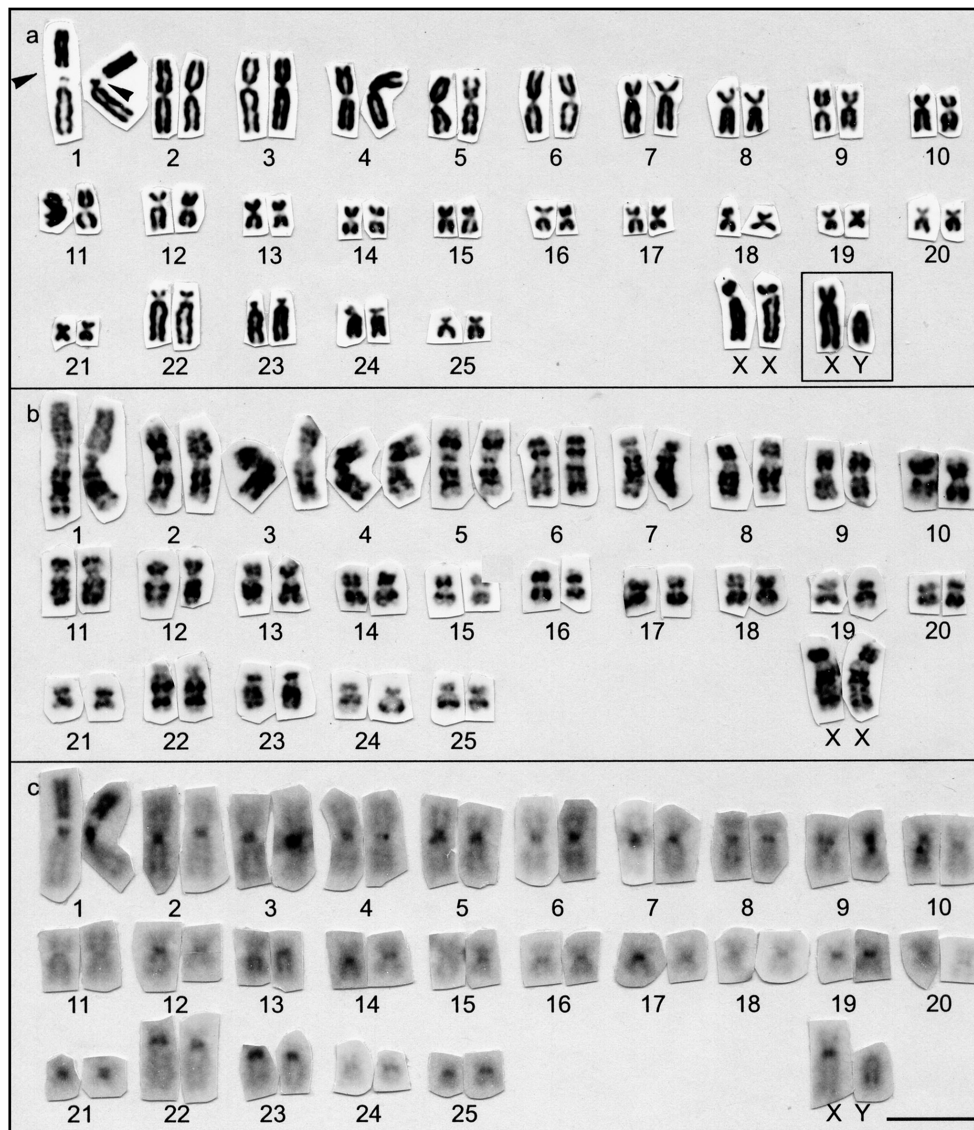

Fig. 1. Conventional (a), G-band (b), and C-band (c) karyotypes of *Chimarrogale himalayica* from Taiwan (THU Lala-2 for a and b; and THU Lala-4 for squared XY chromosome in a and c). Arrowheads indicate the secondary constrictions. The bar at bottom right represents 10 µm.

mens (THU Lala-1, -2, -3, -4) were: body mass (in grams) 28.65 (23.8–36.0), head and body length (in mm) 94.50 (83.37–106.00), and tail length 97.44 (89.15–108.86). The cranial measurements of *C. platycephala* were much larger than those for any subspecies of *C. himalayica*. Within the latter species, cranial measurements of *C. h. leander* from Taiwan were somewhat smaller than those of *C. h. varennei* and *C. h. himalayica* (Table 1).

