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ORIGINAL RESEARCH PAPER

Genetic and phenotypic characterizations of *Xenorhabdus* species (Enterobacteriales: Enterobacteriaceae) isolated from steinernematid nematodes (Rhabditida: Steinernematidae) in Japan

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Abstract The bacterial species of the genus *Xenorhabdus* in the family Enterobacteriaceae have a mutualistic association with steinernematid entomopathogenic nematodes (EPNs), which have been used as biological control agents against soil insect pests. In this study we present the genetic and phenotypic characterizations of the *Xenorhabdus* species isolated from steinernematid nematodes in Japan. The 18 Japanese *Xenorhabdus* isolates were classified into five bacterial species based on 16S ribosomal RNA (16S rRNA) gene sequences: *Xenorhabdus bovienii*, *Xenorhabdus hominickii*, *Xenorhabdus indica*, *Xenorhabdus ishibashii*, and *Xenorhabdus japonica*. There was no genetic variation between the 16S RNA sequences among the three *X. ishibashii* isolates, 0–0.1% variation among the five *X. hominickii* isolates, and 0–0.5% among the eight *X. bovienii* isolates. Phenotypic characterization demonstrated that representative isolates of the five bacterial species shared common characteristics of the genus *Xenorhabdus*, and only *X. hominickii* isolates produced indole. Symbiotic association and co-speciation of *Xenorhabdus* bacteria with *Steinernema* nematodes from Japan are discussed.

The DNA Data Bank of Japan accession numbers for the 16S ribosomal RNA gene are: AB507811-AB507818.

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Introduction

Bacterial species of the genus *Xenorhabdus* Thomas and Poinar 1979 in the family Enterobacteriaceae have a mutualistic association with steinernematid entomopathogenic nematodes (EPNs) (Forst and Clarke 2002). They commonly colonize the nematode intestine, and the steinernematid nematodes show strong insecticidal activities against a diverse group of insect larvae in cooperation with the symbiotic *Xenorhabdus* bacteria (Dowds and Peters 2002). After infective juvenile (IJ) nematodes enter the hemocoel of host insect larvae, the nematodes release *Xenorhabdus* bacteria that are harbored in the nematode intestine into the host hemocoel. The released bacteria multiply and produce a wide variety of metabolites, including toxins that cause death in the host insect. Nematodes grow and reproduce in the insect cadaver by feeding on the propagated bacteria and on the insect tissue digested by the bacteria. Then the reproduced IJs retaining the specific symbiont bacteria in their intestine leave the cadaver in search of a new host. Because of their strong insecticidal activity and lack of toxicity for vertebrates, these nematodes have been used as a biological control agent against soil insect pests (Ehlers 2005).

Since the first description of *Xenorhabdus* bacteria (Poinar and Thomas 1965; Thomas and Poinar 1979), 24 *Xenorhabdus* species have been described worldwide (Akhurst 1983, 1986; Akhurst and Boemare 1988; Ferreira et al. 2013; Kuwata et al. 2013; Lengyel et al. 2005; Nishimura et al. 1994; Somvanshi et al. 2006; Tailliez et al. 2006; 2012). In Japan, more than ten steinernematid

species have been reported, and two EPNs, *Steinernema carpocapsae* (Weiser 1955) Wouts et al. 1982 and *Steinernema glaseri* (Steiner 1929) Wouts et al. 1982, have been introduced as biocontrol agents against several kinds of insect pest (Yoshida 2010). Based on molecular phylogenetic analysis, we previously reported that four *Xenorhabdus* species are distributed in Japan (Kuwata et al. 2006b). After the report, we described a new *Xenorhabdus* species, *Xenorhabdus ishibashii* Kuwata et al. 2013, from Japan and China. Furthermore, the distribution of *Steinernema abbasi* Elawad et al. 1997, in Japan has been reported (Yoshida 2007) but there is no information on the symbiotic bacteria of the nematode. In this study, to understand the phylogenetic relationship of *Xenorhabdus* isolates from Japan and the genetic variations among them, we analyzed 16S ribosomal RNA (16S rRNA) gene sequences from 18 *Xenorhabdus* isolates from ten *Steinernema* nematode species in Japan. In addition, to compare and clarify the phenotypic differences between *Xenorhabdus* isolates, especially the isolates that belong to the same bacterial species but were isolated from different nematode species, we characterized the biochemical and physiological phenotypes of bacterial isolates.

