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【Report Article】

Reexamination of *Crepidotus crocophyllus* (Basidiomycota, Fungi) in Japan, with reference to its phylogenetic placement

Taiga KASUYA[1)], Kunihiko UNO[2)] and Kentaro HOSAKA

Crepidotus crocophyllus, one of the most common species of the genus *Crepidotus* (Basidiomycota, Fungi) in Japan, were morphologically and phylogenetically reexamined based on the materials collected from Choshi, Chiba Prefecture, Central Japan. Morphologically, Japanese *C. crocophyllus* were well match with previous descriptions of the present species based on European and American materials. To reveal the phylogenetic placement of Japanese specimens of *C. crocophyllus*, molecular phylogenetic analysis of the present species were conducted. By maximum parsimony analysis of nuclear rRNA ITS region, materials of *C. crocophyllus* collected from Japan (Choshi, Chiba) and North America (USA) were placed within well-supported, different clades each other. The present analysis revealed for the first time that Japanese and North American specimens of *C. crocophyllus* are polyphyletic, and a taxonomic revision of the species tentatively identified as *C. crocophyllus* is therefore needed.

1. Introduction

Crepidotus (Fr.) Staude is the distinct, well-defined genus having pleurotoid basidiomata of Inocybaceae (Basidiomycota, Fungi) and distributed throughout most of the world[1)]. More than 200 species of this genus have been described[2)] in the world. Modern molecular phylogenetic studies[3~7)] have highlighted the monophyletic status of *Crepidotus*, and the result has been supported also by Garnica et al.[8)] and the multi-gene analyses by Matheny et al.[9)]Although *Crepidotus* has been treated as a member of the family Crepidotaceae based on traditional morphological studies[10)], recent molecular phylogenetic analyses revealed that *Crepidotus* and *Inocybe* (Fr.) Fr. are each other's closest relatives, viz. sister groups[11)].

As above, higher-level classification of *Crepidotus* has recently been revised using molecular phylogenetic analyses. Simultaneously, several studies on species taxonomy of *Crepidotus* have mainly been published focusing on materials from Europe[10,12~15)], Japan[1,16~18)] and Latin America[19~25)]. However, recent studies on species taxonomy of *Crepidotus* have mainly been conducted based on only traditional morphological methods. Molecular phylogenetic analyses provide an effective alternative to evaluate useful morphological characteristics in species taxonomy of fungi. Therefore, taxonomic revisions of *Crepidotus* species using both molecular phylogenetic and morphological methods are needed.

Based on the above backgrounds, the present study made an attempt to reexamine the taxonomy of *Crepidotus* at species level. We focused on the widely distributed species, *C. crocophyllus* (Berk.) Sacc. This species has a wide distribution in Europe[10,14)], Japan[1)], North America[26)], Latin America[24)] and South America[26~27)]. *Crepidotus crocophyllus* is one of the most common species of the genus in Japan, and it has previously been known as *C. badiofloccosus* S. Imai, a synonym of *C. crocophyllus*[1)]. Since the variable morphological characters especially cheilocystidia, and ecologically diverse habitats expanding from cool-temperate areas to tropics[1,10,24)], *C. crocophyllus* probably comprises a species complex. However, no taxonomic reexamination and molecular phylogenetic studies on *C. crocophyllus* have previously

To whom all correspondence; Taiga KASUYA tkasuya@cis.ac.jp
1) Department of Environmental Risk and Crisis Management, Faculty of Risk and Crisis Management, Chiba Institute of Science
2) Department of Botany, National Museum of Nature and Science
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been conducted. Accordingly, phylogenetic relationships of *C. crocophyllus* between geographically different regions have not been known yet. Therefore, it is unclear whether truly *C. crocophyllus* includes multiple species or not. In addition, the possibility of existence of cryptic species in *C. crocophyllus* has not been examined. To resolve these issues, in this article we provide a phylogenetic analysis of Japanese *C. crocophyllus* based on sequences of nuclear rRNA ITS region. Moreover, we also provide descriptions and illustrations of macro- and microscopic characters of Japanese *C. crocophyllus*; their taxonomic status are also discussed.

