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Allozymic Variation in *Rana nigrovittata* (Amphibia: Anura) within Thailand with Special Reference to the Taxonomic Status of *R. mortenseni*

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ABSTRACT.–In order to assess local population differentiation of a ranid frog *Rana nigrovittata* within Thailand and to elucidate its phylogenetic relationship with *R. mortenseni*, we surveyed a total of 72 frogs belonging to eight populations using starch gel electrophoresis. *Rana nigrovittata* is genetically substantially differentiated among populations, and can be divided into two groups with Nei's D of 0.23. *Rana mortenseni* from the Ko Chang Island is closest to the far inland northeastern population in the UPGMA tree and specific separation of this form from *R. nigrovittata* is not supported at least for the Thai population. Genetic differentiation of the two major groups of *R. nigrovittata* is not small compared with those reported for other anuran species. The pattern of geographic variation among populations of *R. nigrovittata* seems to have been strongly affected by the geohistory of their distributional ranges.

KEY WORDS: *Rana nigrovittata*; *Rana mortenseni*; allozymes; geographic variation; Thailand

INTRODUCTION

Rana nigrovittata (Blyth, 1856) occupies a wide range in continental Southeast Asia, including Assam (India) to Yunnan (China), Vietnam and south to Malaya (Frost, 1985). Once the population from Ko Chang Island, Thailand was considered a distinct species *R. mortenseni* (Boulenger, 1903) but the species was synonymized with *R. nigrovittata* long ago (Smith, 1922) and that name has never appeared in the list of Thai fauna (e.g., Taylor, 1962). The name *R. mortenseni*, however, has been resurrected recently (Dubois, 1992), though without any definite reason.

Because *R. nigrovittata* is noted for its immense distribution as mentioned above, the presence of considerable genetic differentiation is expected in this species. Smith (1922) already gave preliminary ideas about pattern of morphological variation of the species within Thailand, but no further studies are available. Likewise, the populational genetic differentiation in *R. nigrovittata* has never been studied so

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TABLE 1. Sampling localities and size of samples used for electrophoretic analysis. Division of biogeographic regions follows Inger (1999).

Pop. no.	Locality (biogeographic region)	N
Rana nigrovittata		
1	Doi Inthanon (northeastern montane region)	5
2	Phu Luang (Thai-Lao dry plateau)	7
3	Erawan (northeastern montane region)	10
4	Phuwua (Thai-Lao dry plateau)	9
5	Khao Srabab (southeast Asian lowlands)	9
6	Ko Samui Island (Tenasserim and Malay Peninsula)	10
7	Khao Chong (Tenasserim and Malay Peninsula)	10
Rana mortenseni		
8	Ko Chang Island (southeastern Asian lowlands)	12

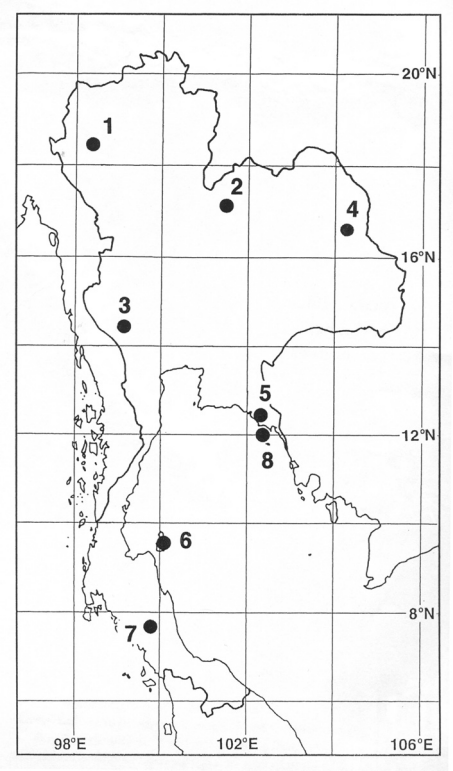

FIGURE 1. A map of Thailand showing sampled localities of *R. nigrovittata* (1-7) and *R. mortenseni* (8) used in the allozymic variation analyses. For population number, refer to Table 1.

far from the analyses of either allozymes or DNA nucleotide sequence.