In PCA using all variables, the first and second principal component axes explained 80.7 and 8.3% of the total variation, respectively. In the first axis, all variables showed similar positive loading between 0.174 and 0.250. In the second axis, BD (0.467) and LIM (-0.352) showed relatively large loading (for others, CIL -0.049, BB 0.328, IOB 0.167, RL -0.077, PPD 0.315, RB 0.216, PL -0.130, PPL -0.004, CG 0.175, UIM -0.165, UPM -0.239, M2W -0.009, ML -0.137, MH

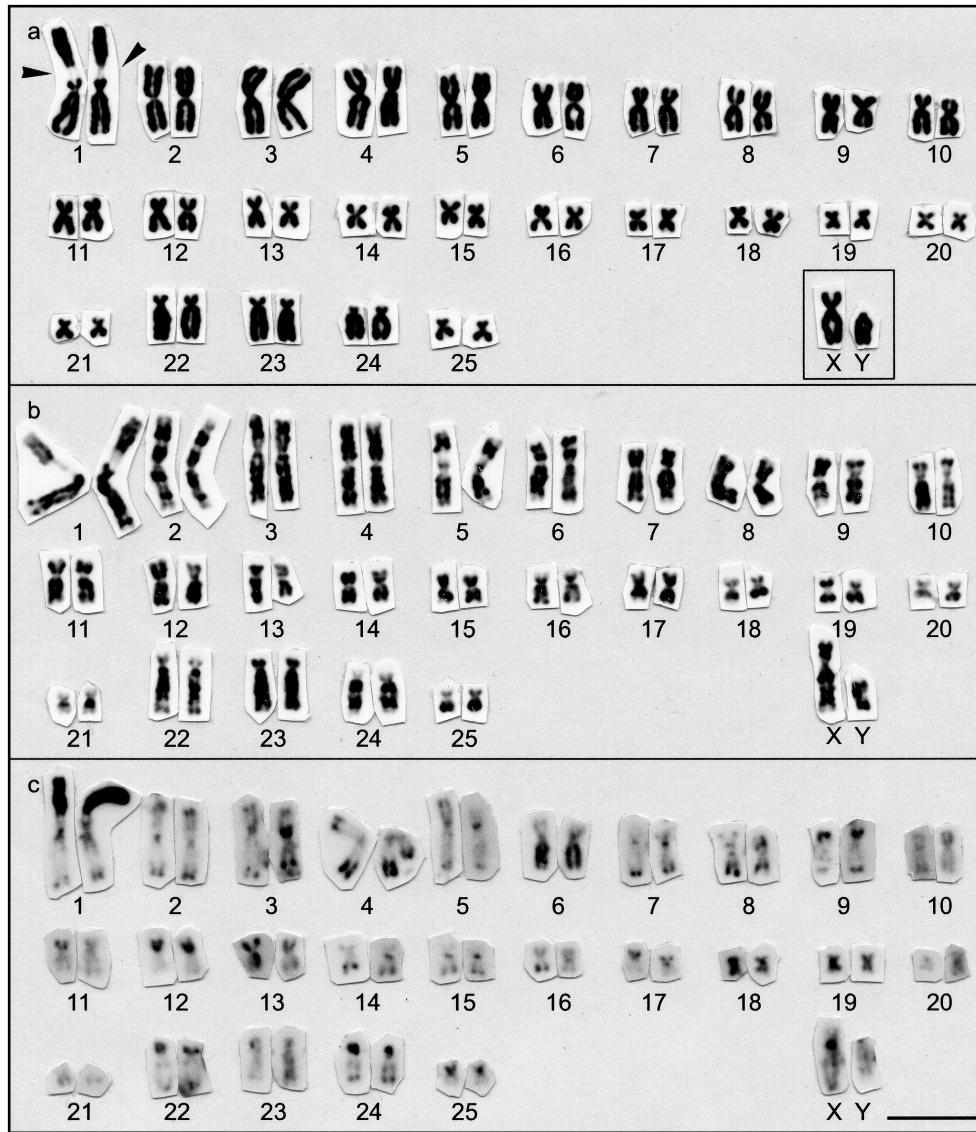

Fig. 2. Conventional (a), G-band (b), and C-band (c) karyotypes of *Chimarrogale platycephala* from Japan (MH 8201 for autosomes of a, MH 5699 for b and c, and squared XY chromosomes of a). Arrowheads indicate the secondary constrictions. The bar at bottom right represents 10 μm.