2. Materials and Methods

2. 1. Collecting sites, collecting scheme, and curation of specimens

Two fresh specimens of *C. crocophyllus* were collected at Tokai Shrine, Takagami-nishi-machi, Choshi, Chiba Prefecture by TK in the year of 2013 (May 18 and 31). A general collection scheme[28] for agaric fungi was followed. Each specimen of *C. crocophyllus* was photographed and macroscopic observation was conducted. Fresh basidiomata of each specimen were dried using a food dehydrator (Snackmaster Express FD-61, Nesco/American Harvest, WI, USA) under 46°C. In addition to dried materials, small fragments of pileal tissue from freshly collected basidiomata were soaked in DMSO (dimethyl sulfoxide) buffer[29] with an addition of 100 mM Tris-HCl (pH 8.0) and 0.1 M sodium sulfite (Na_2SO_3) under 4°C, following the procedures of Hosaka[30], Hosaka and Castellano[31] and Hosaka et al.[32].

More detailed identification and description were conducted after returning to the mycology laboratory at the Faculty of Risk and Crisis Management, Chiba Institute of Science, Japan. Specimens collected during the fieldwork were deposited at the mycological herbarium of the National Museum of Nature and Science, Tsukuba, Japan (TNS). Additionally, for molecular phylogenetic studies, three specimens of Inocybaceae (*Crepidotus* and *Inocybe*) collected from our fieldwork were also investigated. Those specimens were deposited at the mycological herbaria of TNS and Ibaraki Nature Museum, Bando, Japan (INM).

2. 2. Light and scanning electron microscopy

For light microscopic observations, a small portion from the pileus was mounted in water, 3% (w/v) KOH and 30% ethanol solution on glass slides. Those samples were examined with a Leica DM LB microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) under Nomarski interference contrast. More than forty randomly selected ascospores were measured under a light microscope at 1000× magnification. The surface features of the basidiospores were observed by scanning electron microscopy (SEM). For SEM, a small portion from the dried pileus was dusted onto double-sided adhesive tape on a specimen holder and coated with platinum-palladium using an E-1030 Ion Sputter Coater (Hitachi, Tokyo, Japan). They were examined with a S-4200 SEM (Hitachi, Tokyo, Japan) operating at 20 kV.

2. 3 DNA preparation, PCR and sequencing

DNA of the specimens collected by TK was extracted from the pileal tissue fragments stored in DMSO buffer, as mentioned above. DNA extractions used the modified CTAB (cetyl trimethyl ammonium bromide) extraction followed by Glass-Milk (silicon dioxide; Sigma-Aldrich Co., St. Louis, USA) purification methods as summarized by Hosaka[30] and Hosaka and Castellano[31]. Briefly, samples were ground in liquid nitrogen using mortar and pestle, incubated in CTAB buffer at 65°C for 1 hour, and proteins were removed using the mixture of chloroform: isoamylalcohol (24: 1). The materials were further purified using 6 M sodium iodine buffer with glass milk, washed with ethanol/buffer solution, and finally eluted in 100 µl of TE buffer.

DNA sequence data were obtained from the internal transcribed spacer regions (ITS) of the nuclear ribosomal DNA. The nuclear rRNA cistron has been used for fungal diagnostics and phylogenetics for more than 20 years[33]. The eukaryotic rRNA cistron consists of the 18S, 5.8S and 28S rRNA genes transcribed as a unit by RNA polymerase I. Posttranscriptional processes split the cistron, removing two internal transcribed spacers. These two spacers, including the 5.8S gene, are usually referred to as the ITS region[34]. A single fragment of ITS region of fungi is approximately 650-700 bp in length. ITS has formally been proposed for adoption as the universal barcode marker of fungi[34]. For amplifying the ITS region, the primer combination of ITS1f and ITS4[35] was used. PCR reactions were carried out using 20 µl reaction volumes each containing: 1 µl of genomic DNA (10 ng/µl), 1 µl of dNTP, (4 mM), 1 µl of each primer (8 µM), 0.5 units of Taq polymerase (TAKARA), 2µl of $MgCl_2$ (25 mM), 2µl of Bovine Serum Albumin (BSA; 10 µg/µl). Cycling parameters were 1 cycle of 94°C for

3 min, 30 cycles of 94˚C for 1 min, 51˚C for 30 s and 72 ˚C for 1 min, with a final extension at 72˚C for 15 min. PCR products were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light. When amplification of bands were confirmed, PCR products were then purified using the illustra ExoStar (GE Healthcare, UK) and directly sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Norwalk, CT, USA), following the manufacturer's instructions. Five sequences of *Crepidotus* and *Inocybe* were newly generated by above methods and then they were registered to NCBI GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with associated accession numbers (Table 1).