In the present study, we used the starch gel electrophoresis of allozymes to clarify the taxonomic relationships of *R. mortenseni* from Ko Chang Island with respect to *R. nigrovittata*. In doing so, we also tried to investigate the degree of genetic differentiation between populations of *R. nigrovittata* within Thailand through this biochemical method.

MATERIALS AND METHODS

We sampled a total of 72 frogs to investigate genetic variation in *Rana nigrovittata* and *R. mortenseni*: seven populations of *R. nigrovittata* as covering whole Thailand (populations 1-7, N = 60) and a population of *R. mortenseni* from Ko Chang Island (population 8, N = 12) (Table 1; Fig. 1).

Liver and muscle tissues were taken in the field and transported in liquid nitrogen. They were then stored frozen at -84°C until use for electrophoretic assay. Voucher specimens, fixed in 10% formalin and later preserved in 70% ethanol, are currently deposited in the Graduate School of Human and Environmental Studies, Kyoto University (KUHE) (see Appendix). Homogenized tissue extracts were subjected to standard horizontal starch gel electrophoresis (Shaw and Prasad, 1970; Ayala et al., 1972), using Starch Art (Starch Art Corp., Smithville, Texas, USA) and Connaught starch (Connaught Lab., Ontario, Canada) mixed in a 4:1 ratio and then suspended in buffer at a concentration of 11.5%. Enzymes examined, locus designations, tissues, and buffer systems employed are shown in Table 2. Staining methods, genetic interpretations of allozyme data, enzyme nomenclature, enzyme code numbers, abbreviations, and isozyme designations generally follow Nishikawa et al. (2001).

TABLE 2. Enzymes, presumptive loci, tissues (L: liver, M: muscle), and buffer systems used in the analyses of allozyme variations among *Rana nigrovittata* and *R. mortenseni* populations.

Enzymes	E.C. numbers	Locus	Buffer system*	Tissues
Aconitate hydratase	4.2.1.3	mAcoh-A	CAPM6	L
Aconitate hydratase	4.2.1.3	sAcoh-A	CAPM6	L
Aspartate aminotransferase	2.6.1.1	mAat-A	CAPM6	L
Aspartate aminotransferase	2.6.1.1	sAat-A	CAPM6, TC7	L
Creatine kinase	2.7.3.2	Ck-A	CAPM6	M
Fumarate hydratase	4.2.1.1	Fumh-A	TC7	L
Glucose-6-phosphate isomerase	5.3.1.9	Gpi-A	CAPM6	L
Glycerol-3-phosphate dehydrogenase	1.1.1.8	G3pdh-A	TC8	L
Glutamate dehydrogenase	1.4.1.3	Gtdh-A	TBE8.7	L
Isocitrate dehydrogenase	1.1.1.42	mIdh-A	TC7	L
Isocitrate dehydrogenase	1.1.1.42	sIdh-A	TC7	L, M
L-Lactate dehydrogenase	1.1.1.27	Ldh-A	CAPM6, TC7	L, M
L-Lactate dehydrogenase	1.1.1.27	Ldh-B	CAPM6, TC7	L
Malate dehydrogenase	1.1.1.37	mMdh-A	CAPM6, TC8	L, M
Malic enzyme**	1.1.1.40	mMdhp-A	TC7	L
Malic enzyme**	1.1.1.40	sMdhp-A	TC7	L
Peptidase (leucyl-alanine)	3.4.11.-	Pep-la	TBE8.7	L
Peptidase (leucyl-glycine)	3.4.11.-	Pep-lg	TBE8.7	L
Peptidase (leucyl-glycyl-glycine)	3.4.11.-	Pep-lgg	TBE8.7	L
Phosphoglucomutase	5.4.2.2	Pgm-A	TC7	L
Phosphoglucomutase	5.4.2.2	Pgm-C	TC8	L
Phosphogluconate dehydrogenase	1.1.1.44	Pgdh-A	TC7	L
Superoxide dismutase	1.15.1.1	Sod-A	TBE8.7	L

*Buffer systems-CAPM6: Citrate-aminopropylmorpholine, pH = 6.0 (Clayton and Tretiak,1972); TC7: Tris-citrate, pH = 7.0 (Shaw and Prasad,1970); TC8: Tris-citrate, pH = 8.0 (Clayton and Tretiak,1972); TBE8.7: Tris-borate-EDTA, pH = 8.7 (Boyer et al.,1963).

