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Taxonomic re-examination and phylogeny of neglected Japanese black deer truffles, *Elaphomyces miyabeanus* and *E. nopporensis*

クロツチダンゴ *Elaphomyces miyabeanus* およびコクロツチダンゴ *E. nopporensis* の分類学的再検討と系統

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Abstract

Two Japanese black deer truffles, *Elaphomyces miyabeanus* and *E. nopporensis*, were described from Hokkaido in 1929 by Dr. Sanshi Imai. Since then, there have been no reliable, formal records of either species. Both species were collected from the same locality on the same day, and are morphologically similar to each other. We successfully located authentic specimens including syntypes of each species, which had been scattered and lost, and we critically examined their morphology. We also collected fresh ascomata of *E. miyabeanus* / *E. nopporensis* from the type locality and central Honshu. Based on examination of the rediscovered authentic specimens and the fresh ascomata along with the original descriptions by Dr. Sanshi Imai, we concluded that *E. nopporensis* was small, dried, old ascomata of *E. miyabeanus* and should be synonymized into the latter species. Phylogenetic analyses based on the internal transcribed spacer region of the nuclear ribosomal DNA placed *E. miyabeanus* within sect. *Ceratogaster* subsect. *Sclerodermei* along with *E. anthracinus*. The resultant tree also showed that *E. miyabeanus* was conspecific with *Elaphomyces* sp. collected in Norway, suggesting that the species is broadly distributed across northern Eurasia.

要旨

クロツチダンゴ *Elaphomyces miyabeanus* およびコクロツチダンゴ *E. nopporensis* は、今井三子博士により北海道から 1929 年に記載された黒色のツチダンゴ類である。しかし、新種記載以降、両種の確実な報告は無く、その実態は不明である。両種は同日、同一産地（現在の道立自然公園野幌森林公園付近）にて採集されており、形態的に酷似している。著者

らは北海道大学総合博物館および日本きのこセンター菌蕈研究所にて、両種のシンタイプを含む複数の標本を再発見した。さらに、タイプ産地および本州中部（山梨県）にて、両種と形態的特徴の一致する子実体を新たに採集した。これらの標本および原記載の情報から両種の形態的比較検討を行った結果、コクロツチダンゴ *E. nopporensis* は、乾燥の進んだ、古い小型のクロツチダンゴ *E. miyabeanus* 子実体であると結論した。核リボソーム rDNA ITS 領域に基づく分子系統解析の結果、*E. miyabeanus* はツチダンゴ属 *Ceratogaster* 節 *Sclerodermei* 亜節に含まれ、*E. anthracinus* に近縁であること、ノルウェー産の標本と同一種であることが示された。この結果から、本種はユーラシア大陸北部に広く分布していることが示唆された。

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Introduction

The deer truffle genus, *Elaphomyces* T. Nees (Elaphomycetaceae, Eurotiales, Ascomycota) is one of the largest genera composed of truffle-like fungi. Currently, *Elaphomyces* comprises approximately 170 infrageneric taxa (according to the MycoBank database, searched on 3 January, 2022) both from the Northern and Southern Hemispheres. *Elaphomyces* spp. are ectomycorrhizal, which is exceptional in the Eurotiales, and some *Elaphomyces* spp. are also known as hosts of the fungicolous *Tolypocladium* spp.

In Japan, *Elaphomyces* has attracted attention by mycologists since very early in the taxonomic history of Japanese truffle-like fungi, mainly as hosts of mycoparasitic fungi (e.g., Kawamura, 1914; Kobayasi, 1960; Lloyd, 1916; Umemura, 1923; Yasuda, 1919). It was Dr. Sanshi Imai (1900–1976) who revealed a potentially high diversity of Japanese *Elaphomyces* spp. for the first time, describing five new species as well as the widely distributed *E. granulatus* Fr. and *E. variegatus* Vittad. (Imai, 1929, 1934, 1938, 1939). Of these, *E. miyabeanus* S. Imai (Japanese name: Kuro-tsuchidango) and *E. nopporensis* S. Imai (Japanese name: Kokuro-tsuchidango) are the first two black deer truffles described from Japan (Imai, 1929). Both species were collected several times from "Nopporo Forest" (i.e., Nopporo Forest Park as it is known today), Hokkaido Prefecture, on the same day. Some of those specimens were parasitized by *Tolypocladium jezoense* (S. Imai) Quandt, Kepler & Spatafora. Morphologically, both species appear similar but *E. miyabeanus* can be distinguished from *E. nopporensis* by having larger ascomata, a peridium with partial pyramidal warts and slightly larger ascospores (Imai, 1929). It should also be noted that only mature or old ascomata of *E. nopporensis* were collected by S. Imai.

Since the original description, there have been no reliable, formal records of either species although similar black truffles have been sporadically recorded as hosts of *T. jezoense* from Honshu (Shimizu, 1997). Also, it was not clear whether the type specimens of either species were extant. To clarify the true diversity of Japanese black *Elaphomyces* spp., we first need to know the taxonomic entity and phylogeny of the species based on the current systematic framework of *Elaphomyces*. We therefore tried to locate authentic specimens of *E. miyabeanus* and *E. nopporensis* in several Japanese herbaria and made field surveys throughout Japan to locate fresh specimens of both species. As a result, we successfully located several herbarium specimens of *E. miyabeanus* and *E. nopporensis* collected by S. Imai and taxonomically re-evaluated the two species. Furthermore, we obtained fresh materials of *E. miyabeanus* from several localities and examined its phylogenetic position based on the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA).

Materials and methods

Taxon sampling and morphological observation

Authentic herbarium specimens of *E. miyabeanus* and *E. nopporensis* collected by S. Imai were investigated in Hokkaido University Museum (SAPA), National Museum of Nature and Science, Japan (TNS) and Tottori Mycological Institute (TMI). The specimens were photographed and were subjected to microscopic observation.