Materials and methods

Isolation of *Xenorhabdus* bacteria from steinernematid nematodes

Host steinernematid nematodes that retain *Xenorhabdus* bacteria were obtained using a baiting technique from soil samples (Bedding and Akhurst 1975) and were identified based on morphological features and polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) profiles (Yoshida et al. 1998) (Fig. 1). These host nematodes were cultured in vivo using larvae of the greater wax moth, *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae) (Kaya and Stock 1996), and harvested IJs were washed and stored in distilled water at 15 °C.

Approximately 50 IJs of each nematode isolate were immersed in a 0.1% merthiolate solution for 2 h, washed in sterile saline, and crushed in a small amount of sterile Luria-Bertani (LB) broth (pH 7.0) to release bacteria from the nematode intestine. LB broth (200 µL) was added to the suspension, which was spread on an nitroblue tetrazolium agar plate (NBTA) for static culture at 25 °C (Akhurst 1980). Single bacterial colonies were successively picked and streaked on a new NBTA until no contamination was observed. EPNs have basically retained a single bacterial species in their intestine and formed a highly species-specific association (Boemare 2002, Forst and Clarke 2002), and one single colony was picked up as a representative for each bacterial isolate. After obtaining single bacterial colonies, each bacterial isolate was examined for genetic and phenotypic traits.

In this study, 18 Japanese *Xenorhabdus* isolates were used for genetic characterization, and 11 isolates were used for phenotypic characterization (Fig. 1, Table 1). Among the 18 isolates, the 16S rDNA sequences of ten bacterial isolates had already been reported (Kuwata et al. 2006b), and eight bacterial isolates had been newly obtained for this study (Table 1).

Phylogenetic characterization of *Xenorhabdus* bacteria

DNA was extracted from each bacterial isolate as described previously (Kuwata et al. 2006b). Briefly, cells from 5 mL of a 24-h bacterial culture were lysed in 500 µL buffer comprising 0.1 M TRIS–HCl, 0.1 M KCl and 20 mM ethylenediaminetetraacetic acid (EDTA)-Na (pH 8.0) containing 0.1 mg lysozyme and 10 µg RNase A at 37 °C for 20 min. After the addition of 0.25 mL of 10% sarkosyl to the bacterial lysate, the bacterial DNA was purified by phenol/chloroform extraction and ethanol precipitation, and finally, was dissolved in 500 µL of TRIS–EDTA buffer.

The 16S rRNA gene sequences of the 18 Japanese *Xenorhabdus* isolates were determined as described (Kuwata et al. 2006b): PCR amplifications were performed in a final volume of 50 µL containing 1 unit of Takara ExTaq (Takara, Shiga, Japan), 5 µL of 10× PCR buffer, 4 µL of deoxynucleotide triphosphate mixture (2.5 mM), 1 µM of each primer, and 100 ng of template DNA. Nucleotide sequences of the PCR primers were 16S-F (5′-GAA GAG TTT GAT CAT GGC TC-3′) and 16S-R (5′-AAG GAG GTG ATC CAG CCG CA-3′) (Fischer-Le Saux et al. 1999). The PCR amplicons were gel purified using the MonoFas DNA Purification Kit I (Gl Science, Tokyo) and were sequenced directly with six internal primers using the ABI PRISM BigDye Terminator Cycle Sequencing Kit version 1.1 and the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) on both strands. DNA sequences determined in this study were deposited in the DNA Data Bank of Japan under the accession numbers AB507811-AB507818.

Multiple sequence alignment matrices were created using ClustalX in default configuration (Larkin et al. 2007). The substitution model JC69 (Jukes and Cantor 1969) was determined as GTR + I + G using jModelTest 2.1.10 (Darriba et al. 2012). The aligned matrix data were analyzed by maximum likelihood (ML) algorithms using the web server software PhyML 3.0 (Guindon et al. 2010). The tree was represented graphically using the TreeView version 1.6.6 software (Page 2001).

