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Authors: Sakaguchi, Shota, and Ito, Motomi

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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF EST-SSR MARKERS FOR THE *SOLIDAGO VIRGAUREA* COMPLEX (ASTERACEAE) IN THE JAPANESE ARCHIPELAGO[1]

SHOTA SAKAGUCHI[2,3,4] AND MOTOMI ITO[2]

[2]Laboratory of Plant Evolution and Biodiversity, Department of General Systems Studies, Graduate School of Arts and Sciences, University of Tokyo, Meguro, Tokyo 153-0041, Japan; and [3]Research Fellow of the Japan Society for the Promotion of Science, Chiyoda, Tokyo 102-8472, Japan

- *Premise of the study:* We developed simple sequence repeat (SSR) markers from expressed sequence tags (ESTs) for the *Solidago virgaurea* complex, an ecologically and morphologically diverse species complex in the Japanese archipelago, to elucidate population genetic structure and examine taxonomic boundaries.
- *Methods and Results:* Utilizing the RNA sequencing data obtained by next-generation sequencing techniques, 15 polymorphic EST-SSR markers with three to 14 alleles were developed, most of which were transferable to different *Solidago* species native to Eurasia and North America.
- *Conclusions:* The EST-SSR markers developed in this study may be useful for elucidating the population structure and taxonomic delimitation of the species complex, as well as for investigating the population genetics and reproductive ecology of *Solidago* species.

Key words: Asteraceae; expressed sequence tag; genetic structure; microsatellite; *Solidago.*

The *Solidago virgaurea* L. complex (Asteraceae), a deciduous herbaceous perennial ($2n = 18$), is proposed to comprise three species (*S. virgaurea* L., *S. minutissima* (Makino) Kitam., and *S. yokusaiana* Makino) in the Japanese archipelago (Iwatsuki et al., 1995). However, high levels of morphological variation among populations due to its polymorphic nature and plasticity make it difficult to clearly delimit taxonomic boundaries (Hayashi, 1978; Takasu, 1978; Kawano, 1988), and thus the taxonomic treatment of this species complex is still in contention (Kadota, 2008; Semple, 2013). In particular, *S. virgaurea* is the most ecologically and morphologically diverse taxon, and includes five entities inhabiting alpine grassland (subsp. *leiocarpa* var. *leiocarpa* (Benth.) A. Gray), lowland forest and grassland (subsp. *asiatica* var. *asiatica* Nakai ex H. Hara), seashores and lowland hills in northern Japan (subsp. *gigantea* (Nakai) Kitam.), and southern island chains (subsp. *leiocarpa* var. *praeflorens* Nakai and subsp. *asiatica* var. *insularis* (Kitam.) Hara). Despite the apparently differentiated ecological niches, these taxa sometimes occur sympatrically or parapatrically, and in such circumstances intermediate individuals are often found, indicating probable hybridization (gene flow) among taxa (S. Sakaguchi, personal observation). Therefore, there is a great need for molecular studies that can provide new insights into phylogenetic relationships, population genetic structure, and gene flow between taxa and populations of the *S. virgaurea* complex in this region.

EST-SSR (simple sequence repeats in expressed sequence tags) markers are useful in these studies, because highly polymorphic markers can be developed with relative ease using the EST database (e.g., Sakaguchi et al., 2011) and they are less susceptible to null alleles than anonymous SSRs (Ellis and Burke, 2007). Here we developed 15 polymorphic EST-SSR markers for the *S. virgaurea* complex and evaluated their polymorphisms and transferability to other species of *Solidago* native to Eurasia and North America.

