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Cytochrome oxidase subunit I (COI) DNA sequence divergence between two cryptic species of *Oryzias* in South Korea

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Abstract

Oryzias latipes and *Oryzias sinensis* are indigenous species found in Japan, China, and other East Asian countries, including Korea. Based on morphological differences, the species have been classified distinctly. However, the range of morphological characters such as the number of gill rakers, vertebrae, and spots on the lateral body overlaps and is too vague for clear identification, so their classification based on their morphological characteristics remains uncertain. In this study, the mitochondrial cytochrome oxidase subunit I (COI) gene, which is used for DNA barcoding, was applied to clarify interspecific variation of *O. latipes* and *O. sinensis*. Intraspecific genetic diversity was calculated to identify correlations with geographic distributions. We studied two species collected from 55 locations in Korea. All individuals carried a 679-base pair gene without deletion or insertion. Between species, 525 base pairs of the gene were shared. The Kimura two parameter (K2P) distance of *O. latipes* and *O. sinensis* was 0.41% and 1.39%, respectively. Mean divergence within genera was 23.5%. Therefore, the species were clearly different. The distance between *O. latipes* and *O. sinensis* was 14.0%, which is the closest within genera. Interestingly *O. latipes* from the Japanese and Korean group represented 16.5% distant. These results were derived from geohistorical and anthropogenic environmental factors. The *O. latipes* haplotypes were joined in only one group, but *O. sinensis* was divided into two groups, one is found in the Han River and upper Geum River watershed; the other is found in the remaining South Korean watersheds. Further studies will address the causes for geographic speciation of *O. sinensis* haplotypes.

Key words: cytochrome oxidase subunit I, geographic distribution, K2P distance, *Oryzias latipes*, *Oryzias sinensis*

INTRODUCTION

Morphological identification, the most common method for classification, has been employed for many years. Studies involve measuring and comparing the phenotypes of target species, sometimes by microscopic examination. This method is normal and sound when it applies to well-known taxa; however, it is not always efficient for species identification, unless the phenotype is obvious. To overcome this problem, a taxonomic system based on DNA analysis was established in the early 2000s (Tautz et al. 2002, 2003).

Mitochondrial DNA cytochrome oxidase subunit I (mtDNA COI) has been proposed for used in animal bio-identification because the COI gene could differentiate nearly all animal species with only a single gene sequence (Hebert et al. 2003, 2005). In the early 2000s, various taxonomic studies were carried out using the COI gene

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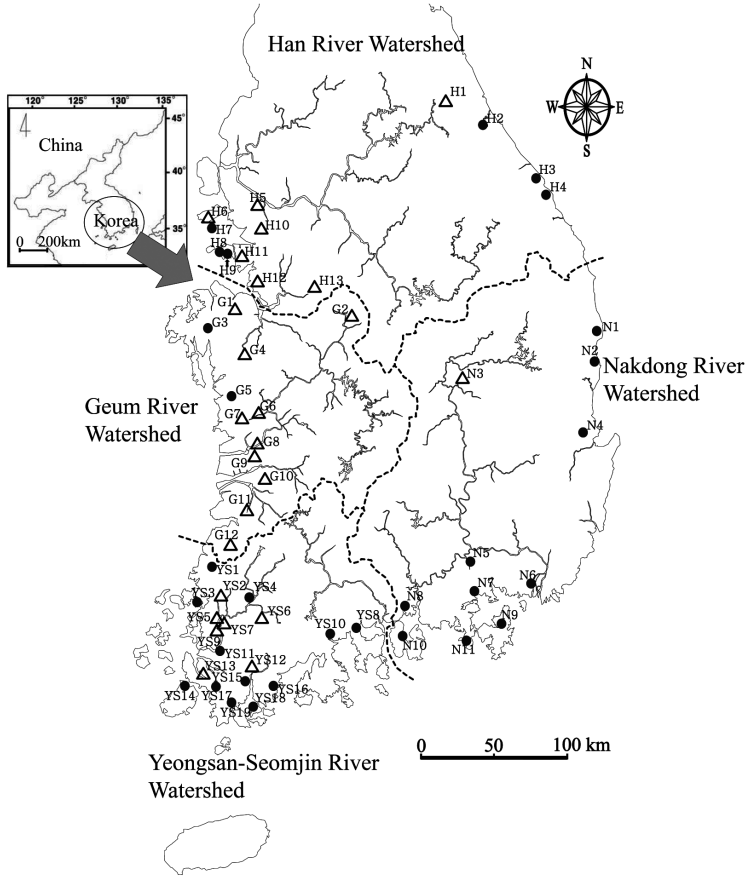

Fig. 1. *Oryzias* collection sites in South Korea.

