Assessment of *Botryosphaeria stevensii* detection by DNA barcoding

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Abstract

Botryosphaeria stevensii is a fungus that infects several tree species and causes cankers. In this study, 139 strains of 12 species in the genus *Botryosphaeria* isolated from infected apple trees were selected for assessing feasibility of identifying *Botryosphaeria stevensii* using DNA barcoding method with three potential marker genes: translation elongation factor 1alpha (*EF-1α*), the internal transcribed spacer (ITS) and *β-tubulin*. By means of intraspecific and interspecific interval and cluster analysis and Wilcoxon rank sum testing, the ranking of the three tested genes for distinguishing intraspecific and interspecific genetic distance in *B. stevensii* is as follows: *EF-1α* > *β-tubulin* > ITS. DNA barcoding using the three tested genes was shown to be suitable for detection of three *Botryosphaeria stevensii* strains isolated from the United States and New Zealand. Therefore, DNA barcoding based on the sequence of the *EF-1α* gene could be used to rapidly identify *B. stevensii* infecting apple trees.

Key words – apple trees – *Botryosphaeria stevensii* – fungus identification

Introduction

Botryosphaeria stevensii Shoemaker (anamorph *Diplodia mutila* (Fr.) Mont.), a fungus found in the United States, Canada, Australia and 20 other countries, is quarantined as a harmful organism once detected on infected plants at the entry to China and Mexico. *B. stevensii* can infect 18 genera of important economic crops, including *Malus*, *Pyrus*, and *Vitis*. Infections can affect the stem, leaf, and fruit, giving rise to symptoms such as cankers, cancerous swelling, necrosis of the stem, yellow, withered leaves, and fruit rot, causing huge economic losses (Farr & Rossman 2018, Wang et al. 2014, <http://www.indexfungorum.org/Names/Names.asp>).

In 2002 in a vineyard in New South Wales, Australia, cankers caused by two fungi, *B. stevensii* and *B. obtusa*, resulted in approximately 97% reduction in production of grape plants and led to nearly total crop failure (Whitelaw-Weckert et al. 2006). In the early 2000s, the value of the annual grape industry output of the state of California in the United States was 2.3 billion dollars,

with more than 260 million dollars of losses caused by stem canker per year (Urbeztorres et al. 2006).

The genus *Botryosphaeria* contains 247 species, of which 12 can infect apple trees. Identification of *Botryosphaeria* species is difficult and time consuming because of their small interspecific differences and the difficulty of inducing sporulation in vitro. With rapid progress in molecular biology in recent years, species that were difficult to identify by traditional morphological and biological methods can now be identified by molecular methods. However, so far there are no reports on rapid identification of *B. stevensii*. DNA barcoding is a rapid and accurate molecular diagnostic technique by which species are identified using a standard DNA sequence (Hebert et al. 2003, Miller 2007). In this study, the *EF-1 α* , ITS and *β -tubulin* genes from 12 species of *Botryosphaeria* that damage apple trees were tested to assess their suitability for DNA barcoding to rapidly identify pathogenic fungi in the genus *Botryosphaeria*. The suitability of the selected DNA barcoding sequences was verified using *B. stevensii* isolates from apples imported from North America and New Zealand.

Materials & Methods

DNA sequences for DNA barcoding screening

First, 10270 sequences of the *EF-1 α* , ITS and *β -tubulin* genes from 12 pathogenic *Botryosphaeria* species that infect *Malus pumila* (apple) were downloaded from the GenBank database (download date was 30 December 2016). Using Mega 5.0 software for alignment to arrange the three genes, each sequence was proof-read and verified to eliminate low quality sequences, sequences for which the number of uncertain base pairs exceeded 10 bp, and sequences shorter than 100 bp, after which reverse complementation and adjustment of sequence direction was performed. The representative strains and sequences of each pathogenic fungus were identified and shown in Table 1 (400 sequences from 139 strains).

Table 1 Sequence information for *EF-1 α* , ITS and *β -tubulin* in 12 *Botryosphaeria* species that infect *Malus pumila*.