Field surveys were conducted in various types of forests in Hokkaido and Honshu. Fresh ascomata were collected using a garden rake and were photographed in the field. After morphological observation, the collected ascomata were air-dried for 2 days at 48°C or freeze-dried and then stored in sterile plastic bags. The specimens are deposited in Kanagawa Prefectural Museum of Natural History, Japan (KPM).

For light microscopy, hand-cut sections of dried specimens were presoaked in 70% ethanol and 3% KOH. After briefly rinsing out those sections with distilled water, they were mounted in lacto-glycerol. A MT5310L microscope (Meiji Techno Co., Ltd., Saitama, Japan) was used for the observation. Photomicrographs were captured using a WRAYMER NOA-2000 camera (WRAYMER Inc., Osaka, Japan). Measurement under the microscope was performed with MicroStudio software (WRAYMER Inc.). Measurement of spores includes outer ornamentation and an outermost layer if any. Scanning electron microscopy (SEM) was performed with a HITACHI TM-4000Plus Tabletop Microscope (Hitachi High-Technologies, Tokyo, Japan).

Small fragments of a dried gleba and spore mass were set on a sample stage with a double-sided carbon tape and were observed at 15 kV.

DNA extraction, PCR amplification and sequencing

DNA was extracted from peridial tissue of a fresh *E. miyabeanus* specimen (KPM-NC 29301) and dried, syntypes of *E. miyabeanus* deposited in TMI using the protocol of Izumitsu et al. (2012). PCR amplification of the ITS of nrDNA followed Orihara et al. (2012) with the exception of reducing the elongation time before the final elongation step to 60S. The primer pair used was ITS1F (Gardes & Bruns, 1993) / ITS4 (White et al., 1990). PCR amplification from the syntypes was not successful. Amplified PCR product was purified with IllustraTM ExoProStarTM (Cytiva, Tokyo, Japan) based on the manufacturer's instructions. Sequencing steps of Orihara et al. (2012) were followed.

Phylogenetic analyses

For the ITS dataset, we retrieved nucleotide sequences that covered all the taxa of sect. *Ceratogaster* shown in Paz et al. (2017) from the International Nucleotide Sequence Database (INSD). We also included sequences of unidentified *Elaphomyces* spp. and environmental sequences from ectomycorrhizal (ECM) roots hit by the BLAST search in the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). ITS sequences used for the phylogenetic analyses are listed in Table 1. Species in sect. *Ascoscleroderma* were selected for outgroups. The dataset was aligned with the online version of MAFFT version 7 (Katoh & Standley, 2013) under default settings (i.e., the alignment algorithm is automatically selected from FFT-NS-1, FFT-NS-2, FFT-NS-i or L-INS-i). Subsequently, the sites with obvious alignment errors were manually adjusted in SeaView version 4 (Gouy et al., 2010). We referred to the results of the Gblocks option (Castresana, 2000) in SeaView to exclude ambiguously aligned sites for the following analyses.

Maximum likelihood (ML) analyses were performed with RAxML 8.2.10 (Stamatakis, 2014) and the online version of PhyML 3.0 (Guindon et al., 2010). In the RAxML analysis, the best substitution model for the whole dataset was estimated by the Akaike Information Criterion (AIC) under jModelTest2 (Darriba et al., 2012; Guindon & Gascuel, 2003). The ITS dataset was partitioned into partial 18S-5.8S-partial 28S and ITS1-ITS2 so that different α-shape parameters, GTR rates, and empirical base frequencies could be assigned to each partition. RAxML analysis was conducted under the GTR+G model with rapid bootstrap replicates set to 1000. PhyML analysis was implemented under default settings with the GTR+G model, which was automatically estimated as a best-fit model based on the SMS algorithm (Lefort et al., 2017). For calculating branch supports in this analysis, the SH-like approximate likelihood-ratio test (SH-aLRT; Anisimova & Gascuel, 2006) was conducted instead of bootstrapping.

Bayesian analyses were conducted with MrBayes 3.2 (Ronquist & Huelsenbeck 2003). The ITS dataset was partitioned in the same pattern as in RAxML analysis, and the resultant two partitions were independently subjected to a substitution-model estimation using jModelTest2 under AIC. The K80+I+G was selected as the best-fit model for the partial 18S-5.8S-partial 28S, and GTR+G was for the ITS1-ITS2 partition. Bayesian posterior probabilities (PPs) were estimated by the Metropolis-coupled Markov chain Monte Carlo method (Geyer, 1991). The two parallel runs were conducted with one cold and seven heated chains each for 3M generations. Temperature parameter of the seven heated chains in both runs was set to 0.20 (as a default). Trees were saved to a file every 1000th generation. We determined that the two runs reached convergence when the average standard deviation of split frequencies (ASDSF) was continuously lower than 0.01. The ASDSF was monitored every 5000 generations. Trees obtained before reaching convergence were discarded as the burn-in, and the remaining trees were used to calculate a 50% majority consensus topology and to determine PP values for individual branches.

Results

Investigation of old *Elaphomyces* specimens in the mycological herbaria

In November 2017, three of the authors (i.e., T. Orihara, M.A. Castellano and K. Hosaka) intensively searched old, unsorted specimens of truffle-like fungi deposited in SAPA, and we found one *E. miyabeanus* specimen parasitized by *Tolypocladium jezoense* (= *Cordyceps jezoensis* S. Imai) and one *E. nopporensis* specimen, both of which were collected and identified by S. Imai (Fig. 1A–C). The former was one of the syntypes of *T. jezoense* and the earliest record of both *E. miyabeanus* and *T. jezoensis* known today. The latter was one of the syntypes of *E. nopporensis*. The first author subsequently searched old specimens collected by S. Imai in TMI in November 2018, and found several additional specimens identified as *E. miyabeanus* and *E. nopporensis* including syntypes of both species (Fig. 1D–I). All of these dried specimens in SAPA and TMI were preserved in good condition. We could not find any specimens of *E. miyabeanus* and *E. nopporensis* in TNS.