2. 4. Phylogenetic analysis

DNA sequences newly generated in the present study (Table 1) were assembled by ATGC ver. 6 (GENETYX, Tokyo, Japan) and then initially aligned using Muscle v.3.6[36~37], followed by manual alignment in the data editor of BioEdit ver. 7.0.1[38]. After the dataset was aligned, ambiguous regions, gaps and introns were excluded from the final alignment using the default parameters in the program Gblocks[39~40].

A phylogenetic analysis of these sequences was conducted in addition to 13 sequences of *Crepidotus* including *C. crocophyllus* collected from USA released at GenBank (Table 2). After alignment of this dataset as mentioned above, maximum parsimony analysis were conducted using PAUP* ver. 4.0b10[41] with the heuristic search option (TBR and MULTREES option on), and 1,000 replicates of random addition sequence. Bootstrap analyses of each node of phylogenetic trees were also conducted with 1,000 BS replicates using the heuristic search option (TBR and MULTREES options on), with 10 random addition sequences. Two taxa of *Inocybe,* viz. *I. lilacina* (Peck) Kauffman and *I. maculata* Boud. were used for outgroups based on the result of Aime[7] (Table 1).

Table 1. Sequence data of nuclear rRNA ITS region newly generated for the present study and associated GenBank accession numbers.

Species		Locality	Date	Collector	Herbarium	Specimen No.	Accession No.
Crepidotus	*crocophyllus*	Japan, Chiba, Choshi, Tokai Shrine	May 18, 2013	T. Kasuya	TNS	Kasuya B950	KF680279
Crepidotus	*crocophyllus*	Japan, Chiba, Choshi, Tokai Shrine	May 31, 2013	T. Kasuya	TNS	Kasuya B983	KF680280
Crepidotus	sp.	Japan, Kochi, Aki, Furui	July 2, 2010	T. Kasuya	TNS	Kasuya B225	KF680278
Inocybe	*Lilacina*	Japan, Ibaraki, Hitachinaka, Mawatari	November 6, 2011	T. Kasuya	INM	2–71759	KF680277
Inocybe	*maculata*	Japan, Chiba, Asahi, Nagabe	June 15, 2013	T. Kasuya	TNS	Kasuya B993	KF680276

Table 2. Sequence data used for the phylogenetic analysis of nuclear rRNA ITS region obtained from GenBank.

Species		Origin	Accession No.
Crepidotus	*crocophyllus*	USA	FJ596821
Crepidotus	*crocophyllus*	USA	FJ596822
Crepidotus	*crocophyllus*	USA	FJ596823
Crepidotus	*crocophyllus*	USA	FJ596824
Crepidotus	*crocophyllus*	USA	FJ596825
Crepidotus	*applanatus*	USA	FJ596803
Crepidotus	*applanatus*	USA	FJ596805
Crepidotus	cf. *applanatus*	USA	DQ202273
Crepidotus	*luteolus*	Italy	JF907963
Crepidotus	*novae-zealandiae*	New Zealand	HQ533046
Crepidotus	*subverrucisporus*	Italy	JF907961
Crepidotus	*sphaerosporus*	Italy	JF907960
Crepidotus	sp.	India	JN113588

3. Results and Discussion

3. 1. Descrpiton of Japanese specimens of *C. crocophyllus*

Crepidotus crocophyllus (Berk.) Sacc., Sylloge Fungorum, 5, 886, 1897 **(**Fig. 1).

Basidiomata (Fig. 1B-C) pleurotoid. Pileus 25–50 mm in diam., campanulate when young, then spathulate, later hemispheric, reniform to flabelliform, finally convex to plano-convex, with incurved, later straight, not sulcate-striate margin, laterally to dorsally attached, surface pallid, pale yellow to ochraceous, densely covered with yellowish to ferruginous squamules when young, later squamules pale brown to brown, subsquamulose, fibrillose to somewhat appressed; at the basal point of pileus tomentose to villose, ferruginous to brown, brittle when dried. Lamellae crowded, subventricose, adnexed, thin, cream when young, later pale yellow to ferruginous, edges fimbriate, whitish, sometimes irregular. Stipe sessile. Pseudostipe absent. Context thin, white, fragile. Taste mild to slightly bitter. Odor indistinct.