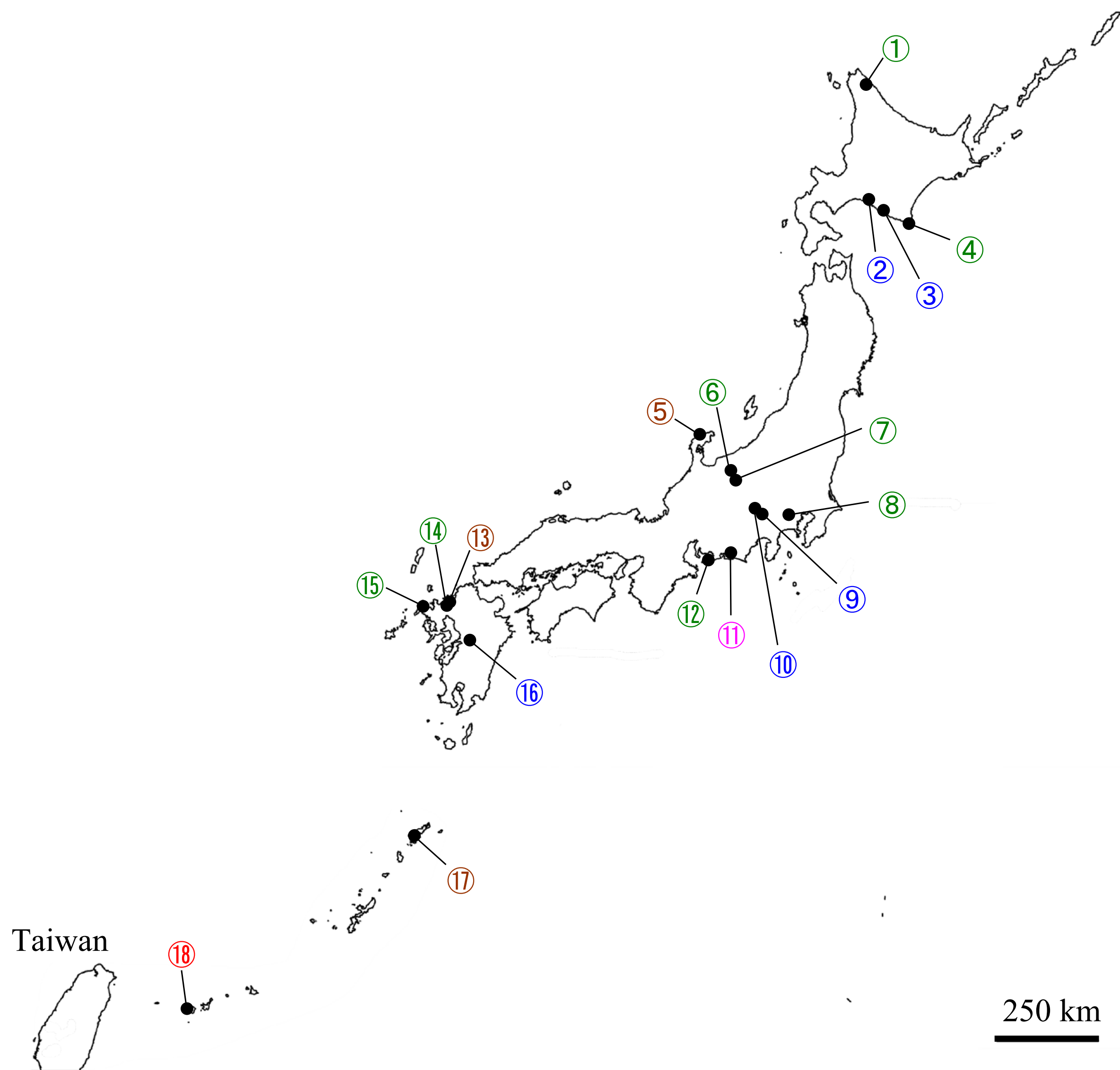

Fig. 1 Collection localities of steinernematid nematodes in Japan. *Numbers at each site* correspond with those shown in Table 1

Biochemical and physiological characterization of *Xenorhabdus* bacteria

Eleven Japanese *Xenorhabdus* isolates were used for phenotypic characterization (Table 1). The biochemical phenotypic test was performed at 28 °C, and the traits were determined after 48 h of incubation. Dye absorption was assayed on NBTA (Kaya and Stock 1996). DNase activity and utilization of citrate were tested on DNase agar and Simmons' citrate agar, respectively (Eiken Chemical, Tokyo). Urease activity was assessed in 5 mL of urea broth (Eiken Chemical). Catalase activity was assayed by dropping of H_2O_2 onto each bacterial colony. Acid production and carbon source assimilation were investigated using API 20E and API 50CH strips (BioMerieux, Marcy-l'Étoile, France) according to the manufacturer's instructions. Following static culture on NBTA, the maximum temperature for growth was determined in LB broth that had been incubated in a water bath with a temperature accuracy of ±0.2 °C for 48 h. To compare the phenotypic traits of the Japanese *Xenorhabdus* isolates with those of previously described *Xenorhabdus* species, we refer to data from the previous report (Tailliez et al. 2006).