Field sampling of fresh ascomata

We conducted field samplings several times in the type locality of *E. miyabeanus* and *E. nopporensis* (i.e., the Nopporo Forest Park,

Table 1. INSD (GenBank) accession numbers of the ITS sequences used for the phylogenetic analyses (UECM: uncultured ectomycorrhizal roots)

表 1. 分子系統解析に用いた ITS 領域塩基配列の INSD (GenBank) 登録番号

Taxa	Locality	Voucher no.	Reference	ITS acc. no.
E. aculeatus	SPAIN, Asturias	IC 27111115 (Herb. pers. A. Paz)	Paz et al. (2017)	KX238821
E. aculeatus	SPAIN, Asturias	IC 10041103 (Herb. pers. A. Paz)	Paz et al. (2017)	KX238844
E. anthracinus	NORWAY, Vestland Co., Stord	O-F-22176	Unpubl. data by Larsson, E. and Molia, A.	KR029773
E. anthracinus	NORWAY, Vestland Co., Stord	O-F-22177	Unpubl. data by Larsson, E. and Molia, A.	KR029774
E. anthracinus	SPAIN, Cantabria	LIP-0001144 (lectotype)	Paz et al. (2017)	KX238803
E. anthracinus	SPAIN, Cantabria	Herb. pers. A. Paz	Paz et al. (2017)	KX238804
E. anthracinus	SPAIN, Cantabria	Herb. pers. A. Paz	Paz et al. (2017)	KX238813
E. anthracinus f. *talosporus*	SPAIN, Asturias	LIP-0001145 (holotype)	Paz et al. (2017)	KX238805
E. foetidus	SPAIN, Caceres	LIP-0001138 (epitype)	Paz et al. (2017)	KX238797
E. leonis	SPAIN, Cantabria	IC 14111101 (Herb. pers. A. Paz)	Paz et al. (2017)	KX238814
E. leucosporus	SPAIN, Cantabria	IC 24051203 (Herb. pers. A. Paz)	Paz et al. (2017)	KX238816
E. leveillei	SPAIN, Asturias	LIP-0001148 (epitype)	Paz et al. (2017)	KX238856
E. maculatus	SPAIN, Cantabria	LIP-0001149	Paz et al. (2017)	KX238799
E. morettii var. *echinatus*	NORWAY, Vest-Agder	IC 27091306 (Herb. pers. A. Paz)	Paz et al. (2017)	KX238854
E. persoonii	SPAIN, Cantabria	IC 18031201 (Herb. pers. A. Paz)	Paz et al. (2017)	KX238828
E. septatus	SPAIN, Cantabria	IC 10041107 (Herb. pers. A. Paz)	Paz et al. (2017)	KX238807
E. spirosporus	SPAIN, Caceres	LIP-0001152 (holotype)	Paz et al. (2017)	KX238796
E. virgatosporus	SPAIN, Asturias	IC 26051213 (Herb. pers. A. Paz)	Paz et al. (2017)	KX238811
E. iuppitercellus	CAMEROON, East Province	M3 (from an ECM root tip)	Castellano et al. (2016)	KT694140
E. miyabeanus	**JAPAN, Yamanashi Pref., Narusawa Village**	**KPM-NC 29301**	**This study**	**OM286885**
Elaphomyces sp.	MEXICO, Mexico State, Temascaltepec	GO-2009-040	Unpubl. data by Garibay Orijel, R. and Pina Paez, C.	KJ594995
Elaphomyces sp.	MEXICO, Mexico State, San Simon de Guerrero	GO-2009-028	Unpubl. data by Garibay-Orijel, R. and Pina Paez, C.	KJ594996
Elaphomyces sp.	ROMANIA, ECM of *Quercus robur*	LM 2779	Suz et al. (2014)	KM576390
Elaphomyces sp.	USA	FLAS_AC727	Unpubl. data by Corrales, A. et al.	MH496798
Elaphomyces sp.	PANAMA	AC-2017a	Unpubl. data by Corrales, A. et al.	KY825739
Elaphomyces sp.	USA, FL	FLAS-F-60353	Unpubl. data by Richter, B. et al.	MF074844
Elaphomyces sp.	USA, FL	FLAS-F-61848	Unpubl. data by Kaminsky, B. et al.	MH399885
Elaphomyces sp. (sp. 6)	NORWAY, Viken, Ullensaker	O-F-22082	Unpubl. data by Larsson, E. and Molia, A.	KR029769
Elaphomyces sp. (sp. 6)	NORWAY, Oslo fylke, Oslo	O-F-245336	Unpubl. data by Larsson, E. and Molia, A.	KR029770
Elaphomyces sp. (sp. 6)	NORWAY, Viken, Ringerike	O-F-245524	Unpubl. data by Larsson, E. and Molia, A.	KR029771
UECM of *Quercus crispula*	JAPAN	M0400	Unpubl. data by Ishida T. A.et al.	KC791026
UECM of *Quercus kelloggii*	USA, CA, Riverside County, James Reserve	JR9	Unpubl. data by Taniguchi et al.	KC791026

Hokkaido Pref.). On 1 October 2018, one of the authors (M. Ohmae) collected a single overmature ascoma that was morphologically identical to *E. nopporensis* (Fig. 2A): the ascoma had a rough, black peridium that became papery due to dehydration and over-maturity, and a shrunken blackish gleba. Subsequently, additional old ascomata were collected at a different site in the type locality on 22 Sep. 2019. Trials of ITS sequencing from both specimens failed.