METHODS AND RESULTS

Assembled RNA sequencing (RNA-seq) data of *S. canadensis* L. (71,433 contigs) was obtained from the Plant OneKP Project repository (https://sites.google.com/a/ualberta.ca/onekp/home), and a similarity search of the contigs against the National Center for Biotechnology Information (NCBI) nr database was conducted using the BLASTX algorithm (Altschul et al., 1990) with an *E*-value cutoff of 1.0E-5. We screened the sequences including microsatellite regions for ≥6 dinucleotide repeats and ≥4 tri- to hexanucleotide repeats using MSATCOMMANDER (Faircloth, 2008) and designed primers using Primer3 software (Rozen and Skaletsky, 2000). A total of 6471 primer pairs bordering microsatellites were designed, and 96 pairs were selected for PCR amplification trials using eight individuals representing the seven taxa collected from a broad range of the Japanese archipelago (Appendix 1). For all the loci, the forward primer was synthesized with one of three different M13 sequences (5′-CACGACGTTGTAAAACGAC-3′, 5′-TGTGGAATTGTGAGCGG-3′, or

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[4]Author for correspondence: sakaguci54@gmail.com

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TABLE 1. EST-SSR markers for the *Solidago virgaurea* complex.[a]

Locus	Primer sequences (5′–3′)[b]	Repeat motif	Allele size (bp)	BLASTX top hit description[c]	*E*-value	Accession no.[d]
Sol_2000155	F: TGTGGAATTGTGAGCGGTTGGTTGACGTTGGGAAGC R: GTTTCTTTCCCTCCAACAGCAATGGG	$(AGAT)_9$	381	Uncharacterized protein	5.0E-52	TEZA-2000155
Sol_2001054	F: CTATAGGGCACGCGTGGTTGGACGGCCATATAATCCTTC R: GTTTCTTAGAGGCTCCTAAAGTGGCG	$(AGC)_8$	432	—		TEZA-2001054
Sol_2001106	F: TGTGGAATTGTGAGCGGCCAGACAGGGATTGGGTCG R: GTTTCTTTTGGGCAACAATGGGCATC	$(AC)_9$	397	Uncharacterized protein	1.0E-68	TEZA-2001106
Sol_2001640	F: CACGACGTTGTAAAACGACGAGGTGGAAGAATCTGTGGC R: GTTTCTTAAAGGGTGCCCTGATCAAC	$(AGT)_8$	443	SKP1-like protein 1	8.0E-75	TEZA-2001640
Sol_2001876	F: TGTGGAATTGTGAGCGGAAGCTCATGGGTCCTCTGC R: GTTTCTTATCAAGCCAAAGCAGCTCG	$(ATC)_8$	547–553	—		TEZA-2001876
Sol_2003053	F: CACGACGTTGTAAAACGACTGAACCGACGGATGGAACC R: GTTTCTTTGGGAGCTGGACATGTTGG	$(GAT)_9$	217–232	—		TEZA-2003053
Sol_2003631	F: TGTGGAATTGTGAGCGGCACCAGGCATGATCTGAAGC R: GTTTCTTCACCCTATCCACAATGCCAC	$(GAT)_{10}$	419–440	Uncharacterized hydrolase YOR131C-like	3.0E-64	TEZA-2003631