Pileipellis a cutis composed of more or less compactly arranged, repent, interwoven, cylindrical to subcylindrical or rarely subventricose, hyaline to pale yellow, 3–6 µm in diam., thin-walled hyphae, mixed with bundles of ascending, yellowish, 10-20 µm, often incrusted, thick-walled hyphae; terminal element of scale-forming hyphae cylindrical, somewhat flexuous, thick-walled. Hymenophoral trama subregular to irregular, not gelatinized, hyphae 4–6 µm in diam., cylindrical to subventricose, hyaline to pale yellowish, thin-walled. Pleurocystidia not seen. Cheilocystidia (9–) 20–40 × (3–) 4–9 µm, numerous, variable in the shape, clavate, subclavate to somewhat utriform or lageniform, rarely subcylindrical to almost cylindrical, apex 8–20 µm in diam., flexuous, subcapitate, hyaline, thin-walled. Basidia 25–40 × 4–8 µm, 4–spored, evanescent, clavate to subclavate, hyaline,

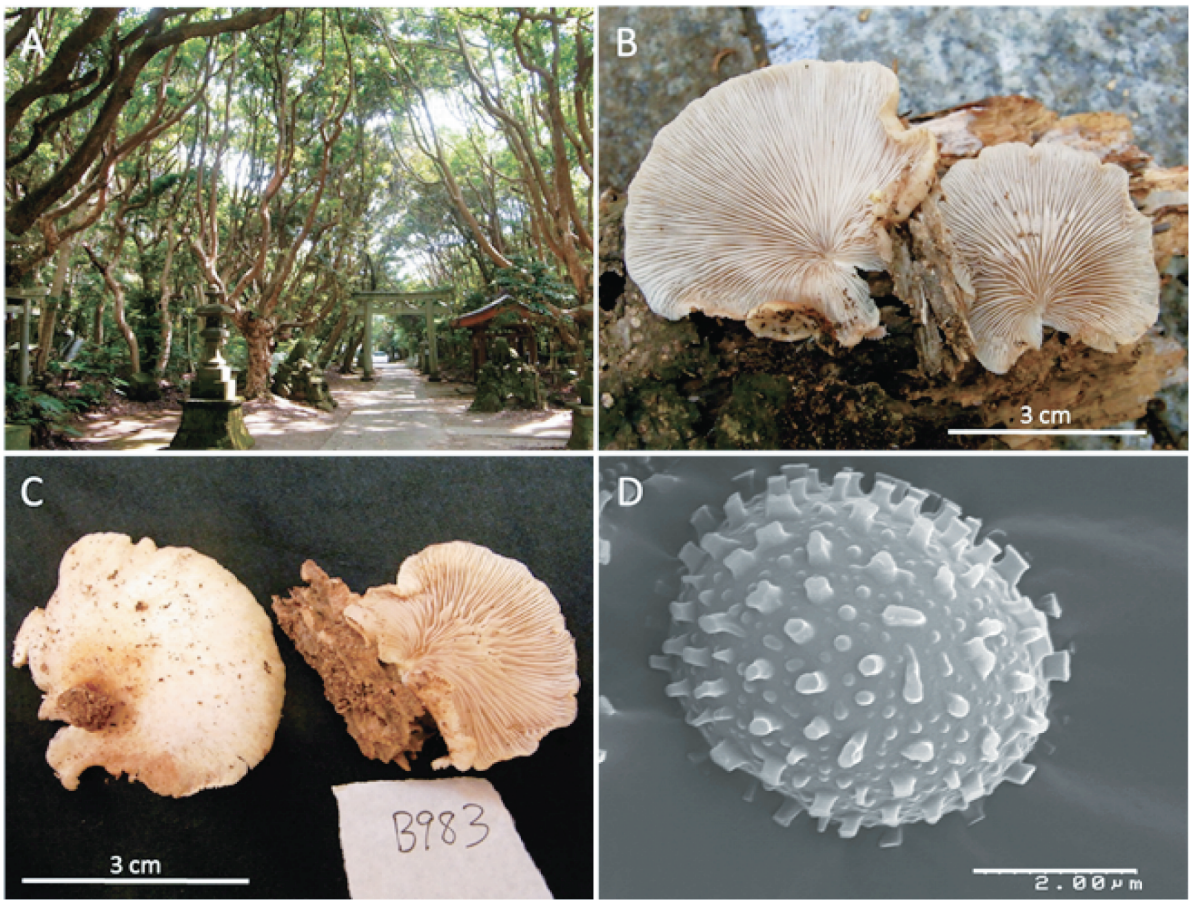

Fig. 1. Habitat and baisidiomata of *Crepidotus crocophyllus*. A: Habitat of *C. crocophyllus* in Choshi. B: Lamellae of mature basidiomata (TNS Kasuya B983). C: Mature basidiomata showing pileal surface and lamellae (TNS Kasuya B983). D: A basidiospore showing baculate ornamentation (TNS Kasuya B950).