We also collected ascomata that were morphologically identical to *E. miyabeanus* from a subboreal coniferous forest in Yamanashi Prefecture, central Honshu (Fig. 2D–H). ITS sequence was successfully obtained from this specimen (KPM-NC 29301; Table 1) and subjected to the following phylogenetic analyses. Additionally, we collected black *Elaphomyces* ascomata that appeared very similar to *E. miyabeanus* from Ehime Prefecture, Shikoku (KPM-NC 29304), but this specimen was different from *E. miyabeanus* in the ascospore morphology (Fig. 2I; see "Notes" below for more details).

Fig. 1. Authentic specimens of *E. miyabeanus* and *E. nopporensis* recorded in Imai (1929) and rediscovered from SAPA and TMI herbaria. A–C: SAPA specimens of *E. miyabeanus* parasitized by *Tolypocladium jezoense* (upper of A and B; syntype of *T. jezoense* [Imai, 1929]) and *E. nopporensis* (lower of A and C; isosyntype of *E. nopporensis*). D–I: TMI specimens Newly designated lectotype of *E. miyabeanus* (D [middle], E, G; TMI 37400) and one of the syntypes of *E. nopporensis* (F, H, I; TMI 37401). Both of the specimens were collected by Dr. S. Imai on 4 July 1926. G: Ascospores of *E. miyabeanus*. H: Ascospores of *E. nopporensis*. Note that the outer warty spore walls and the inner cores are separated in most ascospores. I: SEM photomicrograph of partially angular ascospore (right) of *E. nopporensis* and remnants of spore walls (arrows). Bars: E–F = 1 cm; G–H = 20 µm; I = 10 µm.

図 1. Imai (1929) における記載に用いられたクロツチダンゴ *E. miyabeanus* およびコクロツチダンゴ *E. nopporensis* 標本（北海道大学総合博物館 [SAPA] および日本きのこセンター菌蕈研究所 [TMI] 所蔵）．A–C: SAPA 所蔵のエゾハナヤスリタケ *Tolypocladium jezoense* に寄生された *E. miyabeanus* 標本（A 上部および B；本標本は *Tolypocladium jezoense* のシンタイプに相当）および *E. nopporensis* 標本（A 下部および C；本標本は *E. nopporensis* のアイソシンタイプに相当）．D–I: 新たにレクトタイプ指定された TMI 所蔵の *E. miyabeanus* 標本（D［中央］, E, G; TMI 37400）および *E. nopporensis* シンタイプ標本（F, H, I; TMI 37401）．いずれも今井三子博士により 1926 年 7 月 4 日採集．G: *Elaphomyces miyabeanus* 子嚢胞子．H: *Elaphomyces nopporensis* 子嚢胞子．多くの胞子において、短い刺状突起に覆われた胞子外壁と，その内部の胞子中心部が分離している．I: *Elaphomyces nopporensis* の一部角張った子嚢胞子（右側）および胞子外壁の残骸（矢印）の SEM 画像.