Table 1 Japanese *Xenorhabdus* species and isolates used in this study

Species	Isolates	Host nematode	Location	Site[a]	Accession no.	References (source)
Xenorhabdus bovienii	HkEr36[b]	*Steinernema feltiae*	Erimo, Hokkaido	4	AB243428[c]	Kuwata et al. (2006b), Yoshida (2010)
	HkHm22[b]	*Steinernema kraussei*	Hamatonbetsu, Hokkaido	1	AB243429[c]	Kuwata et al. (2006b), Yoshida (2010)
	AiAt199[b]	*Steinernema litorale*	Atsumi, Aichi	12	AB243430[c]	Kuwata et al. (2006b), Yoshida (2010)
	Mamiya[b]	*Steinernema* sp. MY3	Hachioji, Tokyo	8	AB243431[c]	Kuwata et al. (2006b), Yoshida (1998)
	YBKO	*Steinernema* sp. MY3	Yobuko, Saga	15	AB507817	This work
	NnMt2s[b]	*Steinernema* sp. MY6	Matsumoto, Nagano	7	AB243433[c]	Kuwata et al. (2006b), Yoshida (1998)
	NnOm36[b]	*Steinernema* sp. MY7	Omachi, Nagano	6	AB243434[c]	Kuwata et al. (2006b), Yoshida (1998)
	SM	*Steinernema* sp. MY7	Nijo, Fukuoka	14	AB507818	This work
Xenorhabdus hominickii	HkNk135	*Steinernema monticolum*	Niikappu, Hokkaido	3	AB507814	This work
	YnEn94	*Steinernema monticolum*	Enzan, Yamanashi	9	AB243425[c]	Kuwata et al. (2006b), Yoshida (1998)
	KmYb11[b]	*Steinernema monticolum*	Yabe, Kumamoto	16	AB507815	This work
	HkBt139	*Steinernema ashiuense*	Biratori, Hokkaido	2	AB507816	This work
	YnEn68[b]	*Steinernema ashiuense*	Enzan, Yamanashi	10	AB243432[c]	Kuwata et al. (2006b), Yoshida (2010)
Xenorhabdus indica	OnIr181[b]	*Steinernema abbasi*	Iriomote, Okinawa	18	AB507813	This work
Xenorhabdus ishibashii	IkWj136[b]	*Steinernema aciari*	Wajima, Ishikawa	5	AB243427[c]	Kuwata et al. (2006b), (2013)
	NJ	*Steinernema aciari*	Nijo, Fukuoka	13	AB507811	This work
	KsSu155	*Steinernema aciari*	Setouchi, Kagoshima	17	AB507812	This work
Xenorhabdus japonica	Hamakita[b]	*Steinernema kushidai*	Hamakita, Shizuoka	11	AB243426[c]	Kuwata et al. (2006b), Yoshida (1998)

[a] Numbers corresponding to those in Fig. 1

[b] Bacterial isolates used for phenotypic characterization

[c] Previously reported sequence data (Kuwata et al. 2006b)

Results

Genetic characterization of Japanese *Xenorhabdus* isolates

The 18 *Xenorhabdus* bacterial isolates from ten steinernematid nematode species were put into five bacterial groups (*X. bovienii*, *Xenorhabdus hominickii*, *X. indica*, *X. ishibashii*, and *X. japonica*), each of which showed a species-level concordance rate with known species (Fig. 2). There was 0–0.5% genetic variation between the 16S rRNA gene sequences among the eight *X. bovienii* isolates, 0–0.1% among the five *X. hominickii* isolates, and no genetic variation among the three *X. ishibashii* isolates (Table 2).

Biochemical and physiological characterization of Japanese *Xenorhabdus* isolates

The maximum growth temperature of *X. indica* OnIr181 and *X. ishibashii* IkWj136 was the highest (41 °C) among the Japanese isolates tested, followed by *X. hominickii* YnEz94 and YnEz68 (35 °C), *X. japonica* Hamakita (34 °C), and six isolates of *X. bovienii* (33–35 °C) (Table 3).

Eleven Japanese bacterial isolates showed the following typical characteristics of *Xenorhabdus* species by plate assays and the API20 test: negative to catalase, urease, *o*-nitrophenol *b*-D-galactopyranoside, acetoin (Voges-Proskauer reaction), and H_2S production (Table 3). All bacterial isolates used in this study showed dye absorption

