Genotyping of intraspecies polymorphisms of *Sporothrix globosa* using partial sequence of mitochondrial DNA

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Abstract
Restriction fragment length polymorphism (RFLP) of mitochondrial DNA (mtDNA) had been used for molecular identification of *Sporothrix* spp., which is the causative fungi of sporotrichosis and the most prevalent deep-seated dermatomycosis. Also, mtDNA-RFLP had been used to investigate the molecular epidemiology of sporotrichosis. While the current standard for molecular diagnosis is performed by sequence analysis of the calmodulin gene (CAL), correspondence between the results from CAL and mtDNA is of diagnostic and epidemiological interest. Here, we investigated the correspondence between CAL and mtDNA used for molecular identification of *Sporothrix globosa* and *S. schenckii*, which are two major species. We also investigated and propose molecular markers suitable to describe the epidemiology of *S. globosa*, which is considered as a species with few intraspecific polymorphisms. Eighty-seven strains morphologically identified as *S. schenckii sensu lato* were investigated. They were identified as group A (17 types, 17 strains) or B (14 types, 70 strains) by mtDNA-RFLP. Partial sequences of CAL, internal transcribed spacer, and spacer between atp9 and cox2 genes of mtDNA of these strains were determined. All group A strains corresponded to *S. schenckii*, and group B to *S. globosa*. The sequences of the amplicons targeted on the spacer region in mtDNA of *S. globosa* ranged 510–515 bp in length and exhibited 10 molecular variations, whereas CAL indicated seven molecular variations. In conclusion, most of the *S. schenckii sensu lato* strains isolated from Japanese sporotrichosis patients were confirmed as *S. globosa*, because group B, which comprised the majority of strains, matched perfectly with *S. globosa* by the CAL sequencing study. We proposed sequence variations in the spacer between atp9 and cox2 genes of mtDNA as a suitable molecular epidemiological marker for *S. globosa*.

KEYWORDS
calmodulin gene, genotyping, mitochondrial DNA, *Sporothrix globosa*, *Sporothrix schenckii*

1 | INTRODUCTION

Sporotrichosis is the most predominant and worldwide deep-seated dermatomycosis. The causative fungi, *Sporothrix* spp., which inhabits soil, causes lesions when inoculated into skin or subcutaneous tissue by tiny wounds. *Sporothrix schenckii* had long been regarded as the only species causing sporotrichosis until Marinon et al. conducted molecular characterization of morphologically identified *S. schenckii* isolates using several genes including calmodulin (CAL), and proposed a new taxonomy comprising *S. schenckii* (sensu stricto) with...
some new species, *S. brasiliensis*, *S. globosa*, and *S. mexicana*. Now, the taxonomy morphologically identified as *S. schenckii* is understood to be a species complex (*S. schenckii sensu lato*). Historically, Ishizaki et al. investigated genetic polymorphisms between *S. schenckii sensu lato* strains by restriction enzyme fragment length polymorphisms (RFLP) of mitochondrial (mt)DNA from the late 1980s to early 2000s, and revealed two major groups, A and B, in the species. Later, groups A and B were divided into 17 genotypes and 14 genotypes, respectively, by studies using isolates from many countries in the four continents, Eurasia, the Americas, Africa, and Australia. Although the method using DNA extracted from the mitochondrial fraction recovered from homogenized fungal cells may be considered obsolete, it is still considered the most sensitive method for investigating intraspecific polymorphisms.

Here, we investigated how genotypes defined by RFLP of mtDNA correspond to the latest taxonomy composed of *S. schenckii* and *S. globosa*, the latter being the most important causative species of sporotrichosis in Asia including Japan. We examined a partial sequence of mtDNA, where the existence of diversity was predicted in a previous sequence study, to determine whether it may be used for genotyping *S. globosa* to study the epidemiology of sporotrichosis.