(Hebert et al. 2003, 2004a, 2004b, 2005, Smith et al. 2006, 2007, Bucklin et al. 2010). The universal primer for the COI gene is very stable and attaches at the 5′ end of the animal gene (Folmer et al. 1994, Zhang and Hewitt 1997). The COI provides profound insights into phylogenetic studies more than other mitochondrial genes such as cytochrome b (Simmons and Weller 2001), because changes in the amino acid sequence occur more slowly (Lynch and Jarrell 1993). Moreover, mtDNA extraction and sequencing are easy because the small multi-copy mitochondrial genome is located in each cell. Inter-specific variation is high and intra-specific variation is low, making species classification clears (Jin 2012).

Oryzias latipes and *Oryzias sinensis* (genus *Oryzias*) are indigenous species distributed in South Korea, China, Japan, and East Asia. Both species are shoaling fish that stay near the water surface and favor static, shallow waters such as reservoirs, swamps, and small streams (Kim and Park 2002). They were classified by morphological analysis as different species in the early 1990s; the China-western Korea group is designated *O. sinensis* and the eastern Korea group is *O. latipes* (Kim and Lee 1992, Kim and Kim 1993). However, the range of classification keys, number of gill rakers, vertebrae, and spots on the lateral body overlap and are too vague for clear identification, so debate regarding their classification continues.

In this study, the mitochondrial COI region used for DNA barcoding was applied to clarify the interspecific variation of *O. latipes* and *O. sinensis*. Intraspecific genetic diversity was also calculated to identify the correlation between geographic distributions.

MATERIALS AND MEHTODS

Sample collection and identification of genus *Oryzias*

Sampling was conducted from 2009 to 2011 and sites were selected based on literature and government reports

(Appendix 1). Other sites were chosen based on the possibility of *Oryzias* habitation, even in the absence of records of their presence. Samples were collected at 55 sites, most of which were located in the mid-lower parts of streams (Fig. 1, Appendix 1). At each site, three specimens were chosen for preservation in 99.9% ethanol; other specimens were released at the site of capture. After sample moved to the laboratory, each specimen was identified, labeled for individual identification, and measured total length (mm), standard length (mm) and body weight (g). Spots on the lateral body were used for identification (Park et al. 2006); if spots were distributed on the caudal peduncle, the specimen was classified as *O. latipes* and if not, it was classified as *O. sinensis*.

DNA analysis

We analyzed 165 specimens from 55 sites. DNA extracts were prepared from muscle tissue (about 30 mg) using SolGent™ Genomic DNA Prep Kit (Solgent, Daejoen, South Korea). To amplify the COI gene from mitochondrial DNA, PCR was performed with primers FishF2 (5′-TCAACYAATCAYAAAGATAT-3′) and FishR2 (5′-ACTTCYGGGTGRCCRAARAA-3′) (Ward et al., 2005). The 50 µl PCR mixture included 5 µl 10× reaction buffer, 1 µl each primer (10 pmole/µl), 4 µl dNTP (2.2 mM each), 0.25 µl TaKaRa Ex Taq™ (Takara, Shiga, Japan) and 4 µl template DNA (50 ng/µl). Amplifications were performed using a C1000 gradient thermal cycler (Bio-RAD, Foster City, CA, USA). Cycling included an initial step of 3 min at 95°C followed by 40 cycles of 0.5 min at 95°C, 0.5 min at 55°C, and 1 min at 72°C, followed by elongation for 7 min at 72°C. PCR products were analyzed by 1% agarose gel electrophoresis, and then purified with a *Solg*™ PCR Purification Kit (SolGent, Daejoen, South Korea). Bidirectional sequencing was performed with the FishF2 and FishR2 primers and an 3730xl DNA Analyzer (Applied Biosystems®, Foster City, CA, USA) with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®). Contigs of DNA sequences were generated in CodonCode aligner ver. 3.7.1 (CodonCode Corporation, Dedham, MA, USA). Sequences were aligned with BioEdit ver. 7.0.0 by using the ClustalW Multiple alignment method. Aligned sequences of genus *Oryzias* and references (6 other *Oryzias* species and *Hyporhamphus sajori* as the outgroup) from GenBank (gi208341979; gi253960407; gi314909980; gi253960477; gi253960463; gi253960435; gi253960393; gi254939398; gi253960421) were compared using MEGA5 (Tamura et al. 2011). Neighbor-joining (NJ) was used for phylogenetic analysis. To assess statistical reliability, an NJ tree was generated by bootstrapping with 1000 replications (Felsenstein 1993) and Kimura two parameter (K2P) distances were determined to provide a graphic representation of the patterning of divergence between species. Sequence divergences were calculated using the K2P distance model (Kimura 1980). All *Oryzias* sequences include references calculated with NETWORK ver. 4.6.11 using the median-joining network option for intraspecific relationship (Bandelt et al. 1999) to identify relationships.