Sol_2003944	F: TGTGGAATTGTGAGCGGGGCAGTTACAGTTGCGACC R: GTTTCTTTCCTCTCTCCCGTAATAATATCCTG	$(ATT)_9$	278	UvrABC system protein A	9.0E-15	TEZA-2003944
Sol_2003951	F: CACGACGTTGTAAAACGACAATCACTCGGATCACCGGC R: GTTTCTTTGTGAATCCAGCTGTGACG	$(AC)_{10}$	242	—		TEZA-2003951
Sol_2004040	F: CACGACGTTGTAAAACGACTGGTGAGAGAACCGGACTG R: GTTTCTTCCCTCAAACAAACATGCGTC	$(AT)_{10}$	185	—		TEZA-2004040
Sol_2005892	F: CTATAGGGCACGCGTGGTACATTCATTCCTCGCAATCCC R: GTTTCTTGATTCCGTCAACGGCACAG	$(CTT)_9$	270–312	—		TEZA-2005892
Sol_2005991	F: TGTGGAATTGTGAGCGGTGCGGCTGACAATAATACACC R: GTTTCTTCCCAATTCCCATCTGGGTTC	$(GAT)_{11}$	322–340	Endoglucanase 21	6.0E-156	TEZA-2005991
Sol_2006711	F: TGTGGAATTGTGAGCGGATGAAGACGAGCTTGGCCG R: GTTTCTTGGCAACAAGCACGAACCG	$(ACT)_8$	259	—		TEZA-2006711
Sol_2006931	F: TGTGGAATTGTGAGCGGCTCTGCACCTCTTATCTGGAC R: GTTTCTTAGCCACGTTTCGTCGTTTG	$(AC)_{10}$	325–345	Scarecrow-like protein 23-like	1.0E-28	TEZA-2006931
Sol_2007258	F: TGTGGAATTGTGAGCGGCGGAAGTGGGTTTGGATCG R: GTTTCTTCATGCACGCTATGACTCGG	$(GAT)_{12}$	246–261	—		TEZA-2007258
Sol_2007291	F: TGTGGAATTGTGAGCGGCCTCCGGTCTCCGATGTTG R: GTTTCTTAACCCTAGGCAGCAGTTCC	$(ATC)_9$	376–388	—		TEZA-2007291
Sol_2007556	F: TGTGGAATTGTGAGCGGGCGTCGGCGCTTCATATC R: GTTTCTTTTCCCAACGCCTGAATCCC	$(AAG)_9$	261–285	ras-related protein RABA1f-like	4.0E-83	TEZA-2007556
Sol_2008145	F: CACGACGTTGTAAAACGACTCTCCATAACTTCCGGCCAC R: GTTTCTTAGCCCGTCATCCTATCCAC	$(AG)_{11}$	193	—		TEZA-2008145
Sol_2008565	F: CACGACGTTGTAAAACGACGTACACCAAACCCTCCATCG R: GTTTCTTCAACAGGATCCAAACCGCC	$(ACACAT)_8$	371	Uncharacterized protein	3.0E-74	TEZA-2008565
Sol_2012220	F: CACGACGTTGTAAAACGACGGCCCGGATGGTTGATTTC R: GTTTCTTGCCGAAACACCAAGGCTC	$(AC)_{12}$	410–428	Uncharacterized protein	7.0E-06	TEZA-2012220
Sol_2013037	F: TGTGGAATTGTGAGCGGGCCCTCCTGGGACATCAG R: GTTTCTTCCGTCGGTAATACGCCTGC	$(CT)_{10}$	442	—		TEZA-2013037
Sol_2013075	F: CTATAGGGCACGCGTGGTTCATGTGAAGACACGATCCG R: GTTTCTTCAAGATAAGGCAAGCTCCCAC	$(CT)_{10}$	182–186	—		TEZA-2013075
Sol_2013411	F: CACGACGTTGTAAAACGACTGTTGTGAAGAAAGTGGATACTC R: GTTTCTTCCTTGCCAACAAAGCTTGC	$(GAT)_{10}$	361–373	—		TEZA-2013411
Sol_2013527	F: CACGACGTTGTAAAACGACATCCGATCACCAACGGAGC R: GTTTCTTCCACGAATCTGTAACCGCC	$(GAT)_9$	376	Hypothetical protein	4.0E-103	TEZA-2013527
Sol_2013528	F: TGTGGAATTGTGAGCGGATCCGATCACCAACGGAGC R: GTTTCTTCCACGAATCTGTAACCGCC	$(GAT)_9$	359	Hypothetical protein	2.0E-91	TEZA-2013528