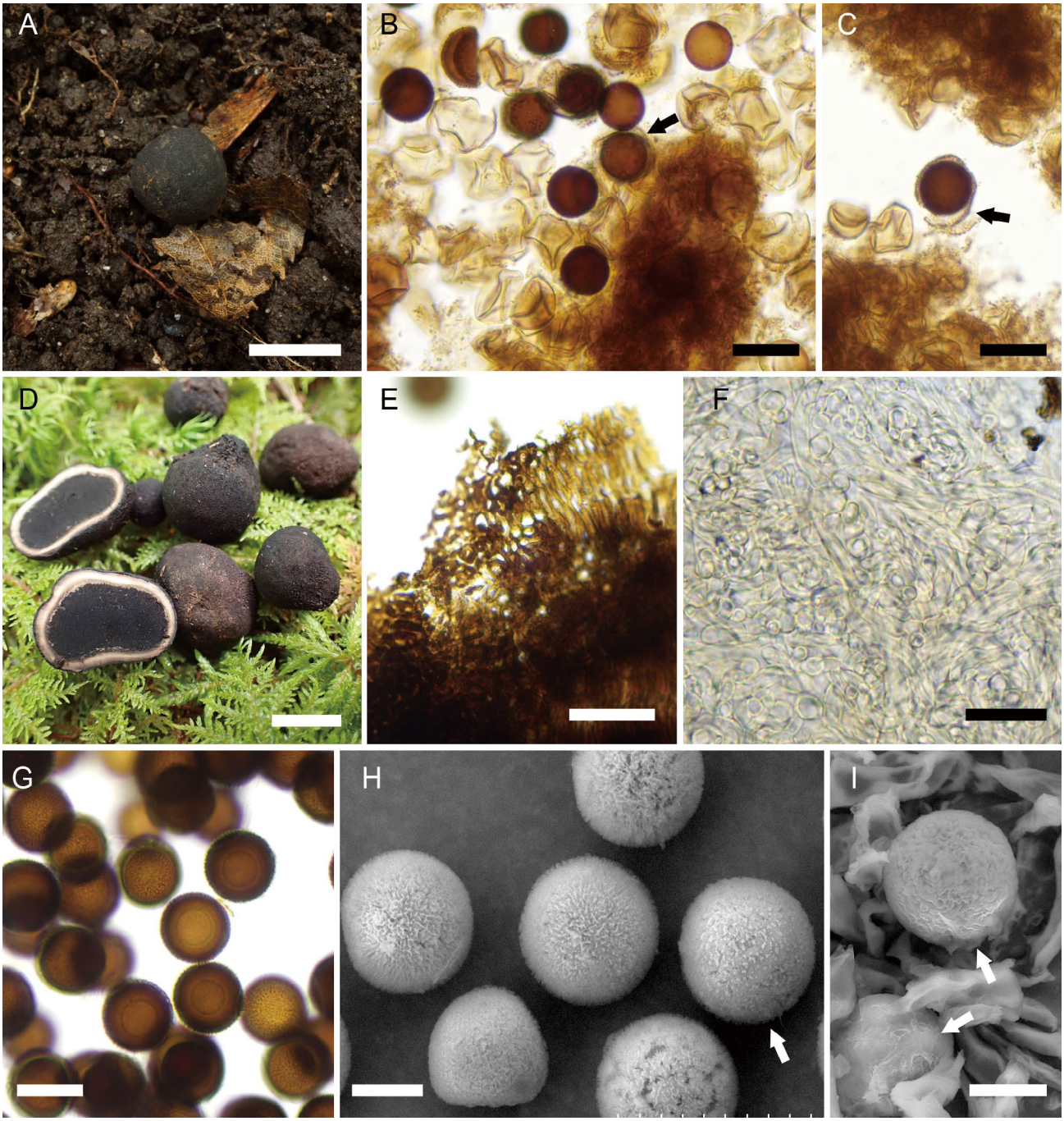

Fig. 2. A–C: *Elaphomyces nopporensis* (= small, old, dried ascoma of *E. miyabeanus*) newly collected from the type locality (KPM-NC 25100). A: Ascoma. B–C: Ascospores; in most ascospores the outer exosporium was shrunken and separated from the inner dark brown core (arrows). D–H: Newly collected *E. miyabeanus* specimens (KPM-NC 29301). D: Ascomata. E: Peridiopellis. F: Inner peridial tissue. G: Ascospores. H. Ascospores in various developmental stages under SEM. Immature ascospores are covered with an evanescent, plate-like, warty layer (arrow). I: SEM photomicrograph of an ascospores of *E. anthracinus* collected from Japan (KPM-NC 29304). Arrows indicate evanescent, outermost veil-like materials. Bars: A, D = 1 cm; B–C, E–G = 20 µm, H–I = 10 µm.

図 2. A–C: タイプ産地（野幌森林公園）で発生が確認されたコクロツチダンゴ *E. nopporensis*（*E. miyabeanus* の乾燥した小型の老菌）標本（KPM-NC 25100）. A: 子嚢果. B–C: 子嚢胞子. ほとんどの子嚢胞子で胞子外壁が収縮し、内部の暗褐色の胞子中心部と分離していることが確認される（矢印）. D–H: 本研究において新たに採集されたクロツチダンゴ *E. miyabeanus* 標本（KPM-NC 29301; 山梨県南都留郡鳴沢村産）. D: 子嚢果. E: 最外殻皮. F: 殻皮実質の菌糸. G: 子嚢胞子. H: 様々な成熟段階の子嚢胞子の SEM 画像. 未熟な胞子はいぼ状突起のある消失性の胞子最外層に覆われる（矢印）. I: 日本産 *E. anthracinus* 子嚢胞子の SEM 画像（KPM-NC 29304；愛媛県北宇和郡鬼北町産）. 矢印は消失性の胞子最外膜を示す.

Phylogenetic analyses

The ITS dataset was composed of 607 aligned nucleotide positions and contained 32 sequences in total, including newly generated sequence of *E. miyabeanus* specimen (KPM-NC 29301; GenBank accession no.: OM286885). In the RAxML analysis, the final ML optimization of log likelihood (lnL) was -3510.721128. In the PhyML analysis, the final lnL was -3570.619692. In the Bayesian inference, the two parallel runs reached convergence after ca. 1.9M generations. Accordingly, we discarded the first 1900 trees in each run, and the remaining 1101 trees in each run were summarized to approximate Bayesian PPs. The total arithmetic and harmonic mean of estimated marginal lnL for runs were -3456.18 and -3490.43, respectively.

The resulting overall ML and Bayesian topologies were identical although statistic support of some branches varied considerably among the three methods (i.e., MLBS/SH-aLRT/PP). We therefore show only the RAxML tree (Fig. 3). The tree mostly recovered overall relationships within *Elaphomyces* sect. *Ceratogaster* shown by Paz et al. (2017) with some minor exceptions (e.g., the relationships among *E. aculeatus* Vittad., *E. virgatosporus* Hollós and *E. anthracinus* Vittad. clades). The *E. miyabeanus* specimen from Yamanashi Pref.,