RESULTS

Analysis of the mtDNA COI gene

The COI sequence length was 679-bp and there were no insertions or deletions in any specimen. The COI gene in *O. latipes* included 629 conserved bp and 23 variable bp. Base compositions were: A, 22.8%; T, 19.5%; G, 30.9%; C, 26.8%. In *O. sinensis*, the COI gene included 620 conserved and 32 variable bp. Base composition was slightly different in *O. latipes*: A, 23.7%; T, 17.9%; G, 31.9%; C, 26.5%. The sequence variation in *O. sinensis* was relatively greater than in *O. latipes*. The overlapping sequence in both species was 525-bp long.

The K2P distance of *O. latipes* was 0.41%, and the distance for *O. sinensis* was 3 times larger, at 1.39%. Mean divergence within genera was 23.5%. Therefore, the species were obviously different. The distance of *O. latipes* and *O. sinensis* was 14.0%, which is the closest within genera. Conversely, *O. latipes* and *O. marmoratus* showed 26.5% distance, so the farthest within genera. Moreover, interestingly *O. latipes* from Japanese group and Korean group represented 16.5% of distance (Table 1).

Molecular phylogeny of genus *Oryzias*

The molecular phylogeny results indicate *O. latipes* and *O. sinensis* in South Korea were clearly branched under 100 bootstrapping (BP) value. *O. latipes* formed a single group including the GenBank reference gi208341979. The *O. sinensis* group with GenBank reference gi314909980 was divided into two groups. The BP value of the *O. sinensis* groups was 99. *O. latipes* in Japan comprise a different branch with a BP value < 89 (Fig. 2). A total of 19 *O. latipes* haplotypes from 30 sites were identified. The estimated number of mutations in the shortest tree within torso was 3, and the estimated number of mutations in the shortest tree was 25. There was only one network of *O. latipes* in South Korea. In contrast, 14 *O. sinensis* haplotypes were