TABLE 1. Continued.

Locus		Primer sequences (5′–3′)[b]	Repeat motif	Allele size (bp)	BLASTX top hit description[c]	*E*-value	Accession no.[d]
Sol_2014047	F:	CTATAGGGCACGCGTGGTTACAATTGGCAGTCGGGTC	$(AC)_{10}$	240	—		TEZA-2014047
	R:	GTTTCTTCCGGCGGTTAAACTCCATAG					
Sol_2014215	F:	TGTGGAATTGTGAGCGGGCACAACCAGACTTGTCCC	$(AAG)_{10}$	181	Homeodomain-like superfamily protein isoform 1	4.0E-73	TEZA-2014215
	R:	GTTTCTTAAAGAGGGTTCCGGTCTTC					
Sol_2015731	F:	CACGACGTTGTAAAACGACCGTTGAAGAATGGCGGGTC	$(GAT)_9$	427	—		TEZA-2015731
	R:	GTTTCTTCCACATCTGCGTTAACATCC					
Sol_2015992	F:	CTATAGGGCACGCGTGGTGACTGGAGCTCTTGGAGGC	$(AT)_{10}$	349–355	Pyrophosphate-energized membrane proton pump 3-like	0.0E+00	TEZA-2015992
	R:	GTTTCTTAAGACCACTCTCCCAAGTCCC					
Sol_2017438	F:	CTATAGGGCACGCGTGGTAGGTTTCCATTGATTCTGGGC	$(GT)_{10}$	398	—		TEZA-2017438
	R:	GTTTCTTCCCAGGTTCTACAAACAGTCAAG					
Sol_2018697	F:	CACGACGTTGTAAAACGACTTTGGCACGTTGTTGACCG	$(ATT)_{10}$	266	ATP-dependent clp protease ATP-binding subunit clpx isoform 2	0.0E+00	TEZA-2018697
	R:	GTTTCTTGGTTCCGTTGCAAGGTAGG					
Sol_2066912	F:	TGTGGAATTGTGAGCGGACATAAGTCACCGAATTTATCAACC	$(AC)_{10}$	428–454	—		TEZA-2066912
	R:	GTTTCTTTCATACGCCATGTTTGCCG					
Sol_2069608	F:	CTATAGGGCACGCGTGGTTTCCAAACCCTAGTCCGCC	$(AT)_{10}$	400	—		TEZA-2069608
	R:	GTTTCTTGTGTTTCTTGTGGCGTTACC					
Sol_2071098	F:	CTATAGGGCACGCGTGGTTCTTGGAGGTGAGGAAAGCC	$(CT)_{11}$	258–294	Conserved hypothetical protein	9.0E-45	TEZA-2071098
	R:	GTTTCTTTGGTGTGCGTTCAAGGTTC					

[a] Annealing temperature in PCR reaction is 60°C for all loci.
[b] Forward and reverse primer sequence (with tag sequence).
[c] Putative functional annotation by the NCBI nr database search.
[d] Accession number in Plant OneKP Project database (https://sites.google.com/a/ualberta.ca/onekp/home).

TABLE 2. Characteristics of the 15 polymorphic EST-SSR markers for the *Solidago virgaurea* complex.[a]

	S. virgaurea subsp. *asiatica* var. *asiatica*[b]			*S. virgaurea* subsp. *leiocarpa* var. *leiocarpa*[b]			*S. virgaurea* subsp. *leiocarpa* var. *praeflorens*[b]			*S. yokusaiana*[b]			All (N = 93)		
Locus	A	H_e	H_o	A	H_e	H_o	A	H_e	H_o	A	H_e	H_o	A	H_e	H_o
Sol_2001876	2	0.187	0.125	3	0.081	0.083	2	0.325	0.318	2	0.287	0.261	3	0.226	0.194
Sol_2003053	3	0.322	0.375	4	0.563	0.625	2	0.351	0.273	4	0.238	0.261	4	0.570	0.387*
Sol_2003631	6	0.590	0.542*	4	0.414	0.083*	3	0.206	0.227	3	0.299	0.261	7	0.583	0.280*
Sol_2005892	7	0.767	0.583	13	0.879	0.542*	9	0.843	0.364*	5	0.681	0.435	14	0.874	0.484*
Sol_2005991	5	0.654	0.792*	3	0.525	0.458	3	0.368	0.273*	2	0.405	0.391	6	0.566	0.484*
Sol_2006931	6	0.642	0.542	8	0.753	0.667	5	0.712	0.773	4	0.635	0.522	10	0.759	0.624*
Sol_2007258	3	0.081	0.083	4	0.120	0.125	1	0	0	1	0	0	5	0.053	0.054
Sol_2007291	5	0.668	0.667	4	0.561	0.583	4	0.652	0.545	4	0.555	0.739	5	0.697	0.634*
Sol_2007556	7	0.365	0.375	5	0.360	0.333*	5	0.682	0.636	2	0.423	0.435	8	0.561	0.441*
Sol_2012220	3	0.468	0.333	4	0.263	0.250	4	0.464	0.273*	1	0	0	5	0.328	0.215*
Sol_2013075	3	0.155	0.167	3	0.119	0.125	3	0.208	0.227	2	0.083	0.087	3	0.142	0.151
Sol_2013411	3	0.155	0.125*	2	0.219	0.167	1	0	0	3	0.299	0.174	5	0.180	0.118*
Sol_2015992	3	0.421	0.333	4	0.640	0.500*	4	0.656	0.636	4	0.541	0.522	4	0.620	0.495
Sol_2066912	3	0.531	0.667	12	0.852	0.750	4	0.665	0.364*	5	0.545	0.435	12	0.799	0.559*
Sol_2071098	6	0.677	0.625	7	0.712	0.750	3	0.615	0.500	3	0.434	0.391	8	0.633	0.570
Average	4.3	0.446	0.422	5.3	0.471	0.403	3.5	0.450	0.361	3.0	0.362	0.328	6.6	0.506	0.379