**NADP-dependent malate dehydrogenase.

We studied 23 presumptive loci encoding 17 enzyme systems (Table 2). Genetic variation for each population was assessed by computing the percentage loci polymorphic (P), the mean heterozygosity by direct count (H), and the mean number of electromorphs per locus (A).

We calculated two genetic distances, Nei's (1978) unbiased genetic distance and modified Rogers' distance (Wright, 1978). Patterns of genetic similarities among populations were inferred from the Nei's (1978) distance clustered according to the UPGMA algorithm (Sneath and Sokal, 1973), and modified Rogers' distance (Wright, 1978) analyzed by the Neighbor-joining (NJ) method (Saitou and Nei, 1987). These analyses were performed by use of BIOSYS-1 (Swofford and Selander, 1981) and PHYLIP vers. 3.5 C computer packages (Felsenstein, 1993).

RESULTS

Of 23 loci scored, 19 (other than Ck-A, Gtdh-A, mIDH-A, and Pgm-A) were polymorphic (Table 3). The most variable locus was sSod-A with five alleles, followed by sMdhp-A, Pgm-C, and Pgdh-A with four alleles.

The mean number of electromorphs per locus (A) varied from 1.2 to 1.6, the percentage loci polymorphic (P) from 17.4 to 52.2, and the mean heterozygosity by direct count (H) from 0.04 to 0.11. The highest and lowest P values were observed in populations 2 and 4, respectively, while the highest and lowest H values in populations 5 and 8, respectively. Thus there was no marked genetic heterogeneity in the population 8 and presence of two genetic morphs was negated.

TABLE 3. Allele frequencies at 19 polymorphic loci of samples examined. For population number, refer to Table 1. A = mean number of alleles per locus; P = percentage of loci; H = mean heterozygosity (direct count).

Pop. (n) Locus	1 (5)	2 (7)	3 (10)	4 (9)	5 (9)	6 (10)	7 (10)	8 (12)
mAcoh-A	b0.900 c0.100	b0.214 c0.786	b0.950 c0.050	b	a0.056 b0.888 c0.056	c	b	b
sAcoh-A	b	b0.071 c0.929	b0.500 c0.500	a	a0.056 b0.888 c0.056	b	b	a0.042 b0.958
mAat-A	a	a	a	a	a0.278 b0.722	a0.800 b0.200	a0.050 b0.850 c0.100	b0.917 c0.083
sAat-A	b	a0.071 b0.929	b	b	b0.944 c0.056	b	b0.950 c0.050	b
Fumh-A	a	a0.929 b0.071	a0.750 b0.250	b	a	b	b	b
G3pdh-A	c	c	c	c	a0.167 c0.833	c	c	b0.083 c0.917
Gpi-A	b	b0.929 c0.071	a0.050 b0.950	b	b0.944 c0.056	b	b	b
sIdh-A	b	a0.071 b0.929	b	b	b	b	b	b
Ldh-A	a	a	a	a	a	a0.950 b0.050	a0.850 b0.150	a
Ldh-B	b0.700 c0.300	b0.929 c0.071	b	a	a	b0.950 c0.050	a	a
mMdh-A	a0.800 b0.200	a0.929 b0.071	a0.800 b0.200	b	b	a0.800 b0.200	a	b
mMdhp-A	a	a	a	a	a	a0.950 b0.050	a0.750 b0.250	a
sMdhp-A	c	b0.143 c0.857	a0.100 b0.850 c0.050	c0.944 d0.056	c0.833 d0.167	c	a	c0.750 d0.250
Pep-la	a	a	a	a	a	a0.950 b0.050	a	a
Pep-lg	a	a	a0.900 b0.100	a	a	a	a	a
Pep-lgg	a	a0.929 b0.071	a0.950 b0.050	a0.833 b0.167	a	a	a	a0.833 b0.167
Pgm-C	a	a	a	a	b0.056 c0.888 d0.056	a	a	a
Pgdh-A	a0.100 c0.900	a0.071 c0.929	a0.050 b0.150 c0.800	a0.111 b0.111 c0.667 d0.111	c	c	c	c
sSOD-A	b0.100 c0.900	b0.071 c0.644 d0.071 e0.214	b0.150 c0.750 d0.050 e0.050	c0.667 d0.333	b0.056 c0.611 d0.333	c0.950 d0.050	a0.050 c0.700 d0.250	c0.125 d0.875
A	1.2	1.6	1.6	1.3	1.6	1.3	1.3	1.3
P	21.7	52.2	43.5	17.4	39.1	30.4	21.7	21.7
H	0.07	0.10	0.09	0.06	0.11	0.05	0.05	0.04