## METHODS

### 2.1 Fungal strains

Thirty-one strains of *S. schenckii sensu lato* maintained in our department (Table 1) were selected. They were identified as *S. schenckii* based on their morphological characteristics when they were registered at our department, and their genotypes were determined by RFLP of mtDNA (Mt-RFLP types). The panel of 31 strains comprised a representative strain of each of 31 Mt-RFLP types; among them, 17 genotypes were classified as group A and 14 as group B. These isolates originated from Japan, the USA, China, Australia, Argentina, Mexico, Venezuela, Costa Rica, South Africa, and India.

An additional 56 group B strains isolated from different regions of Japan were included in this study (Table 2). Overall, 17 strains in group A and 70 in group B were investigated.

### 2.2 Preparation of template DNA

Fungal DNA was extracted from colonies grown on potato dextrose agar slants or plates, as previously described with slight modification. Briefly, small amounts of mycelial mat rinsed with 70% ethanol were ground in 200 μl of lysis buffer (200 mmol/L Tris-HCl, pH 7.5, 0.5% sodium dodecylsulfate, 250 mmol/L NaCl, 25 mmol/L ethylenediaminetetraacetic acid). The homogenates were heated at 100°C for 5 min, followed by the addition of 100 μl of 3 mol/L sodium acetate (pH 7.0), centrifuged, and 300 μl of isopropanol was added to the supernatant. The precipitated DNA pellets were washed in 70% ethanol, dried, and dissolved in 100 μl of 10 mmol/L Tris-HCl (pH 8.0) solution.

### 2.3 Species identification by CAL and internal transcribed spacer (ITS) of ribosome RNA genes

Partial sequence of CAL was determined with primers CL1 and CL2A, and two supplemental primers f1 and r1 designed for 3′- and 5′-ends (Table 3). Sequences near the 3′-end were determined with primers CL2A and f1 and near the 5′-end with primers CL1 and r1, respectively. The polymerase chain reaction (PCR) conditions included an initial cycle of 5 min at 94°C, followed by 35 cycles of 50 s at 94°C, 50 s at 55°C, 1 min at 72°C, and a single extension of 7 min at 72°C. The sequence of ITS of ribosomal RNA gene was determined with primers ITS1 and ITS4 (Table 3) as described. If the strains whose nucleotide sequence did not completely match with the National Center for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov/BLAST/), their conidial shape, assimilation pattern, and limitation of growth temperature were examined for species level identification.

### 2.4 Genotyping using mtDNA

A primer pair 975-8038F and 975-9194R (Table 3) was used for amplification of intergenic spacer region between atp9 and cox2 genes of mtDNA (Figure 1) with the PCR conditions as follows: degeneration at 94°C for 4 min, then 35 cycles of 1 min at 94°C, 2 min at 58°C, and 1.5 min at 72°C. The targeted region revealed the greatest difference between a group A strain (ATCC 10268) and a group B strain (KMU 2052). Amplicons were sequenced and grouped into varieties, and subjected to RFLP with Ase I (New England Biolabs).

## RESULTS

### 3.1 Species identification of the fungal strains based on sequence of CAL

The CAL sequences of 17 strains in group A were 817–822 bp in length, among which 14 were identical to *S. schenckii*. The additional 56 strains in group B were sequenced. Seven variations were found and named Cal-gls 1–7 in this study (Table S1).
Consequently, all group A strains corresponded to *S. schenckii*, and group B to *S. globosa*. No other species such as *S. brasiliensis* and *S. mexicana* were included in the series.

### 3.2 Genotyping based on sequence of mtDNA

Partial sequence of mtDNA of 17 strains belonging to group A, which corresponds to *S. schenckii*, and 70 strains of group B, which corresponds to *S. globosa*, were determined and phylogenetic trees were produced (Figure 3). The topology of each branch on the tree appeared more widely distributed than that on the CAL tree (Figure 2). In detail, the size of the amplicons of 17 strains of group A ranged 513–1116 bp, containing a spacer 343–946 bp in length, comprising 16 variations named Mt-sch 1–16 in this study. The size of the amplicons of group B strains ranged 510–515 bp, containing a spacer 340–345 bp in length, comprising 10 variations named Mt-gl 1–10 in this study (Table S2). The match of 70 strains was: Mt-gl 5, 30 strains; followed by Mt-gl 1, 18 strains; Mt-gl 2, eight strains; Mt-gl 3, five strains; Mt-gl, three strains;