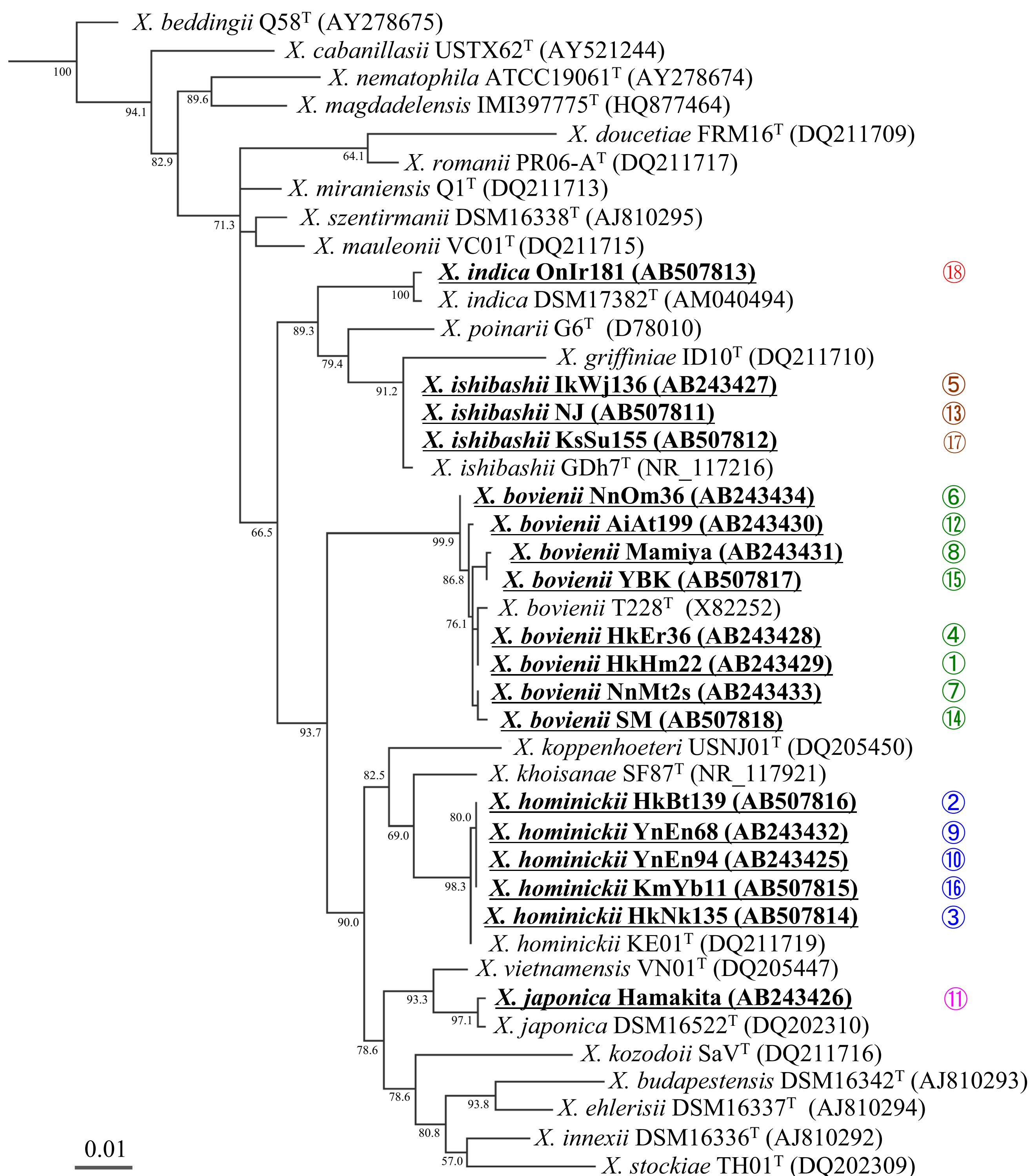

Fig. 2 Phylogenetic maximum likelihood tree among isolates of *Xenorhabdus* bacteria based on 16-S ribosomal RNA (16S rDNA) sequences (bootstrap consensus). The 16S rDNA sequences of the *Photorhabdus asymbiotica* strain CbKj163 (AB222085) and *Photorhabdus luminescens* subsp. *laumondii* strain TT01 (NR_112706) were used as outgroup species (not shown). The Japanese *Xenorhabdus* isolates are *underlined* and in *bold*. *Numbers in parentheses* indicate the GenBank accession number. *Numbers after accession numbers* indicate collection sites shown in Table 1 and Fig. 1. Values > 50% at the branch points indicate in percentages how often the corresponding cluster was found among the 100 intermediate trees. The *bar* represents 0.01 substitutions per site

Table 2 Divergence of the 16S rDNA sequences among Japanese *Xenorhabdus* isolates