As shown in Table 4, the relative magnitudes of pairwise Nei's and modified Rogers' distances were very similar, and only the former will be described below. The highest distance (D = 0.30) was obtained between populations 2 and 8. By contrast, the lowest distance (D = 0.06) was found between populations 1 and 3.

Figure 2 shows the UPGMA tree based on Nei's unbiased genetic distance. Although populations of *R. nigrovittata* and *R. mortenseni* were divided into two distinct groups, the two species were not separated. One group included populations 1-3, and 6, and the other included 4, 5, 7, and 8 (= *R. mortenseni*). The population closest to *R. mortenseni* was population 4 from far distant northeastern inland region. Topology of NJ tree based on modified Rogers' distance was similar to that of UPGMA in that the two major groups were recognized among populations studied, and *R. mortenseni* was closer to populations 4, 5, and 7 than to the remaining populations.

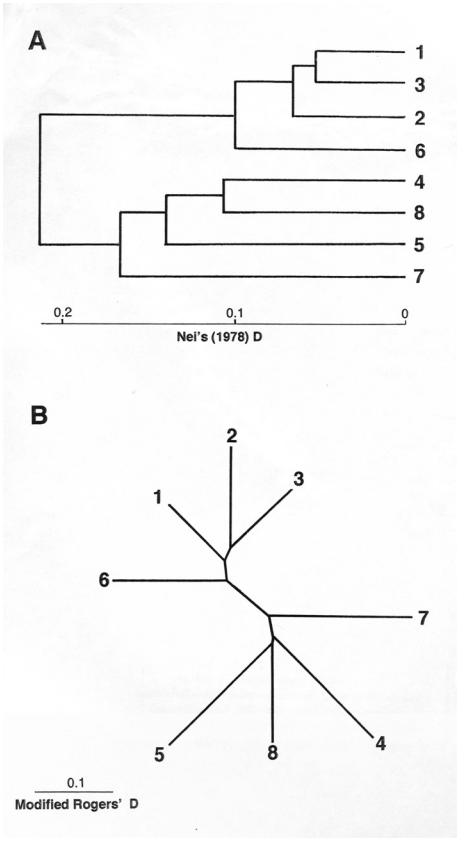

FIGURE 2. UPGMA tree based on Nei (1978) unbiased genetic distances (A), and a NJ network based on modified Rogers' distance (Wright, 1978) (B) among eight populations studied. For population number, refer to Table 1.

DISCUSSION

Boulenger (1893: 331) first identified specimens of ranid frogs from Yado and Thao, northeastern Myanmar as *R. guentheri* originally described from Macau, but later examined specimens collected from Koh Chang (= Ko Chang Island) by Mr. Mortensen and considered these frogs to be specifically different from *R. guentheri*. Boulenger (1903) thus described them as a new species *R. mortenseni*, but did not mention of its type locality. Because Boulenger (1903) dedicated the new name to the collector of Thai specimens, the type locality of *R. mortenseni* should be restricted to Ko Chang Island.

TABLE 4. Matrix of genetic distances, above diagonal: Nei's (1978) unbiased genetic distance, below diagonal: modified Rogers' distance (Wright, 1978). For population number, refer to Table 1.