| No. | KMU number | Origin          | Mt-RFLP types | Mt-RFLP groups | CAL       | ITS       | Mt-seq   |
|-----|------------|-----------------|---------------|---------------|-----------|-----------|----------|
| 1   | 975        | USA             | 1             | A             | LC635382  | LC636163  | LC635763 |
| 2   | 2286       | Central Japan   | 2             | A             | LC635383  | LC636164  | LC635764 |
| 3   | 2500       | Central Japan   | 3             | A             | LC635384  | LC636165  | LC635765 |
| 4   | 2747       | South Japan     | 4             | B             | LC635385  | LC636166  | LC635766 |
| 5   | 3311       | Central Japan   | 5             | B             | LC635386  | LC636167  | LC635767 |
| 6   | 2750       | South Japan     | 6             | B             | LC635387  | LC636168  | LC635768 |
| 7   | 3360       | Central Japan   | 7             | B             | LC635388  | LC636169  | LC635769 |
| 8   | 2741       | West Japan      | 8             | B             | LC635389  | LC636170  | LC635770 |
| 9   | 2760       | South Japan     | 9             | B             | LC635390  | LC636171  | LC635771 |
| 10  | 2763       | South Japan     | 10            | B             | LC635391  | LC636172  | LC635772 |
| 11  | 2687       | South Africa    | 11            | A             | LC635392  | LC636173  | LC635773 |
| 12  | 3314       | Central Japan   | 12            | B             | LC635393  | LC636174  | LC635774 |
| 13  | 2762       | South Japan     | 13            | B             | LC635394  | LC636175  | LC635775 |
| 14  | 3580       | Costa Rica      | 14            | A             | LC635395  | LC636176  | LC635776 |
| 15  | 3504       | USA             | 15            | A             | LC635396  | LC636177  | LC635777 |
| 16  | 3652       | Argentina       | 16            | A             | LC635397  | LC636178  | LC635778 |
| 17  | 3655       | Argentina       | 17            | A             | LC635398  | LC636179  | LC635779 |
| 18  | 3617       | Venezuela       | 18            | A             | LC635399  | LC636180  | LC635780 |
| 19  | 3627       | Venezuela       | 19            | A             | LC635400  | LC636181  | LC635781 |
| 20  | 3621       | Venezuela       | 20            | B             | LC635401  | LC636182  | LC635782 |
| 21  | 3912       | Australia       | 21            | B             | LC635402  | LC636183  | LC635783 |
| 22  | 3492       | USA             | 22            | A             | LC635403  | LC636184  | LC635784 |
| 23  | 3998       | South Africa    | 23            | A             | LC635404  | LC636185  | LC635785 |
| 24  | 4303       | China           | 24            | B             | LC635405  | LC636186  | LC635786 |
| 25  | 4383       | Mexico          | 25            | A             | LC635406  | LC636187  | LC635787 |
| 26  | 4385       | Mexico          | 26            | A             | LC635407  | LC636188  | LC635788 |
| 27  | 4386       | Mexico          | 27            | B             | LC635408  | LC636189  | LC635789 |
| 28  | 4384       | Mexico          | 28            | A             | LC635409  | LC636190  | LC635790 |
| 29  | 4390       | Mexico          | 29            | A             | LC635410  | LC636191  | LC635791 |
| 30  | 4398       | Mexico          | 31            | A             | LC635411  | LC636192  | LC635792 |
| 31  | 4432       | India           | 32            | B             | LC635412  | LC636193  | LC635793 |

Note: KMU number: registration number in Kanazawa Medical University.
Abbreviations: CAL, calmodulin gene; DDBJ, DNA Data Bank of Japan; EMBL, European Molecular Biology Laboratory; ITS, internal transcribed spacer; mtDNA, mitochondrial DNA; RFLP, restriction fragment length polymorphism.