Species	Strain number	GenBank number		
		<i>EF-1α</i>	ITS	<i>β-tubulin</i>
<i>Botryosphaeria australis</i>	CBS 122026	KX464673	KX464161	KX464950
	CBS 114823	KX464671	KX464159	KX464947
	UCR1110	JF271793	JF271758	JF271776
	CMW6853	AY339271	AY339263	AY339255
	CMW3386	AY615157	AY615165	AY615149
	CBS 119046	EU017541	DQ299244	KU198429
<i>Botryosphaeria dothidea</i>	MUCC503	EF591970	EF591918	EF591953
	UCD2432TX	GU294714	FJ790844	GU294728
	SXD1221	KC961072	KC960982	KF005078
	LJL5521	KC961069	KC960978	KC961024
	JCC1311	KC961043	KC960918	KC960996
	BD140	JQ900341	JN545840	JQ900375
	Mht-19	KC864748	KC864746	KC864747
	N14	KM349761	KJ863654	KJ863647
	PD296	GU251236	GU251104	GU251764
	PD315	GU251235	GU251103	GU251763
	PD313	GU251233	GU251101	GU251761
	SNN5711	KC961065	KC960973	KF005077
	EN22931	KC961059	KC960957	KC961015
	LJF2121	KF005081	KC960933	KC960998
	ZY-713	HQ660488	HQ660462	HQ660465
	UF0730	GQ869643	GU595170	JN607154

Table 1 Continued.

Species	Strain number	GenBank number		
		<i>EF-1α</i>	ITS	<i>β-tubulin</i>
<i>Botryosphaeria iberica</i>	zz-1-3-1s	JX462307	JX275785	JX462281
	J531	KC961039	KC960907	KC960993
	SDAU07161	FJ265873	FJ171721	FJ238530
	SENC65	JN607109	JN607086	JN607132
	GS-02s-6	JF441076	JF441086	JF441080
	EW23111	KC961061	KC960959	KC961017
	YZN1311	KC961040	KC960910	KC960994
	HuB10s1	JX462294	JX275786	JX462268
	HeNs1	JX462292	JX275784	JX462266
	LPSU2012127	KX091156	KX091152	KX091155
	CMW8000	AY236898	AY236949	AY236927
	CMW3910	HM176507	HM176522	HM176485
	CMW3903	HM176506	HM176521	HM176484
	MUCC501	EF591969	EF591916	EF591952
	MUCC500	EF591968	EF591915	EF591951
	CBS 113188	EU673278	AY573198	N/A
	UCD1448SLO	EF202023	EF202009	EF202016
	PD257	GU251300	GU251168	GU251828
	CBS 115041	AY573222	NR_111165	N/A
<i>Botryosphaeria lutea</i>	CBS 764.73	KX464692	KX464970	KX464970
	CMW9076	AY339265	AY236946	AY339249
	CBS 110299	AY573217	AY259091	DQ458848
	CMW10310	AY339267	AY339259	AY339251
	UCR1180	JF271798	JF271798	JF271782
	UCR1192	N/A	JF921874	JF921874
	UCD369-Oe	JX515765	JX515719	JX515687
	UCR548	N/A	HQ529761	HQ529731
	CMW10309	AY339266	KF766202	AY339250
	UCR1653	JQ659270	JQ659282	JQ659294
<i>Botryosphaeria obtusa</i>	CBS 119049	DQ458874	DQ458889	DQ458857
	CBS 469.69	KX464611	KX464119	KX464847
	SDZ-01	HQ629958	HQ629954	HQ629956
	CBS 358.68	KX464610	KX464118	KX464846
	CBS 117.82	KX464598	KX464108	KX464834
	UCD1061BC	EU012405	EU012382	EU012434
	UCD621Spa	GU294752	DQ356357	DQ356364
	IRB15	GU121848	GU121890	GU121835
	IRB3	GU121850	GU121878	GU121823
	PPO-45237	KX138559	KX138556	KX138560
	UCR1795	JQ659274	JQ659286	JQ659298
	UCR1687	JQ659273	JQ659285	JQ659297
	CMW41361	KP860694	KP860850	KP860772
	CMW38134	KC769830	KC769919	KC769876
	MUCC673	EU339520	EU339553	EU339483
<i>Botryosphaeria parva</i>	UCD578Spa	GU294748	DQ356359	DQ356366
	CMW1130	AY236890	AY236945	AY236919
	CMW7885	AY236889	AY236944	AY236918
	PD251	GU251256	GU251124	GU251784
	CMW38724	KF923277	KF923244	KF923265
	CMW37263	KF454692	KF432951	KF454706

Table 1 Continued.