Pop.	1	2	3	4	5	6	7	8
1	-	0.066	0.055	0.176	0.147	0.088	0.189	0.208
2	0.256	-	0.064	0.224	0.241	0.090	0.272	0.300
3	0.235	0.249	-	0.195	0.218	0.129	0.183	0.242
4	0.396	0.435	0.408	-	0.171	0.190	0.199	0.107
5	0.363	0.445	0.423	0.386	-	0.249	0.201	0.110
6	0.290	0.291	0.340	0.408	0.456	-	0.178	0.198
7	0.407	0.472	0.396	0.415	0.414	0.395	-	0.109
8	0.425	0.493	0.448	0.313	0.316	0.415	0.316	-

In his description of *R. mortenseni*, Boulenger (1903) did not refer to *R. nigrovittata*. In his later monograph of Asian *Rana* (Boulenger, 1920), he placed *R. mortenseni* remote from *R. nigrovittata* in the key to species. Smith (1921) once considered these two species as distinct on the bases of different conditions of vocal sacs, but later found great variation in that character among populations of *R. nigrovittata*. He finally concluded the two forms to be conspecific (Smith, 1922), and later authors followed this view (e.g., Taylor, 1962).

Our result indicates the presence of two major genetic groups within *R. nigrovittata* from Thailand, of which one included *R. mortenseni* from Ko Chang Island (must be topotypic). As shown above, *R. mortenseni* from Ko Chang was genetically closest to the population of *R. nigrovittata* from Phuwua, locating on far inland Thai-Lao Dry Plateau, with a small genetic distance (Nei's D = 0.11) among all pairs compared.

Although the absolute genetic distances derived from allozymic data themselves are not directly used for outlining species boundaries, relative distances from allozymic data show us a good taxonomic standard (Matsui, 2000). Highton (2000) summarized the published Nei's (1978) D between cryptic amphibian species to be 0.15, which value is larger than that we found between Ko Chang and Phuwua populations (0.11). Thus, *Rana mortenseni* from Ko Chang is genetically not considered to be different from the population of *R. nigrovittata* from Phuwua at the specific level.

Populations of *R. nigrovittata* sister to these two populations (Ko Chang and Phuwua) were one from Khao Srabab, opposite to the Ko Chang Island, and another from Khao Chong, southernmost peninsular Thailand. They altogether formed a major group in opposition to the group including populations from Doi Inthanon and Erawan (northeastern montane region of Inger, 1999), Phu Luang (Thai-Lao dry plateau), and Ko Samui (Tenasserim). These two major groups were divided with a genetic distance of 0.21.

This genetic distance is slightly higher than are found among species of amphibians so far studied as shown above (D > 0.15: Highton, 2000). If there is actually no genetic inter-change between these two groups, they might be split as two distinct species, and the group including Ko Chang population should be called *R. mortenseni*. However, the range of *R. nigrovittata* (sensu lato) is very wide outside of Thailand, and we need further studies using many populations from outside of Thailand. At present, we have no other choice to conclude that *R. mortenseni* Boulenger, 1903, is a subjective junior synonym of *R. nigrovittata* (Blyth, 1856). There is still a possibility that the population of "*R. mortenseni*" from norheastern Myanmar might represent a good species, but if such is the case, it should be given a new name.

Smith (1922), while synonymizing *R. mortenseni* with *R. nigrovittata*, noted the possibility of correlating broad morphological variations of this species with geographical areas. On the basis of the body size, body coloration, and the development of vocal sacs, he recognized three groups in this species: (1) Peninsular Siam, (2) N. E. Siam (= Thailand) and French Laos, and (3) Ko Chang. Our genetic grouping did not agree with Smith's (1922) morphological one, and the populations from southern part of the Peninsula were grouped with either northeastern Thailand or Ko Chang Island as shown above. In order to clarify this dicordance between morphological and genetical patterns, we need more detailed study of morphological variation.

The geohistory of Thailand including the formation of The Isthmus of Kra was supposed to have strongly affected the formation of patterns in distribution and divergence of anurans from Southeast Asia (Inger, 1966, 1999). Until now, the region of the Isthmus of Kra could be considered a geographic barrier that had promoted the divergence of anuran fauna in this region. However, closer genetic similarities of the population from Khao Chong, southern part of the peninsula, to populations from eastern Thailand than to the population from Ko Samui (Tenasserim) demonstrated in

the present study indicate the past interchange between the eastern continental and southernmost peninsular populations through a route over the present Gulf of Siam. This hypothesis should be tested by comparisons with species having similarly wide distribution across regions including Thailand.

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LITERATURE CITED

Ayala, F. J., J. R. Powell, M. L. Tracey, C. A. Mourao and S. Perez-Salas. 1972. Enzyme variability in the *Drosophila willistoni* group. IV. Genetic variation in natural populations of *Drosophila willistoni*. Genetics 70: 113-139.