*Mt-RFLP*: genotypes and groups determined by RFLP of mtDNA.°

*Mt-seq*: partial sequence of mitochondrial DNA determined by primers 975-8038F and 975-9194R.
| No. | KMU number | Geographic background of isolates<sup>a</sup> | Mt-RFLP types<sup>b</sup> | GenBank/EMBL/DDBJ accession no | Genotypes | Cal-gl<sup>d</sup> | Mt-gl<sup>e</sup> |
|-----|------------|---------------------------------|----------------|-----------------|-----------|----------|----------|
| 1   | 2679       | Central                         | 4             | LC635794        | LC635952  | 1        | 4        |
| 2   | 2688       | West                            | 4             | LC635795        | LC635953  | 1        | 4        |
| 3   | 2747       | South                           | 4             | LC635385        | LC635766  | 1        | 4        |
| 4   | 3021       | Central                         | 4             | LC635796        | LC635954  | 1        | 4        |
| 5   | 3112       | North                           | 4             | LC635797        | LC635955  | 1        | 4        |
| 6   | 3191       | Central                         | 4             | LC635798        | LC635956  | 1        | 7        |
| 7   | 3392       | South                           | 4             | LC635799        | LC635957  | 1        | 4        |
| 8   | 3479       | West                            | 4             | LC635800        | LC635958  | 1        | 4        |
| 9   | 3877       | West                            | 4             | LC635801        | LC635959  | 1        | 4        |
| 10  | 4061       | Central                         | 4             | LC635802        | LC635960  | 1        | 7        |
| 11  | 4078       | Central                         | 4             | LC635803        | LC635961  | 1        | 4        |
| 12  | 4131       | West                            | 4             | LC635804        | LC635962  | 1        | 4        |
| 13  | 4193       | Central                         | 4             | LC635805        | LC635963  | 1        | 7        |
| 14  | 4230       | South                           | 4             | LC635806        | LC635964  | 1        | 4        |
| 15  | 4257       | West                            | 4             | LC635807        | LC635965  | 1        | 4        |
| 16  | 4526       | Central                         | 4             | LC635808        | LC635966  | 1        | 4        |
| 17  | 4670       | South                           | 4             | LC635809        | LC635967  | 1        | 4        |
| 18  | 6488       | Central                         | 4             | LC635810        | LC635968  | 1        | 7        |
| 19  | 6799       | South                           | 4             | LC635811        | LC635969  | 1        | 4        |
| 20  | 2746       | South                           | 5             | LC635812        | LC635970  | 4        | 1        |
| 21  | 2778       | South                           | 5             | LC635813        | LC635971  | 5        | 1        |
| 22  | 2824       | North                           | 5             | LC635814        | LC635972  | 4        | 1        |
| 23  | 3041       | Central                         | 5             | LC635815        | LC635973  | 5        | 1        |
| 24  | 3308       | North                           | 5             | LC635816        | LC635974  | 4        | 1        |
| 25  | 3311       | Central                         | 5             | LC635836        | LC635767  | 1        | 1        |
| 26  | 3341       | Central                         | 5             | LC635817        | LC635975  | 1        | 1        |
| 27  | 3874       | Central                         | 5             | LC635818        | LC635976  | 1        | 1        |
| 28  | 4073       | Central                         | 5             | LC635819        | LC635977  | 4        | 1        |
| 29  | 4244       | West                            | 5             | LC635820        | LC635978  | 4        | 1        |
| 30  | 4453       | North                           | 5             | LC635821        | LC635979  | 1        | 1        |
| 31  | 4669       | South                           | 5             | LC635822        | LC635980  | 1        | 1        |
| 32  | 4710       | Central                         | 5             | LC635823        | LC635981  | 1        | 1        |
| 33  | 6326       | Central                         | 5             | LC635824        | LC635982  | 5        | 1        |
| 34  | 6637       | Central                         | 5             | LC635825        | LC635983  | 4        | 1        |
| 35  | 6705       | Central                         | 5             | LC635826        | LC635984  | 4        | 1        |
| 36  | 6798       | South                           | 5             | LC635827        | LC635985  | 1        | 1        |
| 37  | 2750       | South                           | 6             | LC635387        | LC635768  | 1        | 4        |
| 38  | 3376       | Central                         | 6             | LC635828        | LC635986  | 1        | 4        |
| 39  | 3515       | Central                         | 6             | LC635829        | LC635987  | 1        | 2        |
| 40  | 3604       | West                            | 6             | LC635830        | LC635988  | 1        | 4        |
| 41  | 3693       | West                            | 6             | LC635831        | LC635989  | 1        | 4        |
| 42  | 3705       | Central                         | 6             | LC635832        | LC635990  | 1        | 4        |
| 43  | 4130       | West                            | 6             | LC635833        | LC635991  | 6        | 4        |
| 44  | 4238       | South                           | 6             | LC635834        | LC635992  | 1        | 7        |