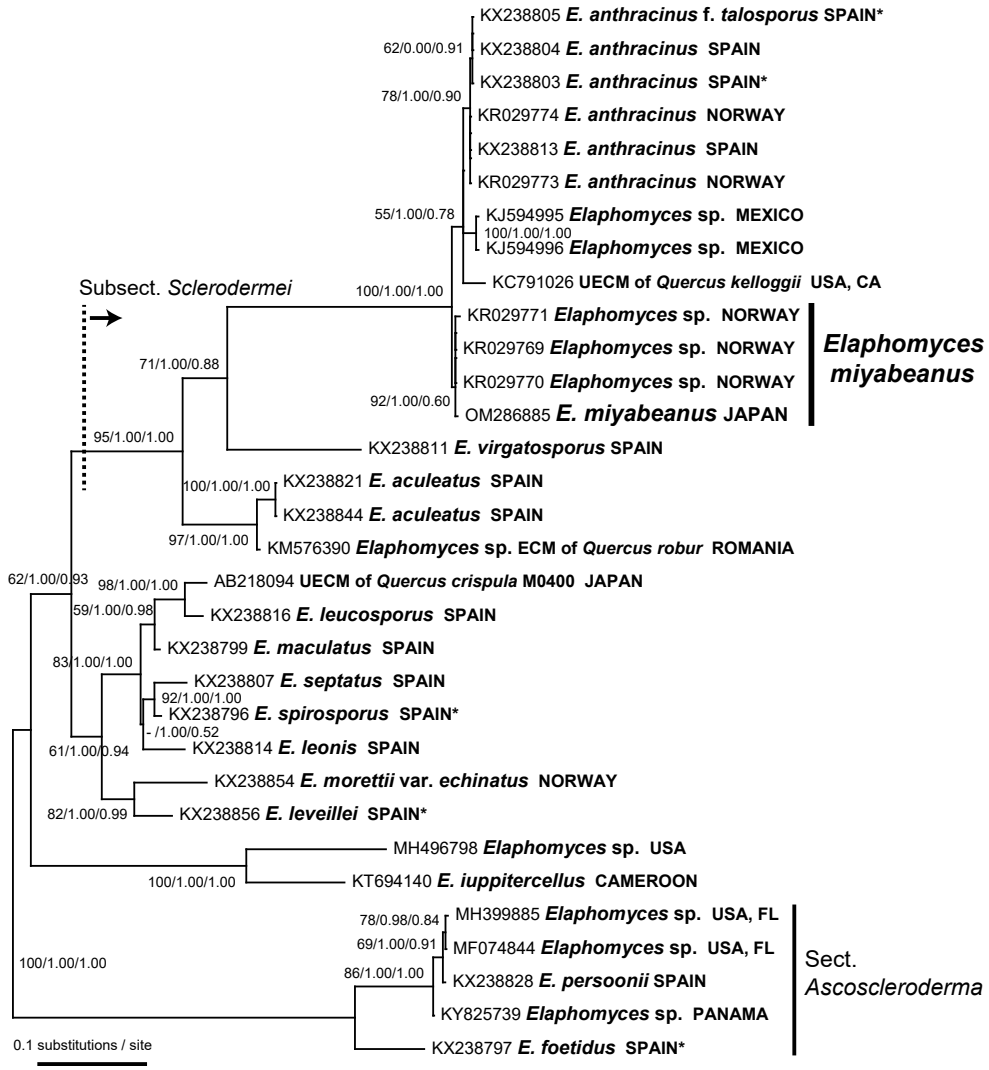

Fig. 3. RAxML Maximum likelihood (ML) tree of *Elaphomyces* sect. *Ceratogaster* based on the nuclear rDNA ITS dataset. Sequence of *E. miyabeanus* specimen (OM286885) was generated for this study. Values of RAxML rapid bootstrapping (MLBS), the SH-aLRT test by PhyML and Bayesian PPs are designated above or below branches or at nodes (MLBS/SH-aLRT/PP). The GenBank accession numbers are shown followed by taxon names as registered in GenBank. Sequences from holotype, lectotype or epitype specimens are indicated as asterisks (*). Species of *Elaphomyces* sect. *Ascoscleroderma* are selected as outgroups. UECM: uncultured ectomycorrhizal root.

図 3. 核 rDNA ITS 領域のデータセットに基づく，RAxML によるツチダンゴ属 *Ceratogaster* 節の最尤系統樹．クロツチダンゴ *E. miyabeanus* 標本から新たに得られた配列（OM286885）を含む．RAxML 高速ブートストラップ法（MLBS），PhyML による SH 補正近似尤度比検定（SH-aLRT test），およびベイズ事後確率（PP）の値を各分岐に示す（MLBS/SH-aLRT/PP）．本節の姉妹群に相当する，ツチダンゴ属 *Ascoscleroderma* 節に含まれる種を外群に用いた．UECM: uncultured ectomycorrhizal root.

Japan was placed within sect. *Ceratogaster* subsect. *Sclerodermei* (Vittad.) Bellanger & P.-A. Moreau and was shown to be closely related to European *E. anthracinus*, *Elaphomyces* sp. from Mexico and an environmental ECM sample from the USA. The ITS1-5.8S-ITS2 nucleotide identities between *E. miyabeanus* and *E. anthracinus* were 95.46% (589 bp / 617 bp; query coverage: 100%). *Elaphomyces miyabeanus* formed a species-level clade with unidentified *Elaphomyces* sp. sequences from Norway (i.e., "*Elaphomyces* sp. 6" according to the taxon-name label by Larsson E. & Molia A.) although the support by Bayesian PP was unexpectedly low (i.e., PP = 0.60). The ITS1-5.8S-ITS2 nucleotide identities between *E. miyabeanus* and one of "*Elaphomyces* sp. 6" were 98.54% (608 bp / 617 bp; query coverage: 100%).

Taxonomy

Taxonomic relationship between *E. miyabeanus* and *E. nopporensis*

Imai (1929) noted that he repeatedly collected *E. miyabeanus* and *E. nopporensis* on the same days in the same locality ("Nopporo Forest"). However, the number of *E. nopporensis* specimens were relatively small, and no young ascomata of the species were found (Imai, 1929). The peridium of the species is papery, which is a typical character of dried, old ascomata of black deer truffles, and the surface of both species are more or less rough although in *E. miyabeanus* large pyramidal warts partially remain on the apical or rarely on the basal part (Imai, 1929). According to Imai (1929), the ascospores of *E. nopporensis* are smaller than those of *E. miyabeanus* but their sizes overlap within the range of those of *E. miyabeanus* (i.e., 15–17.5 µm in *E. nopporensis* vs. 15–22 µm in *E. miyabeanus*).

Our observation of the rediscovered syntypes of both the species confirmed that the descriptions by Imai (1929) were mostly accurate, except for the ascospore size of *E. nopporensis*. In some ascomata the ascospores were shrunken and the exosporium and outermost ornamentation were often collapsed or separated from the inner core. As a result, many of the remaining ascospores were smaller in size than those of the *E. miyabeanus* specimens, but in well-preserved ascomata of *E. nopporensis* the ascospore size was larger than the original description and close to that of *E. miyabeanus* (i.e., 15.2–21.9 µm; n = 30).