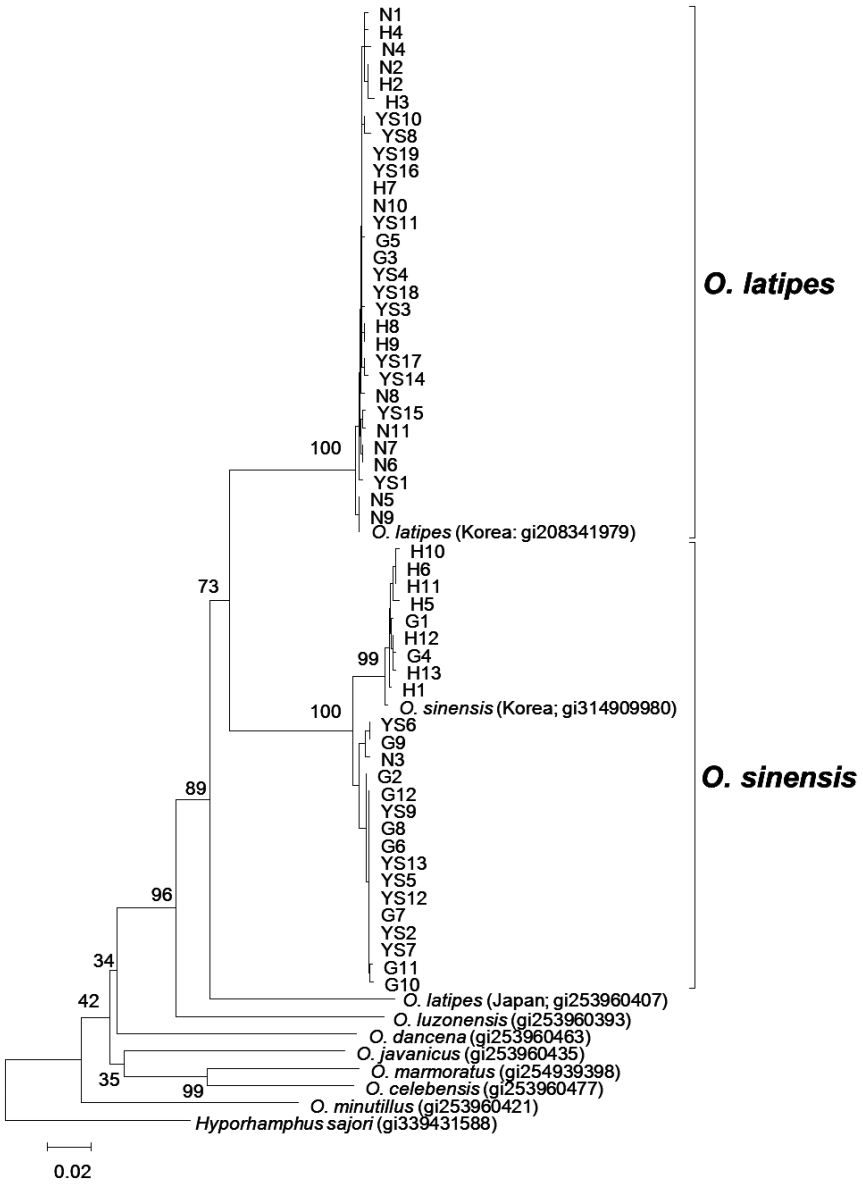

Fig. 2. Molecular phylogeny of *Oryzias* species using the Neighbor-joining method in MEGA5.

Table 1. Matrix of K2P (Kimura 2 Parameter) for 9 *Oryzias* species. *Hyporhamphus sajori* is an outgroup

Species	1	2	3	4	5	6	7	8	9	10
1 *O. latipes* (Korea)	0.000									
2 *O. latipes* (Japan)	0.165	0.000								
3 *O. sinensis* (Korea)	0.140	0.180	0.000							
4 *O. celebensis*	0.248	0.260	0.242	0.000						
5 *O. dancena*	0.232	0.253	0.246	0.235	0.000					
6 *O. javanicus*	0.243	0.245	0.239	0.212	0.242	0.000				
7 *O. luzonensis*	0.194	0.215	0.195	0.264	0.255	0.217	0.000			
8 *O. marmoratus*	0.243	0.264	0.250	0.145	0.256	0.230	0.265	0.000		
9 *O. minutilus*	0.253	0.235	0.237	0.217	0.222	0.253	0.264	0.251	0.000	
10 *Hyporhamphus sajori*	0.273	0.252	0.248	0.265	0.264	0.225	0.249	0.274	0.227	0.000