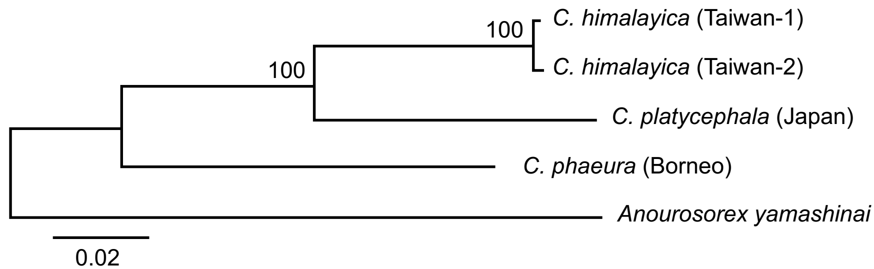

Fig. 3. The neighbor-joining tree for *Chimarrogale* species inferred from the mitochondrial cytochrome *b* gene sequences (930 base-pairs). *Anourosorex squamipes* was used as an outgroup. Nodal values indicate percent support for branches in 1000 bootstrap replications.

Table 1. Cranial measurements (mm) of *Chimarrogale himalayica* and *C. platycephala*. Values given are mean (range) and sample size. For *C. h. leander*, sample size was 6 (Taiwan) and 1 (Fujian). [1] Variables follow the criteria of Ruedi (1995), [2] excluding auditory ring, [3] from the anterior surface of the first incisor to the posterior portion of the palate, [4] variables follow that of Motokawa *et al*. (1996), [5] length between canine and third molar, [6] greatest length, [7] mandible characters used for principal component analysis.

Character	Acronym	*C. h. leander*		*C. h. varennei*		*C. h. himalayica*		*C. platycephala*	
		Taiwan Mean (range)	Fujian Mean	Mean (range)	*n*	Mean (range)	*n*	Mean (range)	*n*
Condylo-incisive length[1]	CIL	25.77 (23.85–26.71)	25.05	26.28 (25.37–27.16)	6	27.25 (26.73–27.93)	3	28.33 (26.75–29.20)	4
Braincase breadth[1]	BB	13.35 (12.07–13.98)	–	13.20 (13.06–13.35)	5	13.45 (13.28–13.68)	3	15.16 (14.33–15.62)	4
Interorbital breadth[1]	IOB	5.82 (5.37–6.07)	5.59	5.75 (5.58–6.08)	6	6.09 (6.00–6.21)	5	6.55 (6.27–6.80)	5
Rostral length[1]	RL	10.78 (9.87–11.32)	10.38	11.10 (10.4–11.66)	7	11.49 (11.02–11.88)	5	12.00 (11.16–12.42)	6
Postpalatal depth[1]	PPD	4.69 (4.21–4.98)	4.55	4.50 (4.25–4.79)	7	4.67 (4.4–4.96)	5	5.24 (4.88–5.40)	6
Braincase depth[2]	BD	6.92 (6.32–7.24)	6.51	6.66 (6.47–6.86)	5	6.65 (6.38–7.14)	3	7.48 (6.99–7.76)	4
Rostral breadth[1]	RB	3.63 (3.28–3.85)	3.58	3.54 (3.47–3.75)	6	3.71 (3.43–3.82)	5	4.06 (3.82–4.37)	6
Palatal length[3]	PL	12.24 (11.48–12.90)	11.96	12.48 (11.78–13.11)	7	13.03 (12.4–13.34)	5	13.34 (12.40–14.00)	6
Postpalatal length[1]	PPL	11.01 (10.08–12.59)	10.07	11.01 (10.33–11.81)	6	11.20 (11.09–11.28)	3	11.69 (11.31–12.17)	4
Condyle to glenoid length[1]	CG	11.11 (9.97–11.88)	10.72	11.20 (11.01–11.42)	5	11.36 (10.94–11.77)	3	12.06 (11.45–12.29)	4
Upper toothrow length[1]	UIM	11.60 (10.85–12.27)	11.16	11.88 (11.07–12.40)	7	12.47 (12.18–12.75)	4	12.76 (11.94–13.44)	6
Upper molariform teeth length[1]	UPM	6.79 (6.46–7.13)	6.31	6.79 (6.32–7.11)	7	7.19 (6.95–7.40)	4	7.29 (7.02–7.52)	6
Greatest width between M2s[1]	M2W	7.85 (7.23–8.29)	7.68	7.88 (7.38–8.33)	7	8.23 (7.85–8.56)	4	8.89 (8.43–9.30)	6
Mandible length[4, 7]	ML	16.22 (15.50–16.89)	15.85	16.63 (15.73–17.74)	7	17.19 (16.28–17.76)	5	17.85 (16.78–18.38)	6
Mandible height[4, 7]	MH	5.90 (5.22–6.24)	6.01	6.09 (5.63–6.28)	7	6.38 (5.92–6.91)	5	6.71 (6.42–7.18)	6
Lower toothrow length[4, 7]	LIM	10.48 (10.09–10.86)	10.36	10.86 (9.93–11.41)	7	11.17 (10.73–11.62)	5	11.51 (11.18–12.02)	6
Lower toothrow[5, 7]	LCM	7.60 (7.33–7.81)	7.44	7.84 (7.17–8.25)	7	8.07 (7.75–8.4)	5	8.33 (8.11–8.65)	6
Lower molar row length[4, 7]	LM	5.50 (5.29–5.60)	5.22	5.51 (5.07–5.84)	7	5.70 (5.52–5.95)	5	5.92 (5.73–6.13)	6
Condyle[6, 7]	CD	4.12 (3.69–4.34)	4.17	4.10 (3.61–4.40)	7	4.27 (4.08–4.38)	5	4.94 (4.50–5.24)	6