Note: A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals genotyped.

[a]Vouchers representing each population, except for the Nagano population, are deposited at the Kyoto University Herbarium (KYO; accession numbers KYO 00019876 [Fukushima], KYO 00019877 [Tokyo], and KYO 00019878 [Hyogo]).

[b]Locality information and number of individuals genotyped: var. *asiatica* (Fukushima, N = 24), var. *leiocarpa* (Nagano, N = 24), var. *praeflorens* (Tokyo, N = 22), *S. yokusaiana* (Hyogo, N = 23).

*Denotes significant deviation from Hardy–Weinberg equilibrium tested with 1000 randomizations ($P < 0.01$).

5′-CTATAGGGCACGCGTGGT-3′), and the reverse primer was tagged with a PIG-tail sequence (5′-GTTTCTT-3′) to promote full adenylation (Brownstein et al., 1996). Plant DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). The PCR reaction was carried out following the standard protocol of the QIAGEN Multiplex PCR Kit (QIAGEN, Hilden, Germany), in a final volume of 10 μL, which contained approximately 5 ng of DNA, 5 μL of 2× Multiplex PCR Master Mix, and 0.01 μM of forward primer, 0.2 μM of reverse primer, and 0.1 μM of M13 primer (fluorescently labeled with Beckman Dye, Beckman Coulter, Brea, California, USA). The PCR thermal profile involved denaturation at 95°C for 3 min; followed by 35 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min; and a final 7-min extension step at 72°C. PCR products were loaded onto an autosequencer (GenomeLab GeXP, Beckman Coulter) to assess fragment lengths using Fragment Analysis Software version 8.0 (Beckman Coulter). For the 34 primer pairs that exhibited clear microsatellite peaks, extracted DNA of 93 individuals of the *S. virgaurea* complex (from four populations in Fukushima [37°41′02″N, 140°27′09″E], Nagano [36°19′59″N, 137°39′34″E], Tokyo [34°13′18″N, 139°09′28″E], and Hyogo [34°51′28″N, 135°18′53″E]; see also Table 1) was used to evaluate allelic polymorphism. In addition, transferability among the other *Solidago* species (*S. minutissima* [N = 2] from Yakushima Island, Japan [30°20′07″N, 130°30′17″E]; *S. altissima* L. [N = 2], diploid individuals from Minnesota, USA [46°51′12″N, 92°01′52″W]; *S. canadensis* [N = 1], diploid individual from Jena, Germany [50°54′40″N, 11°34′02″E]; *S. hispida* Muhl. ex Willd. [N = 1], diploid individual from Minnesota, USA [46°47′52″N, 92°04′43″W]) was tested using the same PCR conditions described above. To characterize each EST-SSR marker, three summary statistics were calculated using GenAlEx 6.5 (Peakall and Smouse, 2012): number of alleles per locus (A), expected heterozygosity (H_e), and observed heterozygosity (H_o). In addition, the significance of Hardy–Weinberg equilibrium and genotypic equilibrium were tested by 1000 randomizations with adjustment of resulting P values by sequential Bonferroni correction, using FSTAT 2.9.3 (Goudet, 1995).