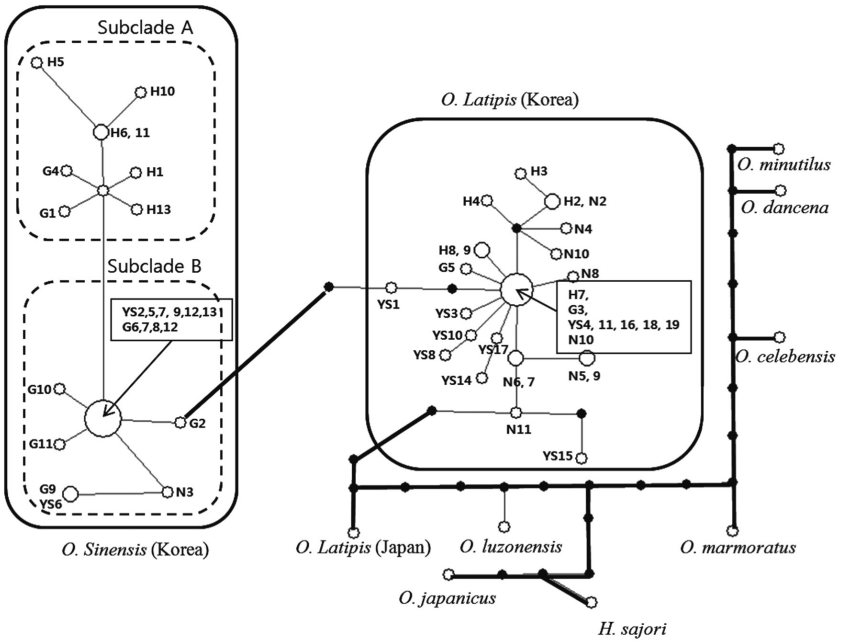

Fig. 3. Intraspecific relationship between haplotypes in Korea constructed by median-joining networks with *Oryzias latipes* and *O. sinensis*. Circles indicate each haplotype and geographical location; bold lines represent mutated regions.

identified across 25 sites; the estimated number of mutations in the shortest tree was 32. Thus, the *O. sinensis* group is divided into two subclades by the Charyeong mountain chain; subclade A is from the Han River watershed to the northern part of the Geum River watershed and subclade B is from the southern part of the Geum River watershed to the Yeongsan and Nakdong River watershed (Fig. 3).

DISCUSSION

COI region for species identification for *Oryzias* species

O. latipes and *O. sinensis* are considered different species; they have a different number of chromosomes (Park et al. 2006) and are reproductively incompatible (Kim and Kim 1993). Recent results from mitochondrial DNA cytochrome b and allozyme confirmed the biogeographic and reproductive isolation of these species (Sakaizumi and Jeon 1987, Matsuda et al. 1997, Takehana et al. 2004). These results are consistent with karyotype analyses (*O. latipes*: eastern Korea grout, 2n = 48 and *O. sinensis*: China-western Korea group, 2n = 46) (Uwa 1986, Kim and Moon 1987, Uwa and Jeon 1987). Haplotype analysis of the mtDNA control region (Kang et al. 2005) revealed a similar distribution of *O. sinensis*.

In this study, we clearly classified two species using the mitochondrial COI region. Thus, the COI region could be used to supplement current taxonomic keys that are largely focused on morphological characteristics. In many cases, morphological classification generates identification errors due to vague criteria. Therefore, species classification using DNA markers such as the COI region (DNA barcodes) is an appropriate alternative.

NJ trees clearly differentiated *O. latipes* and *O. sinensis* independently. The COI sequences provided strong support for the monophyletic origin of *O. latipes* and *O. sinensis*. BP of each species was the same for 100. Thus, the species are completely different. The K2P distance of *O. sinensis* was higher than that of *O. latipes*, indicating the intraspecific variation of *O. sinensis* is greater. Two clusters occurred within *O. sinensis*, and K2P distance was 2.17%. We suspect there is more variation within *O. sinensis*. The early study conducted by Min (1997) indicated a similar result, suggesting the genetic variation of *O. sinensis* groups was about 2.5 times higher than that of *O. latipes*. Genetic variations are normally accumulated through many factors such as stability, group and habitat size, etc. (Soule 1972, 1976, Valentine 1976, Stonecking et al. 1981). Thus, we suspect differences in habitat or environmental conditions exist.