(Continues)
Mt-\textit{gl} typing and Mt-\textit{RFLP} typing revealed incompatibility. However, only Mt-\textit{gl} 1 corresponded exactly to Mt-\textit{RFLP} type 5. These sequence variations were examined by RFLP analysis, but only five polymorphisms were detected among \textit{S. schenckii} strains and none among \textit{S. globosa} strains (Figure S2). The variations of \textit{S. globosa} strains could not be detected using commercially available restriction enzymes in silico (data not shown).

**Table 2** (Continued)

| No. | KMU number | Geographic background of isolates$^b$ | Mt-RFLP types$^b$ | GenBank/EMBL/DDBJ accession no | Genotypes |
|-----|------------|--------------------------------------|------------------|-------------------------------|-----------|
|     |            |                                      | CAL          | Mt-seq$^c$               | Cal-gl$^d$ | Mt-gl$^e$ |
| 45  | 6084       | North                                | 6             | LC635835                 | 1          | 4         |
| 46  | 6429       | West                                 | 6             | LC635836                 | 1          | 9         |
| 47  | 2647       | Central                              | 7             | LC635837                 | 5          | 8         |
| 48  | 3360       | Central                              | 7             | LC635388                 | 1          | 2         |
| 49  | 3507       | Central                              | 7             | LC635838                 | 1          | 2         |
| 50  | 4115       | North                                | 7             | LC635839                 | 1          | 2         |
| 51  | 4129       | South                                | 7             | LC635840                 | 1          | 2         |
| 52  | 4256       | West                                 | 7             | LC635841                 | 1          | 7         |
| 53  | 4648       | South                                | 7             | LC635842                 | 1          | 2         |
| 54  | 6085       | West                                 | 7             | LC635843                 | 1          | 2         |
| 55  | 6684       | South                                | 7             | LC635844                 | 1          | 2         |
| 56  | 6796       | South                                | 7             | LC635845                 | 7          | 8         |
| 57  | 2741       | West                                 | 8             | LC635389                 | 1          | 5         |
| 58  | 2736       | West                                 | 9             | LC635846                 | 1          | 10        |
| 59  | 2760       | South                                | 9             | LC635390                 | 1          | 4         |
| 60  | 3398       | West                                 | 9             | LC635847                 | 1          | 4         |
| 61  | 4132       | West                                 | 9             | LC635848                 | 1          | 7         |
| 62  | 4219       | Central                              | 9             | LC635849                 | 1          | 4         |
| 63  | 2763       | South                                | 10            | LC635391                 | 1          | 4         |
| 64  | 3314       | Central                              | 12            | LC635393                 | 1          | 1         |
| 65  | 2762       | South                                | 13            | LC635394                 | 1          | 4         |

Note: KMU number: registration number in Kanazawa Medical University.

Abbreviations: CAL, calmodulin gene; DDBJ, DNA Data Bank of Japan; EMBL, European Molecular Biology Laboratory; mtDNA, mitochondrial DNA; RFLP, restriction fragment length polymorphism.

$^a$Geographic background of isolates: the regions of Japan geographically divided into four parts: Central (central Japan; central to eastern Honshu), West (western Japan; Shikoku, western Honshu), South (southern Japan; Kyushu), and North (northern Japan; northern Honshu, Hokkaido).

$^b$Mt-RFLP: genotypes and groups determined by RFLP of mitochondrial DNA.$^{3-6}$

$^c$Mt-seq: partial sequence of mitochondrial DNA determined by primers 975-8038F and 975-9194R.