As noted above, the newly collected ascomata from the type locality (KPM-NC 25100, 27903) represented typical macroscopic features of *E. nopporensis*. Similar to the *E. nopporensis* syntypes, in most ascospores the outermost spore wall was shrunken and separated from the inner spore core (Fig. 2B–C). In other cases, ascospores looked heavily depressed or somewhat angular, or only the outermost layer remained (Figs. 1H–I, 2B).

Based on these observations, we conclude that *E. nopporensis* is a small, dried, old, overmature ascoma of *E. miyabeanus* and should be synonymized into the latter species. The characteristic large warts on the surface of *E. miyabeanus* ascomata apparently fall off on old ascomata (= *E. nopporensis*) or are absent on smaller ascomata. We herein designate lectotype of *E. miyabeanus* from the syntype specimens preserved in TMI. The lectotype specimen (TMI 37400), which was collected on 4 July 1926 by S. Imai, consists of many ascomata of various developmental stages and is morphologically one of the most well-preserved syntype specimens. Although we successfully obtained an ITS sequence from the specimen collected from central Honshu (KPM-NC 29301), we will not designate epitype of *E. miyabeanus* until fresh ascomata in good condition with its nucleotide sequences including the barcoding region are obtained near the type locality.

***Elaphomyces miyabeanus* S. Imai**, Transactions of the Sapporo Natural History Society 11: 35 (1930).
[MycoBank ID: 271439]
= *Elaphomyces nopporensis* S. Imai, Transactions of the Sapporo Natural History Society 11: 36 (1930).
[MycoBank ID: 272439].

Figs. 1, 2A–H.

Lectotype (designated here!): JAPAN, Hokkaido Pref., Ishikari Prov., Nopporo Forest (the present Nopporo Forest Park), 4 Jul. 1926, *S. Imai*, TMI 37400 [MycoBank Typification ID: 10006470].

Description: Ascomata solitary or gregarious, 3–30 mm in diam., rigid, hard, globose to subglobose, often with a slight apical projection and irregular depression, surface black, almost smooth to the naked eye but more or less rough under loupe or stereomicroscope, often with pyramidal-obtuse warts around the apical projection and basal depression. Mycelia on the surface of ascomata inconspicuous. Outer peridium thin, up to 0.4 mm thick, black, rigid, fragile, separable from inner peridium. Inner peridium up to 2 mm thick, off-white to pale brown with a reddish tinge, more or less firm, rubbery, evanescent when dry and overmature. Gleba filled with a black spore mass with white sterile veins running more or less radially. The outermost part of gleba appears as a pale reddish brown hyphal layer. Spore mass more or less firm when immature, becoming powdery at maturity. Odor not distinct.

Outer peridium and warts of thick-walled (0.7–3 µm), filamentous or palisade-like, dark brown hyphae 2.5–5.5 µm broad and firmly adherent to one another, running in several directions. Inner peridium of whitish to pale brown, filamentous hyphae 2.5–10 µm broad, running perpendicularly in two directions, walls 0.6–1.5 µm thick.

Glebal hyphae of densely interwoven, evanescent, partially inflated, dark brown, thin-walled (<1.1 µm) filamentous hyphae 3–10 µm broad, with inflated, clavulate, terminal cells. Asci not seen, but Imai (1929) described them as 8-spored. Ascospores 15.2–25.5 µm in diam. including ornamentation (average size in lectotype: 21.8 µm; standard deviation: 1.57 [n = 50]), globose, composed of multiple wall layers: outermost walls 0.8–2 µm thick, dark brown, surface finely reticulate under SEM, densely covered with blunt to acute spines 0.6–2.5(–3) µm long partially adhered to each other to form a minute, incomplete reticulum, often collapsed or separated from the inner wall in old, overly dried specimens; in young ascospores the spiny ornamentation is completely or partially covered with evanescent, plate-like, warty layer; inner walls 4–5 µm thick, apparently composed of 4–5 layers, but sometimes not distinct especially in old, overmature specimens, dark to medium brown.

Habitat, distribution and season: mostly hypogeous but occasionally sub-epigeous under various ectomycorrhizal tree species such as *Quercus* spp. and *Abies veitchii*; Japan and Norway, but potentially broadly distributed across northern Eurasia; summer to winter.

Japanese name: *Kuro-tsuchidango* ("*kuro*" = black, "*tsuchidango*" = deer truffle in Japanese).

Other specimens examined: JAPAN, **Hokkaido Pref.**, Ishikari Prov., Nopporo Forest (the present Nopporo Forest Park), parasitized by *T. jezoense*, 8 Oct. 1923, *S. Imai*, deposited in SAPA; *ibid*, 15 Nov. 1925, *S. Imai*, TMI 37402; *ibid*, 4 Jul. 1926, *S. Imai*, a syntype of *E. nopporensis*, TMI 37401 (duplicate in SAPA); *ibid*, parasitized by *T. jezoense*, 7 Oct. 1927, *S. Imai*, a syntype of *E. nopporensis*, TMI 37403; *ibid*, 21 Oct. 1928, *S. Imai*, TMI 37404; *ibid*, parasitized by *T. jezoense*, 17 Oct. 1929, *S. Imai*, TMI 37405; *ibid*, 30 Oct. 1938, *S. Imai*, TMI 37406; Ebetsu-shi, Nishi-nopporo, Nopporo Forest Park, under *Quercus crispula*, 1 Oct. 2018, *M. Ohmae*, KPM-NC 25100; Sapporo-shi Atsubetsu-ku, Atsubetu-cho-konopporo, Nopporo Forest Park, near Hokkaido Museum and the Centennial Memorial Tower, under *Q. crispula*, 22 Sep. 2019, *S. Hatakeyama*, KPM-NC 27903; **Yamanashi Pref.**, Narusawa Village, northeastern slope of Mt. Fuji, under *Abies veitchii*, alt. *ca*. 2100 m, 19 Sep. 2021, *Y. Kaneko*, KPM-NC 29301.