Fifteen primer pairs (Table 1) were shown to be polymorphic, with A ranging from three to 14 alleles, while H_e and H_o ranged from 0.053 to 0.874 and 0.054 to 0.634, respectively (Table 2). Significant departures from Hardy–Weinberg equilibrium were detected in eight loci in the four populations, but most are specific to one or two populations (Table 2). No significant genotypic equilibrium for any pair of loci was detected. Of the 34 EST-SSR primer pairs tested, 33 were successfully PCR amplified for *S. minutissima* and 30 for each North American species of *S. altissima*, *S. canadensis*, and *S. hispida* (Table 3).

TABLE 3. Transferability of the 34 EST-SSR markers for the Eurasian and North American *Solidago* species.[a]

Locus	*S. minutissima* ($2n$, N = 2)	*S. altissima* ($2n$, N = 2)	*S. canadensis* ($2n$, N = 1)	*S. hispida* ($2n$, N = 1)
Sol_2000155	No	No	No	No
Sol_2001054	m	No	No	No
Sol_2001106	m	m	m	p
Sol_2001640	m	m	p	m
Sol_2001876	p	p	m	m
Sol_2003053	p	p	p	p
Sol_2003631	m	m	m	m
Sol_2003944	m	m	m	No
Sol_2003951	m	m	p	m
Sol_2004040	m	p	p	p
Sol_2005892	p	p	m	m
Sol_2005991	p	p	p	m
Sol_2006711	m	m	m	p
Sol_2006931	m	p	p	p
Sol_2007258	m	m	m	m
Sol_2007291	m	p	m	m
Sol_2007556	m	p	p	m
Sol_2008145	m	m	m	m
Sol_2008565	m	m	p	m
Sol_2012220	m	No	No	m
Sol_2013037	m	m	p	p
Sol_2013075	m	p	m	p
Sol_2013411	m	p	p	p
Sol_2013527	m	m	m	No
Sol_2013528	m	m	m	m
Sol_2014047	m	No	No	m
Sol_2014215	m	m	m	p
Sol_2015731	m	m	m	m
Sol_2015992	m	p	m	m
Sol_2017438	m	p	m	m
Sol_2018697	m	m	p	m
Sol_2066912	p	p	p	m
Sol_2069608	m	p	m	m
Sol_2071098	p	m	p	m
	33/34 (6)	30/34 (14)	30/34 (13)	30/34 (9)

Note: No = no PCR amplification; m = monomorphic (only one allele was detected); p = polymorphic (more than one allele was detected).

[a]Vouchers for these samples are not available.

CONCLUSIONS

We developed 15 polymorphic EST-SSR markers for the *S. virgaurea* complex, most of which are transferable in different *Solidago* species. These markers may be useful for evaluating the population structure and taxonomic delimitation of the *S. virgaurea* complex, as well as providing useful markers to investigate the population genetics and reproductive ecology of *Solidago* species.

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Appendix 1. Voucher and locality information of the plant samples used for the initial PCR amplification trials.

Taxon	Locality	GPS coordinates	Voucher no.
S. virgaurea subsp. *asiatica* var. *asiatica* Nakai ex H. Hara	Fukushima, Fukushima, Japan	37°41′02″N, 140°27′09″E	KYO 00019876
S. virgaurea subsp. *asiatica* var. *asiatica* Nakai ex H. Hara	Sakaide City, Kagawa, Japan	34°20′35″N, 133°53′24″E	KYO 00019881
S. virgaurea subsp. *asiatica* var. *insularis* (Kitam.) Hara	Kunigami-gun, Okinawa, Japan	26°29′38″N, 127°54′49″E	KYO 00019882
S. virgaurea subsp. *gigantea* (Nakai) Kitam.	Akita City, Akita, Japan	39°48′53″N, 140°04′03″E	KYO 00019883
S. virgaurea subsp. *leiocarpa* var. *leiocarpa* (Benth.) A. Gray	Azumino, Nagano, Japan	36°19′59″N, 137°39′34″E	Not available
S. virgaurea subsp. *leiocarpa* var. *praeflorens* Nakai	Kodu, Tokyo, Japan	34°13′18″N, 139°09′28″E	KYO 00019877
S. yokusaiana Makino	Takarazuka, Hyogo, Japan	34°51′28″N, 135°18′53″E	KYO 00019878
S. yokusaiana Makino	Yakushima, Kagoshima, Japan	30°15′55″N, 130°34′49″E	KYO 00019880