thin-walled, clamped at the base, transformed into amorphous globose to subglobose materials or cystidioid-like structures when mature. Basidiospores 5–6.5 × 5–6 μm (mean = 5.7 × 5.2 μm), Q = 1.0–1.06 (mean = 1.03), globose to subglobose, warty to spinulose-verruculose, yellowish brown to rusty brown, thick-walled under LM, when observed under SEM, surface baculate, roughly covered with baculiform to somewhat echinulate spines up to 0.5 μm long (Fig. 1D).

Habitat: Gregarious or solitary on decayed trunks and fallen branches of evergreen broad-leaved trees, especially on *Castanopsis sieboldii* (Makino) Hatus. ex T. Yamaz. & Mashiba and *Machilus thunbergii* Sieb. & Zucc. (Fig. 1A).

Japanese name: Kurige-no-cha-hiratake[42].

Specimens examined: JAPAN, Chiba Pref., Choshi-shi, Takagami-nishi-machi, Tokai Shrine, 18 May, 2013, leg. & det. T. Kasuya (TNS Kasuya B950); same locality, 31 May, 2013, leg. & det. T. Kasuya (TNS Kasuya B983).

Remarks: Remarkable characteristics of the studied Japanese specimens are: (1) pilei densely covered with ferruginous to brownish squamules, (2) pale yellow to ferruginous lamellae, (3) globose to subglobose, baculate (SEM) basidiospores, (4) pileipellis composed of two types of hyphae, (5) high variability in the shape of cheilocystidia, and (6) evanescent basidia transformed into amorphous globose to subglobose materials or cystidioid-like structures when mature. From our detailed macro- and microscopic observations as well as from the aforementioned important characteristics of the studied Japanese specimens we see a good agreement with previous descriptions of *C. crocophyllus*[1,10,14,24,26]. Morphologically, Japanese *C. crocophyllus* cannot be distinguishable from those of European and American specimens.

3. 2. Phylogenetic and taxonomic implications

By the maximum parsimony analysis, all sequences of ITS regions of Japanese *C. crocophyllus* were placed within a strongly supported clade (BS = 100%; Fig. 2). Our two sequences from Japanese *C. crocophyllus* differed each other at only one base each other. We treat this single difference as intraspecific variation because there are no morphological differences between two Japanese specimens used for the phylogenetic analysis. A clade including the North American (USA) group of *C. crocophyllus* (Fig. 2) was resolved as the sister group of Japanese *C. crocophyllus* in the strict consensus of equally parsimonious trees with strong support (Fig. 2).