Species	No.	Isolate name	Number																	
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
X. bovienii	1	HkEr36 [a]	-	0.0	0.2	0.3	0.3	0.1	0.3	0.3	2.7	2.8	2.8	2.8	2.8	3.8	3.2	3.2	3.2	3.1
	2	HkHm22 [a]	0	-	0.2	0.3	0.3	0.1	0.3	0.3	2.7	2.8	2.8	2.8	2.8	3.8	3.2	3.2	3.2	3.1
	3	AiAt199 [a]	3	3	-	0.4	0.3	0.2	0.2	0.3	2.6	2.7	2.7	2.7	2.7	3.7	3.1	3.1	3.1	3.0
	4	Mamiya [a]	5	5	6	-	0.1	0.2	0.5	0.3	2.9	3.0	3.0	3.0	3.0	3.7	3.1	3.1	3.1	3.0
	5	YBK	4	4	5	1	-	0.3	0.4	0.4	2.9	2.9	2.9	2.9	2.9	3.7	3.2	3.2	3.2	2.9
	6	NnMt2s [a]	2	2	3	3	4	-	0.3	0.1	2.8	2.9	2.9	2.9	2.9	3.8	3.1	3.1	3.1	3.1
	7	NnOm36 [a]	4	4	3	7	6	4	-	0.4	2.7	2.7	2.7	2.7	2.7	3.6	3.1	3.1	3.1	2.9
	8	SM	4	4	5	5	6	2	6	-	2.8	2.9	2.9	2.9	2.9	3.8	3.1	3.1	3.1	3.1
X. hominickii	9	HkNk135	41	41	39	44	43	42	40	42	-	0.1	0.1	0.1	0.1	3.3	3.1	3.1	3.1	2.7
	10	YnEn94 [a]	42	42	40	45	44	43	41	43	1	-	0.0	0.0	0.0	3.3	3.1	3.1	3.1	2.7
	11	KmYb11	42	42	40	45	44	43	41	43	1	0	-	0.0	0.0	3.3	3.1	3.1	3.1	2.7
	12	HkBt139	42	42	40	45	44	43	41	43	1	0	0	-	0.0	3.3	3.1	3.1	3.1	2.7
	13	YnEn68 [a]	42	42	40	45	44	43	41	43	1	0	0	0	-	3.3	3.1	3.1	3.1	2.7
X. indica	14	OnIr181	57	57	55	56	55	57	54	57	49	50	50	50	50	-	2.1	2.1	2.1	3.2
X. ishibashii	15	IkWj136 [a]	48	48	47	47	48	46	47	46	46	46	46	46	46	32	-	0.0	0.0	3.7
	16	NJ	48	48	47	47	48	46	47	46	46	46	46	46	46	32	0	-	0.0	3.7
	17	KsSu155	48	48	47	47	48	46	47	46	46	46	46	46	46	32	0	0	-	3.7
X. japonica	18	Hamakita [a]	46	46	45	45	44	46	43	46	40	41	41	41	41	48	56	56	56	-

Above diagonal indicates the percentage of nucleotide differences and below diagonal indicates the number of nucleotide differences. Dotted line separates the bacterial species, and gray boxes indicate intraspecific variation

[a] The 16S rDNA sequences of these isolates are previously reported (Kuwata et al., 2006b)

on NBTA and DNase activity on DNase agar. Some characteristics of fermentation and carbon source assimilation were different among the five isolates of *X. bovienii*: acid production from ribose, maltose, and trehalose and assimilation of ribose and citrate (Table 3). Only the *X. hominickii* isolates YnEz94 and YnEz68 produced indole.