Species	Strain number	GenBank number		
		<i>EF-1α</i>	ITS	<i>β-tubulin</i>
	DL2611	KC961032	KC960892	KC960987
	CDZ1-1s2	HQ859955	HQ859951	HQ859953
	CMW14141	EU821890	EU821920	EU821860
	IRN12	GU121874	GU121902	GU121847
	PD39	GU251276	GU251144	GU251804
	5B602	KF779053	KF778863	KF778958
	CMW14082	EU821877	EU821907	EU821847
	UCD577Spa	GU294747	DQ356358	DQ356365
	CMW9081	AY236888	AY236943	AY236917
	MFLUCC11-0184	JX646860	JX646795	JX646843
<i>Botryosphaeria quercuum</i>	CCF109	KC507809	KC507812	KC507806
	MFLUCC14-0459	N/A	KU848199	N/A
	96-186	N/A	AF243399	N/A
	CBS 177.89	N/A	N/A	DQ026404
<i>Botryosphaeria rhodina</i>	UCD1060BC	EU012396	EU012376	EU012428
	7E88	KC357297	KC357279	KC357288
	HD1332	KU712503	KU712502	KU712504
	CMW9074	AY236901	AY236952	AY236930
	MFLUCC14-0512	KM409631	KM396893	KM510351
	81.I	KC964552	KC964548	KC964551
	UCD2430TX	GU294713	FJ790831	GU294727
	SEFL28b	JN607115	JN607092	JN607139
	UCD1028BC	EU012394	EU012374	EU012426
	PD270	GU251253	GU251121	GU251781
	CBS 287.47	KX464639	KX464145	KX464914
	CMW41360	KP860686	KP860841	KP860764
	FXPZ	KR232660	KR232666	KR232674
	MBAi184AG	KP721663	KJ596529	KP721701
	CMM3647	KF226704	KF234548	KF254932
	SEAL9a	JN607113	JN607090	JN607137
	MFLUCC11-0508	JX646864	JX646799	JX646847
	CMW7773	DQ235142	AY236936	AY236907
	MUCC247	EU339513	EU301028	EU339476
	SEGA5	JN607121	JN607098	JN607145
<i>Botryosphaeria ribis</i>	PD288	GU251259	GU251127	GU251787
	H73-1	HQ392755	HQ392733	HQ392754
	UCD2579MO	HQ288272	HQ288230	HQ288309
	CMW7772	AY236877	AY236935	AY236906
	PD289	GU251260	GU251128	GU251788
	SENC34a	JN607124	JN607101	JN607148
	MUCC125	EU339514	EU339525	EU339477
	SENC29	JN607123	JN607100	JN607147
	PD254	GU251258	GU251126	GU251786
	SEGA8	JN607122	JN607099	JN607146
<i>Botryosphaeria sarmentorum</i>	SEAL2	JN607120	JN607097	JN607144
	CBS 174.53	KX464620	KX464128	KX464883
	PD78	GU251301	GU251169	GU251829
	CBS 165.33	AY573225	AY573208	KX464882
	CBS 120.41	AY573224	AY573207	KX464880
	CMW39370	KF575049	KF575011	KF575107
	CMW39364	KF575048	KF575010	KF575106

Table 1 Continued.

Species	Strain number	GenBank number		
		<i>EF-1α</i>	ITS	<i>β-tubulin</i>
<i>Botryosphaeria</i>	cfcc82346	KU221235	KT343257	N/A
<i>sinensia</i>	CGMCC 3.17723	KU221233	N/A	N/A
<i>Botryosphaeria</i>	UCR1670	JQ659272	JQ659284	JQ659296
<i>stevensii</i>	CBS 112553	AY573219	AY259093	DQ458850
	JL375	DQ458871	DQ458887	DQ458852
	UCROK1429	JQ512121	JQ411412	JQ411443
	UCD127-Oe	JX515786	JX515745	JX515674
	CBS 230.30	DQ458869	DQ458886	DQ458849
	UCD1965SB	EU012413	DQ233599	DQ233620
	CMW7060	AY236904	AY236955	AY236933
	CBS 112554	DQ458870	AY259095	DQ458851
	CMW7781	N/A	AY972107	AY972122
	BEI36	N/A	KT954166	KT954167
	UCD147-Oe	JX515787	JX515746	JX515675
	UCROK1425	JQ517318	N/A	JQ411460
	CBS 431.82	N/A	KU198424	KU198426

Materials for DNA barcoding verification

Materials for DNA barcoding verification were obtained from *B. stevensii* isolated from apples imported from the United States and New Zealand, which had been morphologically identified. The *EF-1 α* , ITS and *β -tubulin* genes were sequenced and submitted to the GenBank database. See details in Table 2.

Table 2 *EF-1 α* , ITS and *β -tubulin* genes from *Botryosphaeria stevensii* isolates from apples.

No.	Host	Source	Collection time	Gene sequence		
				<i>EF-1α</i>	ITS	<i>β-tubulin</i>
iso8438	Apple	USA	20160603	KY465923	KY465921	KY644559
iso2953	Apple	New Zealand	20160701	KY465922	KY465920	KY644558
iso50624	Apple	New Zealand	20160715	KY465924	KY465919	KY644560

Isolation and culture

Tissues showing signs of disease were collected from rotted fruit and used to inoculate potato dextrose agar (PDA) plates. After 3–5 d of culture at 25 °C, the growth conditions of conidia were observed.