Notes: We also collected several black ascomata that appeared quite similar to *E. miyabeanus* from Ehime Prefecture (Kitauwa-gun Kihoku-cho, Narukawa Gorge, under *Castanopsis cuspidata* and *Quercus* spp., 13 Dec. 2021, *T. Teramoto*, *T. Orihara & K. Yamamoto*, KPM-NC 29304). The ascomata were 10–22 mm in diam., and the surface was almost smooth to the naked eye and lacked large warts. Ascospores were almost the same in size as *E. miyabeanus* ascospores (i.e., 18.5–23.1 µm; average 20.5 µm [n = 25]), but SEM observation revealed that the ascospores had a nearly complete reticulum fully or partially covered with an evanescent vail-like material (Fig. 2I). Those characters match well with the description of the *E. anthracinus* by Castellano et al. (2017). We therefore tentatively identify the specimen KPM-NC 29304 as *E. anthracinus*.

Discussion

Taxonomy of black *Elaphomyces* species, which mostly belong to sect. *Ascoscleroderma* (Clémencet) Bellanger & P.-A. Moreau, *Ceratogaster* and *Malacodermei* (Vittad.) Tul. & C. Tul., have long been neglected in Japan since Kobayasi (1960) despite the recent remarkable progress on global biodiversity of *Elaphomyces* (e.g., Castellano et al., 2011, 2012a, b, c, 2016, 2018, 2021; Paz et al., 2017). However, Hatakeyama & Orihara (2020) reported *E. asahimontanus* Kobayasi, currently endemic to Japan, for the first time since Kobayasi (1960), and gave detailed morphology and ecology of the species. In this study, we focused on two historically forgotten black deer truffles, *E. miyabeanus* and *E. nopporensis*, and concluded that they were identical species. It should be noted that both species had not been collected for more than 80 years. Most known Japanese *Elaphomyces* spp. were described more than a half century ago, and require critical reassessment and modern descriptions to incorporate them into the current framework of *Elaphomyces* systematics. This study is an important step to understand the diversity of black *Elaphomyces* species in East Asia.

Both *E. miyabeanus* and *E. nopporensis* were originally described by Imai (1929). They were, therefore, published on the same date and have equal taxonomic priority (cf. Article 11.5 of the current version of the International Code of Nomenclature for algae, fungi, and plants [Shenzhen Code, 2018]; https://www.iapt-taxon.org/nomen/pages/main/art_11.html). We adopt the name *E. miyabeanus* for the combined species because *E. nopporensis* is only represented by an overly dried, old ascomata and does not represent its typical morphology. As a result, *E. miyabeanus* will be treated as having a priority over *E. nopporensis* according to Article 11.5 of the Shenzhen Code.

Elaphomyces miyaeanus shares many morphological characteristics with *E. anthracinus* such as an almost smooth black peridial surface, blackish spore mass, medium-sized ascospores (20–24 µm) with low ornamentation (Castellano et al., 2018). However, *E. miyabeanus* differs from the latter species in the peridium being partially covered with large, pyramidal warts and the ascospore ornamentation forming an incomplete reticulation or more or less isolated spines. In addition, the young ascospores of *E. miyabeanus* are covered with a plate-like, warty layer (Fig. 2H). Our ITS phylogeny also supported that

these two species were closely related but genetically distinct (Fig. 3). Kobayasi (1960) and Japanese Society of Cordyceps Research (2014) recorded *E. anthracinus* from Japan, but they did not provide a detailed morphological description. We collected ascomata morphologically identical to *E. anthracinus* from Ehime Prefecture, Japan, and confirmed that *E. anthracinus* and *E. miyabeanus* are morphologically distinguishable (Fig. 2I).

Paz et al. (2017) proposed *E. anthracinus* f. *talosporus* A. Paz & Lavoise for the *E. anthracinus* specimens that have globose-polyhedral ascospores, but no particular genetic divergence was found between the two forms. As noted above, the ascospores of dried, old ascomata of *E. miyabeanus* in poor condition tend to become somewhat angular-polyhedral or shrunk. We consider that this tendency is not related to genetic divergence, but could be caused by several environmental and/or biological factors. For example, such shrinkage may be caused by the parasitism by *T. jezoence* because the glebal hyphae of *E. nopporensis* are often intermingled with alien, off-white hyphae (Fig. 1F). It is quite possible that morphology of *E. anthracinus* ascospores are also heavily influenced by environmental and/or biological factors. Critical observation of the ascospores of *E. anthracinus* f. *talosporus* in relation to ascomata conditions will be necessary to clarify the cause of the spore deformation.

The ITS phylogeny indicates that *E. miyabeanus* is potentially conspecific with *Elaphomyces* sp. from Norway (Fig. 3). Paz et al. (2017) previously reported that those Norwegian specimens were genetically divergent from *E. anthracinus s.str.*. However, they tentatively retained them within *E. anthracinus* (as "*E. anthracinus* f. *talosporus*" presumably based on the morphology of the shrunken, irregular-shaped ascospores) because they could not ascertain any distinct species-level morphological difference. Additional Japanese records of *E. miyabeanus* are from both deciduous broad-leaved and coniferous forests in subboreal regions. These results suggest that *E. miyabeanus* may be widespread in northern Eurasia, and is possibly misidentified as more widely known *E. anthracinus*. This is an interesting example that clarifying the entities of historically overlooked truffle-like fungi in Asia can sharpen our understanding of the distribution and systematics of another taxon in a different region of the world.

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