0.003, LCM -0.325, LM -0.253, and CD 0.219). In the first axis, *C. platycephala* had larger value than *C. himalayica* subspecies. In the second axis, the values were large for *C. platycephala*, *C. h. leander*, and *C. h. varennei*. Two dimensional plots (Fig. 4a) show *C. platycephala* well differed from the 3 subspecies of *C. himalayica*. There was no overlapping between *C. h. leander* from Taiwan and *C. h. himalayica*. In PCA using mandible variables, the first and second princi-

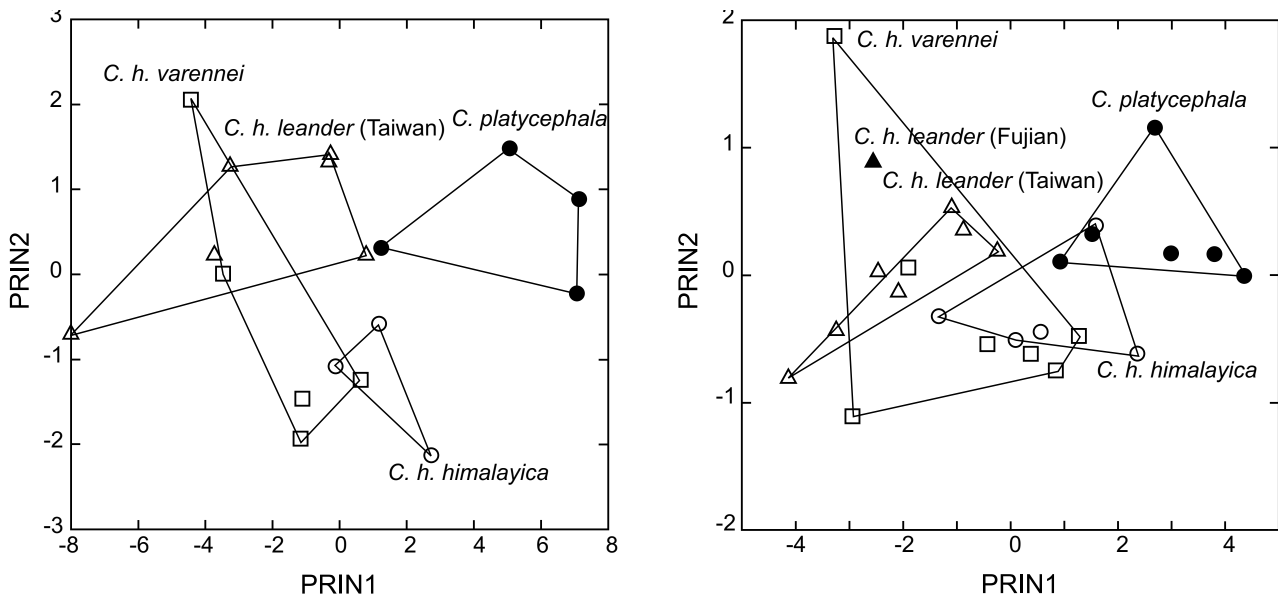

Fig. 4. Scatter plots of the scores on the first and second principal component axes based on all (a) and 6 selected (b) cranial characters of *Chimarrogale himalayica* and *C. platycephala*.

pal component axes explained 86.4 and 7.5% of the total variation, respectively. In the first axis, all variables showed similar positive loading between 0.381 and 0.427. In the second axis, CD (0.624) and MH (0.466) showed relatively large loading (for others, ML 0.069, LIM -0.321, LCM -0.377, and LM -0.380). In the first axis, the *C. platycephala* had a larger value than the *C. himalayica* subspecies. In the second axis, *C. h. leander* and *C. platycephala* were larger than *C. h. himalayica*. Two dimensional plots (Fig. 4b) show no overlap between *C. h. leander* and *C. h. himalayica*.

Discussion

Chimarrogale himalayica from Taiwan was first described by Jones and Mumford (1971), and then by Arai *et al*. (1985). After this there were no additional reports for *Chimarrogale* captured from Taiwan. Our 4 specimens from the Lala River were collected in 2003.

Karyotypes of *C. himalayica* and *C. platycephala* are similar to each other, except for the size heteromorphism of the short arm of no. 1 chromosome pair in *C. platycephala*. This was already reported by Obara and Tada (1985), Satoh and Obara (1995), and Obara *et al*. (1996). This size heteromorphism was observed in one of two individuals of *C. platycephala*, but not evident in the two *C. himalayica* specimens. Because of limits in sample size, we cannot conclude whether this heteromorphism indicated individual variation within *C. platycephala*, or distinguishes between *C. platycephala* and *C. himalayica*. The large FNa values of *C. himalayica* and *C. platycephala* (FNa=100) are characteristics of the tribe Neomyini as discussed by Motokawa *et al*. (1998).