Geographic distribution of genus *Oryzias*

Oryzias latipes is also distributed in China and Japan, inhabiting streams that flow into the eastern part of Korea and the islands part of southwestern Korea. In contrast, *O. sinensis* is distributed in the streams and islands of western Korea and China (Kim and Moon 1987, Kim and Lee 1992). Through allozyme studies of *O. latipes* in Japan, northern and southern groups were distinguished and a relationship was identified between the China and southern groups (Sakaizumi 1986). An isozyme study indicated the Korean and Japanese *O. latipes* groups are genetically different species (Min 1997). Such distribution could be generated by geohistorical and environmental factors. *O. sinensis* flowed into western Korea from southern China when the Korean peninsula was connected via the paleo-Hwangho (Yellow River) during the regression period of the Pliocene. *O. latipes* originated from the paleo-Amur River (Kim et al. 2005). Geographic characteristics of the Korean peninsula also support the distinct distribution of genus *Oryzias*. The Taebaek Mountains divide South Korea vertically into eastern and western regions, serving as a physical barrier. Stream morphology and fish species significantly differ between these regions. Thus, although similar species flowed from different sources into the Korean peninsula, they have maintained their original genetic identities because of the geographic characteristics of the Korean peninsula. In this study, *O. latipes* intruded into the western part of Korea. We believe this is due to anthropogenic causes, rather than natural displacement. Unintentional human releases are a significant cause for fish displacement. Also this newly found *O. sinensis* inhabitation sites (N3, YS 12 and YS 13). As we demonstrated, *O. latipes* from Japan and Korea are incorporated into different clades and the K2P distance was 16.50%. Thus, the phylogeny of these groups should be reconsidered for classification. In addition, overall consideration of the genetic relationship between species in Korea, China, and Japan is required for establishing genetic divergence in Asian countries.

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Appendix 1. Collection sites information of genus *Oryzias* (H: Han River Watershed, G: Geum River Watershed, YS: Yeongsan-Seomjin River Watershed, N: Nakdong River Watershed)