Discussion

Xenorhabdus bovienii

Bacterial species of *Xenorhabdus bovienii* (Akhurst 1983) Akhurst and Boemare 1993 have been isolated from several steinernematid species worldwide (Boemare 2002; Fischer-Le Saux et al. 1998). The phylogenetic analysis in this study showed that the eight bacterial isolates from six steinernematid nematode species *Steinernema feltiae* (Filipjev 1934) Wouts et al. 1982, *Steinernema kraussei* (Steiner 1923) Travassos 1927, *Steinernema litorale* Yoshida 2004, and undescribed nematode species of *Steinernema* spp. MY3, MY6, and MY7 (Yoshida et al. 1998) (Table 1) formed a cluster with *X. bovienii* (Fig. 2). Interestingly, all of those nematodes, except *Steinernema* sp. MY7, belong to the feltiae group (Kuwata et al. 2006a; Yoshida et al. 1998). *Steinernema* sp. MY7 is phylogenetically different from them and related to *Steinernema affine* (Bovien 1937) Wouts et al. 1982 and *Steinernema intermedium* (Poinar 1985) Mamiya 1988 belonging to the intermedium group (Kuwata et al. 2006a; Yoshida et al. 1998). *X. bovienii* is also the symbiotic bacteria from *S. affine* and *S. intermedium* (Tailliez et al. 2006). Thus, phenotypic characterization was compared. The maximum growth temperatures of *Xenorhabdus bovienii* isolates in this study range from 33 to 35 °C and are lower than those of *Xenorhabdus ehlersii*, *Xenorhabdus griffinae*, *Xenorhabdus ishibashii*, and *Xenorhabdus indica* (Table 3), which agrees with previous findings that *X. bovienii* typically grows at relatively low temperatures (Akhurst 1983; Boemare 2002). These data corresponded with the fact that some host nematodes, *S. feltiae*, *S. litorale*, *Steinernema* spp. MY3 and MY6 also show high pathogenicity at low temperature (< 20 or 25 °C) (Yoshida 2010). Moreover, *S. kraussei* is known as a EPN active at cold temperatures (Long et al. 2000). There were no remarkable differences between the biochemical characteristics and genetic variations of the bacterial isolate NnOm36 from *Steinernema* sp. MY7 and other *X. bovienii* isolates. As mentioned in Boemare (2002), the nematodes of the "feltiae group and the intermedium group are phylogenetically different but both form mutualistic associations with the same bacterial symbionts of *X. bovienii*. Further study is necessary to clarify the mechanisms of the non cospeciated adaptation of the bacterial species of *X. bovienii*.

The present study revealed that the bacteria *X. bovienii* associates with different steinernematid nematode species, which are widely distributed in Japan; *S.*

Table 3 Phenotypic characteristics of Japanese *Xenorhabdus* isolates

Characteristics	*Xenorhabdus* species or isolates																
	X. bovienii[a]	HkEr36	HkHm22	AiAt199	Mamiya	NnMt2s	NnOm36	*X. hominickii*[a]	YnEz94	YnEz68	*X. indica*[a]	OnIr181	*Xenorhabdus ehlersii*[a]	*Xenorhabdus griffinae*[a]	*X. ishibashii* IkWj136[b]	*X. japonica*[a]	Hamakita
Maximum growth (ºC)	32–33	33	33	35	34	33	33	33–35	35	35	39–41	41	38–40	39	41	34	34
Dye absorption	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dnase activity	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease activity	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Simmons' citrate	v(−)	−	−	−	w	−	−	−	−	w	−	−	v(−)	+	+	−	−
Indole production	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−
Acid production from																	
Ribose	v(−)	+	−	+	−	−	+	v(−)	+	+	−	−	v(+)	+	−	+	−
Inositol	v(−)	+	+	+	+	+	+	−	+	+	+	w	−	−	−	−	−
Sorbitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Maltose	v(+)	+	+	+	−	+	+	v(+)	+	+	−	−	v(+)	−	−	+	+
Trehalose	v(−)	+	+	+	+	−	+	−	w	w	−	−	−	−	−	−	w
Gluconate	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5-Ketogluconate	v(−)	+	w	+	w	+	+	+	+	+	+	−	v(+)	w	+	+	−
Assimilation of																	
Fructose	v(+)	+	+	+	+	+	+	v(−)	w	+	v	w	v(−)	−	−	+	+
Maltose	v(+)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ribose	v(−)	−	−	−	w	−	+	v(−)	−	−	−	−	v(−)	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−	−	+	w	−	w	−	+	w
Citrate	v(−)	w	w	−	−	w	−	−	−	−	−	−	v(+)	+	+	−	−

+ 90–100% Positive, (+) 76–89% positive, d 26–75% positive, (−) 11–25% positive, − 0–10% positive, *v* variable, *w* weakly positive

[a] Characteristic data of the *Xenorhabdus* species from Tailliez et al. (2006)

[b] Characteristic data of the *Xenorhabdus* species from Kuwata et al. (2013)

litorale occurs in Kyushu, Honshu and Hokkaido, *S. feltiae* and *S. kraussei* in Hokkaido, *Steinernema* sp. MY3 in Kyushu, Shikoku, Honshu and Hokkaido, and *Steinernema* sp. MY7 in Kyushu, Honshu and Hokkaido (Yoshida et al. 1998; Yoshida 2003a, b, 2004, 2007, 2010). These results suggest that *X. bovienii* is highly adaptive to steinernematids and environmental changes, and appears to be the most successful and predominant *Xenorhabdus* species in the cool to temperate zones in Japan.