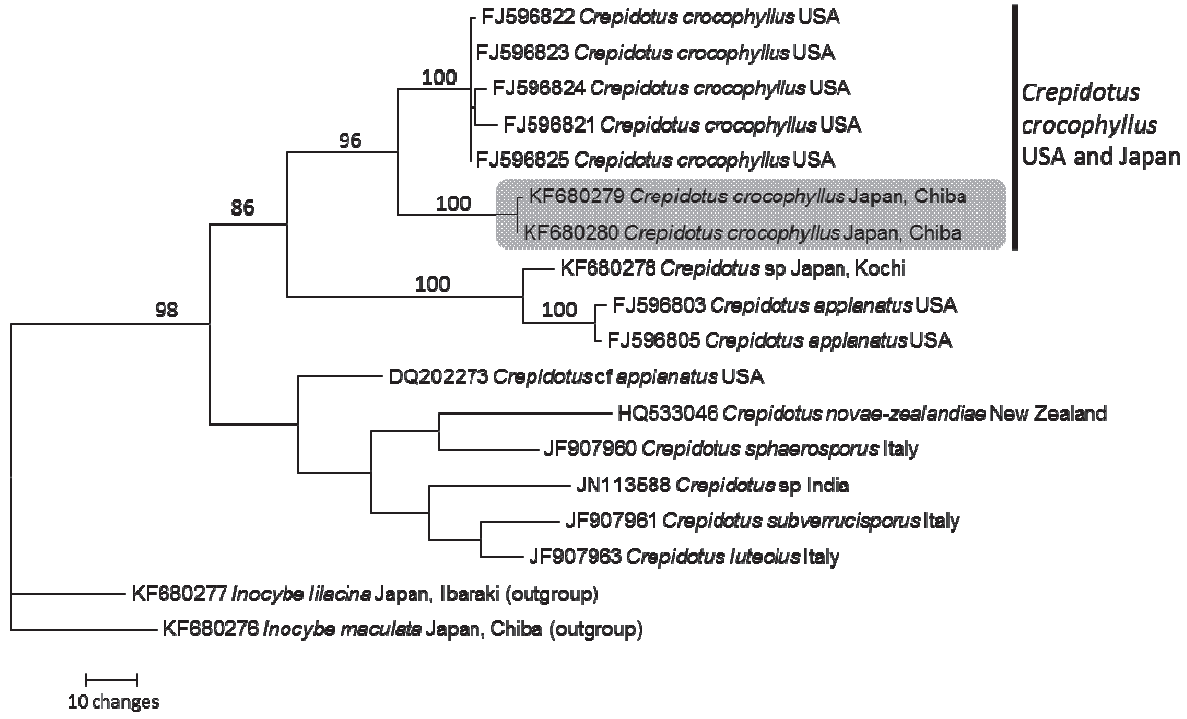

Fig. 2. One of two parsimonious trees of *Crepidotus crocophyllus* derived from maximum parsimony analysis based on the nuclear rRNA ITS region. Numbers along branches are nodal supports (parsimony bootstrap values).

The present phylogenetic analysis reveals that taxa tentatively identified as "*C. crocophyllus*" turned out to be polyphyletic (Fig. 2) for the first time. Therefore, *Crepidotus* species previously recognized as "*C. crocophyllus*" comprises species complex. The type specimen of *C. crocophyllus* was collected from Ohio, USA[43]. While several sequences of *C. crocophyllus* from North America were included in the present analysis, we could not obtain any sequence data of the holotype. Moreover, our analysis includes only two sequences of Japanese *C. crocophyllus*. Previously, *C. crocophyllus* has been reported from multiple continents such as Eurasia, North America and South America[1,10,14,24,26]. Therefore, further phylogenetic analyses of *C. crocophyllus* sensu lato including the holotype and specimens from geographically different regions are required to evaluate taxonomic status of this species complex.

The present study showed the polyphyly of *C. crocophyllus* sensu lato and suggests it includes multiple taxa. However, Japanese specimens of *C. crocophyllus* sensu lato cannot be distinguished from European and American ones by traditionally used morphological characters for taxonomy of *Crepidotus*. Therefore, further analyses of relationships of clades detected by *C. crocophyllus* sensu lato between molecular phylogeny and morphology are needed for taxonomic revision of this species complex.

3. 3 Phylogeographic implication

In our analyses, sequence data of Japanese *C. crocophyllus* sensu lato were obtained from only two specimens. Moreover, samples from the other areas including Europe, North America, Latin America and South America have not been collected. Although the present study newly suggests the monophyly of Japanese *C. crocophyllus* sensu lato (Fig. 2), we could not conclude at this time whether the Japanese group is truly monophyletic due to a few taxon sampling. Further comprehensive sampling of *C. crocophyllus* from multiple continents will clarify detailed traits of its polyphyly and evolutionary process.

It is noteworthy to mention that *C. crocophyllus* sensu lato is saprotrophic. In addition, nearly all taxa within *Crepidotus* are reported as saprotrophic[1,10]. Saprotrophic fungi are generally more widespread in distribution than ectomycorrhizal fungi because they do not require the presence of compatible host plants[31]. However, in spite of its saprotrophic habit of *Crepidotus*, distinct phylogenetic clades of *C. crocophyllus* sensu lato were recognized between USA and Japan (Fig. 2). Presumably, this fact shows that long distance dispersal of *Crepidotus* species may not occur frequently, and despite its saprotrophic habits, distribution and species dispersal of *Crepidotus* may be restricted by geographic and environmental conditions such as climate, soil and vegetation.

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