Site code	Collection site	GPS coordination		Collection date
		latitude	longitude	
H1	Dongsong, Cheorwon, Gangwon	38°12′36.00″N	128°14′47.03″E	2011.10.03
H2	Sonyang, Yangyang, Gangwon	38°05′11.00″N	128°39′11.08″E	2011.10.02
H3	Gangdong, Gangneung, Gangwon	37°43′53.02″N	128°57′29.05″E	2011.10.16
H4	Okgye, Gangneung, Gangwon	37°37′42.05″N	129°02′19.04″E	2011.10.16
H5	Dongyang, Gyeyang, Incheon	37°32′51.04″N	126°45′38.03″E	2011.09.18
H6	Eurwang, Jung, Incheon	37°26′44.09″N	126°23′37.06″E	2009.07.28
H7	Muui, Jung, Incheon	37°23′23.02″N	126°25′41.05″E	2009.07.28
H8	Yeongheung, Ongjin, Incheon	37°15′51.02″N	126°28′56.07″E	2011.09.18
H9	Yeongheung, Ongjin, Incheon	37°14′17.05″N	126°31′37.04″E	2009.05.30
H10	Hajung, Siheung, Gyeonggi	37°23′21.08″N	126°47′54.09″E	2011.09.18
H11	Seongam, Danwon, Ansan, Gyeonggi	37°13′45.06″N	126°38′37.06″E	2011.09.04
H12	Ujeong, Hwaseong, Gyeonggi	37°03′36.06″N	126°46′36.02″E	2011.09.13
H13	Daedeok, Anseong, Gyeonggi	37°02′09.08″N	127°12′51.02″E	2011.09.17
G1	Jeongmi, Dangjin, Chungcheongnam	36°52′47.04″N	126°36′06.03″E	2011.09.25
G2	Chopyeong, Jincheon, Chungcheongbuk	36°51′23.39″N	127°30′22.87″E	2011.09.15
G3	Buseok, Seosan, Chungcheongnam	36°45′34.07″N	126°23′08.05″E	2011.09.25
G4	Geumma, Hongseong, Chungcheongnam	36°36′04.06″N	126°41′19.00″E	2011.09.25
G5	Naehang, Boryeong, Chungcheongnam	36°20′34.06″N	126°35′04.02″E	2011.09.24
G6	Nam, Buyeo, Chungcheongnam	36°13′33.09″N	126°47′40.05″E	2011.09.24
G7	Misan, Boryeong, Chungcheongnam	36°11′30.07″N	126°40′04.07″E	2011.09.24
G8	Hwayang, Seocheon, Chungcheongnam	36°02′30.09″N	126°47′03.09″E	2011.09.24
G9	Gaejeong, Gunsan, Jeollabuk	35°57′46.01″N	126°46′290.2″E	2011.09.24
G10	Baeksan, Gimje, Jeollabuk	35°49′11.01″N	126°51′19.07″E	2011.11.06
G11	Julpo, Buan, Jeollabuk	35°36′39.09″N	126°42′49.08″E	2011.11.06
G12	Mujang, Gochang, Jeollabuk	35°23′16.06″N	126°35′32.07″E	2011.11.06
N1	Pyeonghae, Uljin, Gyeongsangbuk	36°44′46.09″N	129°27′43.01″E	2010.03.18
N2	Byeonggok, Yeongdeok, Gyeongsangbuk	36°33′19.93″N	129°24′23.16″E	2011.05.08
N3	Dain, Uiseong, Gyeongsangbuk	36°27′37.00″N	128°21′37.00″E	2011.11.12
N4	Heunghae, Pohang, Gyeongsangbuk	36°06′49.00″N	129°18′18.00″E	2009.09.11
N5	Gaya, Haman, Gyeongsangnam	35°17′20.07″N	128°23′59.02″E	2011.10.23
N6	Bongnim, Gangseo, Busan	35°09′26.06″N	128°53′36.06″E	2011.11.13
N7	Jinjeon, Changwon, Gyeongsangnam	35°05′51.04″N	128°26′46.04″E	2011.10.23
N8	Jingyo, Hadong, Gyeongsangnam	35°01′39.01″N	127°54′41.04″E	2011.10.23
N9	Yeoncho, Geoje, Gyeongsangnam	34°54′11.00″N	128°38′45.00″E	2009.05.01
N10	Namhae, Namhae, Gyeongsangnam	34°49′54.62″N	127°54′09.02″E	2010.05.01
N11	Sanyang, Tongyeong, Gyeongsangnam	34°48′24.08″N	128°23′09.00″E	2011.10.23
YS1	Gunseo, Yeonggwang, Jeollanam	35°15′11.04″N	126°28′09.06″E	2011.11.06
YS2	Hampyeong, Hampyeongn, Jeollanam	35°04′00.00″N	126°31′36.03″E	2011.10.08
YS3	Hyeongyeong, Muan, Jeollanam	35°03′37.01″N	126°22′08.02″E	2011.10.22
YS4	Noan, Naju, Jeollanam	35°03′39.00″N	126°44′31.09″E	2011.10.22
YS5	Mongtan, Muan, Jeollanam	34°55′34.02″N	126°30′25.02″E	2011.10.08
YS6	Dado, Naju, Jeollanam	34°55′23.00″N	126°50′30.02″E	2009.10.11
YS7	Donggang, Naju, Jeollanam	34°53′47.04″N	126°32′26.06″E	2011.10.08
YS8	Yulchon, Yeosu, Jeollanam	34°52′46.00″N	127°35′07.00″E	2011.11.12
YS9	Illo, Muan, Jeollanam	34°51′12.00″N	126°30′31.03″E	2011.10.08
YS10	Beolgyo, Boseong, Jeollanam	34°50′33.08″N	127°21′26.04″E	2011.11.12
YS11	Samho, Yeongam, Jeollanam	34°43′12.04″N	126°30′07.03″E	2011.10.08
YS12	Gangjin, Gangjin, Jeollanam	34°37′33.00″N	126°46′09.04″E	2011.10.09
YS13	Hwangsan, Haenam, Jeollanam	34°34′05.02″N	126°24′04.03″E	2011.10.09
YS14	Gunnae, Jindo, Jeollanam	34°32′57.06″N	126°18′19.03″E	2011.10.09
YS15	Sinjeon, Gangjin, Jeollanam	34°31′19.02″N	126°43′36.04″E	2011.10.09
YS16	Hoejin, Jangheung, Jeollanam	34°29′53.09″N	126°56′41.00″E	2011.10.22
YS17	Hwasan, Haenam, Jeollanam	34°29′19.09″N	126°30′24.02″E	2011.10.09
YS18	Gogeum, Wando, Jeollanam	34°25′14.05″N	126°48′55.07″E	2011.10.22
YS19	Gunoe, Wando, Jeollanam	34°22′39.08″N	126°38′44.09″E	2011.10.09