Xenorhabdus hominickii

Xenorhabdus hominickii Tailliez et al. 2006 was described as a bacterial symbiont of *Steinernema karii* Waturu et al. 1997 from Kenya, *Steinernema monticolum* Stock et al. 1997 from Korea, and *Steinernema ashiuense* Phan et al. 2006 from Japan (Kuwata et al. 2006b, Yoshida et al. 1998). The present study revealed that all five *X. hominickii* isolates (HkNk135, HkBt139, YnEn94, YnEn68, and KmYb11) from *S. monticolum* and *S. ashiuense* were genetically close (Table 2). Only YnEn94 and YnEn68 among the *Xenorhabdus* isolates tested produced indole, which is characteristic for *X. hominickii* among the described *Xenorhabdus* species (Tailliez et al. 2006). Furthermore, the upper temperature limit to growth and biochemical characteristics, except for utilization of inositol, were almost identical among the two bacterial isolates and *X. hominickii* (Table 3). Based on these 16S rDNA sequences and phenotypic data, we conclude that the symbionts associated with *S. monticolum* and *S. ashiuense* are *X. hominickii* with very similar characteristics. The two nematode species were phylogenetically closely related (Phan et al. 2006), and *S. monticolum* and *S. ashiuense* appear to have co-speciated from a common ancestor associated with *X. hominickii*.

Xenorhabdus indica

Xenorhabdus indica Somvanshi et al. 2006 was initially described from *Steinernema thermophilum* Ganguly and Singh 2000 in India, which was a junior synonym of *Steinernema abbasi* (Elawad et al. 1997). After the first description of *X. indica*, this species has also been isolated from *S. abbasi* in Oman (Tailliez et al. 2006) and Taiwan (Tsai et al. 2008) and from *Steinernema yirgalemense* Nguyen et al. 2005 in South Africa (Ferreira et al. 2016). The bacterial isolate, OnIr181, from *S. abbasi* in Japan formed a cluster with *X. indica* in phylogenetic analysis (Fig. 2) and showed three nucleotide differences compared with the type strain 28^T (=DSM 17382^T; GenBank accession no. AM040494) in the 16S rRNA gene sequences. Phenotypic traits of the bacterial isolate OnIr181, including a higher upper-growth-limit temperature of 41 °C, were similar to those of *X. indica* (Table 3). The host nematode from Japan also shows high pathogenicity at a high temperature, i.e. > 25 °C (Yoshida 2007, 2010). Because *X. indica* was isolated only from *S. abbasi*, which has been isolated only from the most southern area of Japan, the distribution of *X. indica* in Japan seems to be limited to this subtropical area.

Xenorhabdus ishibashii

Xenorhabdus ishibashii Kuwata et al. 2013 has been described as a symbiont of *Steinernema aciari* Qiu et al. 2005 from China and Japan. There was no intraspecific genetic variation in the 16S rRNA gene sequences among the three bacterial isolates (IkWj136, NJ, and KsSu155, Table 2), and all three showed a two nucleotide substitution from the Chinese strain, $GDh7^T$ (Kuwata et al. 2013). Their low genetic diversity and limited distribution in East Asia suggests that the *S. aciari*-*X. ishibashii* complex is relatively new and has spread from East Asia relatively recently.

Xenorhabdus japonica

Xenorhabdus japonica Nishimura et al. 1995 was isolated from *Steinernema kushidai* Mamiya 1988 in Japan. *S. kushidai* was also detected in the coastal regions of Kagoshima (Yaku-shima Island), Kochi, Wakayama, Aichi, and one mountainous site in Nagano (Yoshida et al. 1998; Yoshida 2010). Two isolates from Kochi were investigated for their pathogenicity against white grubs, and also showed pathogenicity similar to *S. kushidai*'a Hamakita (M. Yoshida, unpublished data).

Conclusion

In this study, we have described the genetic and phenotypic characterization of five Japanese *Xenorhabdus* species: *X. bovienii*, *X. hominickii*, *X. indica*, *X. ishibashii*, and *X. japonica*. Our present study and Yoshida (2010) indicate the geographic distribution of these bacterial species in Japan; *X. bovienii* and *X. hominickii* are widespread from northern (Hokkaido) to southern (Kyusyu Island) parts of Japan, *X. ishibashii* and *X. japonica* are distributed mainly in southwestern parts of Japan, and *X. indica* is limited to Okinawa Island. The distribution of the host nematodes and their symbiotic bacteria appears to be highly influenced by the ambient temperatures of their habitats. The present study may provide useful information for the use of indigenous EPNs and their symbiotic bacteria as biocontrol agents under different climate conditions in Japanese agricultural systems.

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