$^d$Cal-gl: genotypes based on variations of sequence of calmodulin gene.

$^e$Mt-gl: genotypes based on variations of sequence of mitochondrial DNA determined by primers 975-8038F and 975-9194R.

**Table 3** Primers used in this study

| Target                  | Primers | Sequence                   |
|-------------------------|---------|----------------------------|
| Calmodulin gene, partial$^1$ | CL1     | GA(GA)/T(AT)CAAGGAGGCTTCTC |
|                         | CL2A    | TTTTGTGATCATGAGTTGAC       |
| Near the 3’-end          | f1      | AACAACGGCCACCATTGACTT      |
| Near the 5’-end          | r1      | GTGACCTCGTGTGTACATGT       |
| Internal transcribed spacer$^{10}$ | ITS1    | TCCGTAAGTTAACTTCCCTCG      |
|                         | ITS4    | TCCTCCGCTATTGATATGC        |
| Mitochondrial DNA, partial$^8$ | 975-8038F | GCTAGAAATCTTCTTTAAGAGGAGC |
|                         | 975-9194R | CTTCTCATTAGGTTAGTGC       |

 Mt-gl 6, two strains; and of Mt-gl 7, Mt-gl 8, Mt-gl 9, and Mt-gl 10, one strain each. The Mt-gl typing and Mt-RFLP typing$^{3-6}$ revealed incompatibility. However, only Mt-gl 1 corresponded exactly to Mt-RFLP type 5.

These sequence variations were examined by RFLP analysis,$^5$ but only five polymorphisms were detected among \textit{S. schenckii} strains and none among \textit{S. globosa} strains (Figure S2). The variations of \textit{S. globosa} strains could not be detected using commercially available restriction enzymes in silico (data not shown).

**4** **| DISCUSSION**

The present study revealed that groups A and B of \textit{S. schenckii sensu lato} classified by RFLP of mtDNA$^{3-6}$ correspond to \textit{S. schenckii} and \textit{S. globosa}, respectively. The molecular epidemiology of 257 strains isolated before 1990 in Japan had comprised 14 group A strains,
and 243 group B strains. Therefore, it can be regarded that 14 of 257 strains (5.4%) were S. schenckii, and 243 of 257 (94.6%) S. globosa. A previous molecular epidemiological study using CAL and ITS found nine strains (3.0%) of S. schenckii and 291 (97.0%) of S. globosa among 300 Japanese isolates collected independently. The present study indicated that the major causative species of sporotrichosis is S. globosa. No causative species other than S. schenckii and S. globosa has been found among Japanese strains so far.

Sporotrichosis has distinctive characteristics and is known as an endemic mycosis, which is widespread. In Japan, sporotrichosis tends to be concentrated in specific regions such as large river basins, but such regions exist in geographically distant locations. In addition, human activities involving contact with wood, plants, moss, and so forth have sometimes been associated with outbreaks of sporotrichosis, which may affect the epidemiological distribution of Sporothrix spp. Since a case of simultaneous infection in a human by genetically distinct strains was reported, molecular markers that can detect polymorphisms within a species are useful to study epidemiology.

Several molecular markers have been applied to track and monitor sporotrichosis. In particular, S. globosa is known to have low diversity and considered to require sensitive markers. Intraspecific polymorphisms of CAL or ITS have been detected in only a few varieties among S. globosa strains. Amplified fragment length polymorphism (AFLP) analysis, which detects differences in the length of fragments sandwiched between restriction enzyme cleavage sites, divided 225 clinical isolates of S. globosa into eight distinct clusters. Multilocus microsatellite analysis is another sensitive method and microsatellite markers have been reported for genotyping of S. globosa which enabled amalgamation of 120 isolates from China into three distinct clusters. However, peaks for microsatellite markers sometimes shift due to differences in electrophoresis conditions and primer modification processes, and special attention is needed in inter-laboratory comparison. The most sensitive marker is RFLP analysis of mtDNA, which albeit a non-PCR-based complicated and time-consuming method, found 14 polymorphisms among S. globosa strains. However, the RFLP analysis was sometimes difficult to compare banding profiles and could be confused by bands of similar size or conditions of electrophoresis. In recent days, nucleotide sequence analysis has become easier, and highly variable regions of genes are targeted as molecular markers. As one candidate for this purpose, Kawasaki et al. proposed the intergenic region between atp9 and cox2 genes based on sequence comparison of completely determined mtDNA of KMU975 (group A) and KMU2052 (group B) (Figure 1). Using the primer pair 975-8038F and 975-9194R, 10 polymorphisms were detected among 70 strains, which is fewer variations than that of RFLP analysis of whole molecule of mtDNA, yet more sensitive than sequence analysis of CAL which revealed seven variations among these strains. In addition, it is easier to sequence the partial mtDNA gene compared to CAL due to their smaller size. This marker may contribute to understanding the route of transmission of Sporothrix, especially when the source was assumed to be in the environment such as plants and soil, pet animals, or in family onset cases.