In contrast to the similarity of karyotypes, mitochondrial cytochrome *b* gene sequences diverged between *C. himalayica* and *C. platycephala*. There was 10% sequence differences, and these 2 species were more closely related to each other than to *C. phaeura* in Borneo. Based on Bradley and Baker (2001), this large cytochrome *b* gene divergence would be very unusual between conspecific populations of mammals. Therefore, we support the view that *C. himalayica* and *C. platycephala* represent separate species. Hoffmann (1987) considered both *C. platycephala* and *C. phaeura* (including *hantu* and *sumatrana* as junior synonyms) as super-

species of *himalayica*. The present results indicate that this superspecies if valid involves as much as 15% of the sequence differences in the cytochrome *b* gene. The *himalayica* superspecies concept should also be reevaluated in the light of our molecular results.

Overall cranial size distinguished between *C. himalayica* and *C. platycephala*, and also supports species status. Hoffmann (1987) reported that the subspecies *C. h. leander* from southeastern China and Taiwan is characterized by its small size compared to other *C. himalayica* subspecies. Our morphometric analysis suggests the occurrence of extensive intraspecific variation within *C. himalayica*. The average size of the Taiwan shrew was slightly smaller than those for *C. h. himalayica* and *C. h. varennei*. However, the first author examined the nonmetric characters of *C. himalayica* specimens including holotypes of *himalayica*, *leander*, *varennei* and of its congeners (10 BMNH specimens of *C. hantu*, *C. phaeura*, *C. sumatrana*, and *C. styani* including their holotypes), and he could not find any discrete differences to validate any of subspecies within *C. himalayica*. To clarify the subspecies systematics of *C. himalayica*, we recommend further study of continental populations using karyological and genetic techniques.

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