Blyth, E. 1856. Report for October meeting, 1855. J. Asiatic Soc. Bengal 24(7): 711-723.

Boulenger, G. A. 1893. Concluding report on the reptiles and batrachians obtained in Burma by Signor L. Fea, dealing with the collectionmade in Pegu and the Karin Hills in 1887-88. Ann. Mus. Civ. Stor. Nat. Genova Ser. 2, 13: 304-347+pls.vii-xii.

Boulenger, G. A. 1903. On a new frog from upper Burma and Siam. Ann. Mag. Nat. Hist. Ser. 7, 12: 219.

Boulenger, G. A. 1920. A monograph of the South Asian, Papuan, Melanesian, and Australian frogs of the genus Rana. Rec. indian Mus. 20: 1-126.

Dubois, A. 1992. Notes sur la classification des Ranidae (Amphibiens: Anoures). Bull. Mens. Soc. Linn. Lyon 61: 305-352.

Felsenstein, J. 1993. PHYLIP (phylogeny inference package) Version 3.5 C. Distributed by the author. Department of Genetics, University of Washington, Seattle.

Frost, D. R. (ed.) 1985. Amphibian Species of the World: A Taxonomic and Geographical Reference. Allen Press, Lawrence, Kansas.

Highton, R. 2000. Detecting cryptic species using allozyme data. In: R. C. Bruce, R. G. Jaeger and L. D. Houck (eds.), The Biology of Plethodontid Salamanders, pp. 215-241. Kluwer Academic /Plenum Publishers, New York.

Inger, R. F. 1966. The systematics and zoogeography of the Amphibia of Borneo. Fieldiana: Zoology 52: 1-402.

Inger, R. F. 1999. Distribution of amphibians in southern Asia and adjacent islands. In: W. E. Duellman (ed.), Patterns of Distribution of Amphibians, pp. 445-482. Johns Hopkins Univ. Press, Baltimore.

Matsui, M. 2000. Batrachology of Japan and adjacent regions - a systematic review. Comp. Bioch. Physiol. B 126: 247-256.

Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.

Nishikawa, K., M. Matsui, S. Tanabe and S. Sato. 2001. Geographic enzyme variation in a Japanese salamander, *Hynobius boulengeri* Thompson (Amphibia: Caudata). Herpetologica 57(3): 281-294.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Shaw, C. R. and R. Prasad. 1970. Starch gel electrophoresis of enzymes - a compilation of recipes. Biochem. Genet. 4: 297-330.

Smith, M. A. 1921. New or little-known reptiles and batrachians from southern Annam (Indo-China). Proc. Zool. Soc. London 1921: 423-440+pls.1-2.

Smith, M. A. 1922. Notes on reptiles and batrachians from Siam and Indo-China, (No.1). J. Nat. Hist. Soc. Siam 4: 203-214.

Sneath, P. H. A. and R. R. Sokal. 1973. Numerical Taxonomy. W.H. Freeman, San Francisco.

Swofford, D. L. and R. B. Selander. 1981. BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. J. Hered. 72: 281-283.

Taylor, E. H. 1962. The amphibian fauna of Thailand. Univ. Kansas Sci. Bull. 63: 265-599.

Wright, S. 1978. Evolution and the Genetics of Populations, Vol. 4. Variability within and among natural populations. University of Chicago Press, Chicago.

APPENDIX

Specimens used for electrophoresis. Voucher specimens are stored at the Graduate School of Human and Environmental Studies, Kyoto University (KUHE).

Population 1: KUHE 19115-19116, 19151-19152, 19158; population 2: KUHE 19238-19239, 19246-19248, 19263, 19274; population 3: KUHE 20049, 20051, 20055, 20057, 20063, 20066, 20069-20071, 20074; population 4: KUHE 22087-22088, 22163-22165, 22195, 22257-22258, 22260; population 5: KUHE 20320-20321, 20329-20330, 20335, 20344, 20353, 2 KUHE unnumbered; population 6: KUHE 19545, 19548-19549, 19595-19601; population 7: KUHE 23245-23247, 23299-23300, 23323, 23325-23326, 23391, 23393; population 8: KUHE 20207, 20211-20212, 20220-20223, 20229, 20238, 20241-20242, 20262.

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