We tried to find correspondence of the present Mt-gl types with the geographic origins of S. globosa. The 65 Japanese strains were isolated from four provinces of Japan: southern Japan (Kyushu), western Japan (Shikoku, western Honshu), central Japan (central to eastern Honshu), and northern Japan (northern Honshu, Hokkaido). However, the strains in each of the four provinces were found to be genetically polymorphic; namely 18 strains from southern Japan comprised five genotypes (Mt-gl 1, 2, 4, 7, 8), 16 strains from western Japan seven genotypes (Mt-gl 1–5, 9, 10), 25 from central Japan five genotypes (Mt-gl 1, 2, 4, 7, 8), and six strains from northern Japan three genotypes (Mt-gl 1, 2, 4).
Genotype Mt-gl 4, the most common, was found in 27 among 65 strains, and isolated from all four provinces in Japan. Mt-gl 4 was also found among isolates from China, Mexico, and Australia, suggesting global distribution. Genotypes Mt-gl 1 (18 strains), and Mt-gl 2 (eight strains) were also found in all four Japanese provinces. The proportion of Mt-gl 1 among genotypes was low in western Japan but high in central-east Japan. The proportion of Mt-gl 4 among the isolates was higher in southern and western Japan, and lower in central and northern Japan.

However, no particular genotype was responsible for the endemic in Japan. In China, AFLP genotyping was reported to reflect regional differences,\(^1\) but in Japan, many people inhabit relatively small areas and farming was prevalent, so it is postulated that genotypes were affected by human activities. In addition, 18 strains of Mt-gl 1 isolated from Japan have three types of CAL variations, and combining these markers makes more detailed genotyping of \(S.\) globosa possible.

The relationship between genotypes and virulence is of clinical interest. In a few strains belonging to Mt-gl 1 and Mt-gl 4, we attempted to find differences in thermotolerance and minimum inhibitory concentration (MIC) for some antimycotics, which may influence their pathogenicity (Table S3), but comprehensive studies of a larger number of samples are needed to make any reliable conclusion. We would like to determine the genotype as an attribute of the maintained culture collection for further study.

In conclusion, the present study revealed that groups A and B of \(S.\) schenckii sensu lato classified by RFLP of mtDNA corresponded to \(S.\) schenckii and \(S.\) globosa, respectively. Twelve variations were found among group A strains and three among representative strains in group B. The mtDNA RFLP types are shown in parentheses. Neighbor-joining method.

**FIGURE 2** Phylogenetic tree of \(Sporothrix\) schenckii sensu lato based on partial sequence of calmodulin gene. All 17 strains from each mitochondrial (mt)DNA restriction fragment length polymorphism (RFLP) type in group A were clustered with type strain \(S.\) schenckii CBS 359.36, and all 14 in group B with ex-type strain \(S.\) globosa, CBS 120340, respectively. Twelve variations were found among group A strains and three among representative strains in group B. The mtDNA RFLP types are shown in parentheses. Neighbor-joining method.
and 975-9194R has indicated higher discriminatory power than that of CAL, and we propose to adopt this region for a useful marker for molecular epidemiology of *S. globosa*.

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CONFLICT OF INTEREST
None declared.

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