DNA amplification and sequencing

The mycelia were scraped from the PDA plates, ground to a powder in liquid nitrogen, and transferred to a 1.5 mL EP tube. The QIAGEN kit was used for DNA extraction. A 25 μ L reaction system was used for PCR amplification: 10 $\times$ PCR buffer, 0.1 μ M of each primer, 0.25 mM dNTPs, 1 U *Taq* DNA polymerase, and 10–100 ng template DNA. The primer sequences for *EF-1 α* , ITS and *β -tubulin* are listed in Table 3. The reaction conditions were as follows: 35 cycles of 95 °C denaturation for 30 s, annealing for 30s, and extension at 72 °C for 30 s, followed by a 72 °C extension for 10 min. The annealing temperatures used for *EF-1 α* , ITS and *β -tubulin* amplification were 55 °C, 57 °C and 58 °C, respectively. The amplification products were sent to a company for sequencing after purification. All samples were subjected to two-way sequencing.

Data processing

SeqMan 5.0 software was adopted for sequence splicing and manual correction. After correction, the sequences were deposited in the GenBank database and subjected to a Blast search for homology comparison.

Table 3 Primer sequences used for PCR

Gene fragment	Primers	Sequence (5'–3')	Reference
<i>EF-1α</i>	EF1-728F	CATCGAGAAGTTCGAGAAGG	Carbone & Kohn 1999
	EF1-986R	TACTTGAAGGAACCCTTACC	
ITS	ITS4	TCCTCCGCTTATTGATATGC	White et al. 1990
	ITS5	GGAAGTAAAAGTCGTAACAAGG	
<i>β-tubulin</i>	T1	AACATGCGTGAGATTGTAAGT	O'Donnell & Cigelnik 1997
	Bt-2b	ACCCTCAGTGTAGTGACCCTTGGC	Glass & Donaldson 1995

DNA barcoding analysis

Comparison of intraspecific and interspecific differences

The intraspecific and interspecific genetic distances were determined by calculating a similarity matrix by means of inputting the selected genes into DNASTAR software and using the Kimura-2-parameter distance (K2P) model implemented in the MEGA 5.0 software package. The frequency distribution was analyzed using Microsoft Excel.

Statistical testing of differences in intraspecific and interspecific genetic distance

SPSS 18.0 software was used to perform the Wilcoxon rank sum test to compare differences between gene sequences.

Results

Screening of DNA barcoding genes of *B. stevensii*

The distribution of intraspecific and interspecific genetic distances was determined by calculating each paired genetic distance of *EF-1α*, ITS and *β-tubulin* from *B. stevensii* and 11 closely related species, extracting intraspecific genetic distance data for all sequences in *B. stevensii*, and performing statistical analysis. The intraspecific and interspecific genetic distances of *EF-1α* are shown in Fig. 1. There was no overlap between the intraspecific and interspecific genetic distance, which made it easy to distinguish the target species, *B. stevensii*, from other *Botryosphaeria* species that infect *M. pumila*. The intraspecific and interspecific genetic distances of the ITS fragment are shown in Fig. 2. There was an overlap between the intraspecific and interspecific genetic distance between 0 and 0.02, which mainly consisted of the overlap between *B. stevensii* and closely related species *B. obtusa* and *B. quercuum*. The intraspecific and interspecific genetic distances of *β-tubulin* are shown in Fig. 3. There was an overlap in the intraspecific and interspecific genetic distance between 0 and 0.03, which mainly consisted of the overlap between *B. stevensii* and *B. australe*, *B. australis*, *B. iberica*, *B. obtusa*, and *B. parva*.

Intraspecific and interspecific genetic distance

The minimum and average intraspecific and interspecific genetic distances for three genes expressed by *B. stevensii* and 11 other *Botryosphaeria* species are shown in Table 4. For *EF-1α*, ITS and *β-tubulin*, the average interspecific genetic distance was far greater than the intraspecific genetic distance. The maximum and average intraspecific and interspecific genetic distances of *EF-1α* were the greatest of the tested genes, followed by *β-tubulin*, whereas ITS had the smallest values.

Fig. 1 – Intraspecific and interspecific genetic distance distribution based on the *EF-1α* gene.

Fig. 2 – Intraspecific and interspecific genetic distance distribution based on the ITS fragment.

Fig. 3 – Intraspecific and interspecific genetic distance distribution based on the *β-tubulin* gene.

Table 4 Intraspecific and interspecific genetic distance.

Gene	Intraspecific genetic distance			Interspecific genetic distance		
	Maximum	Minimum	Average	Maximum	Minimum	Average
<i>EF-1α</i>	0.037	0.000	0.017	0.467	0.091	0.267
ITS	0.019	0.000	0.005	0.057	0.002	0.037
<i>β-tubulin</i>	0.027	0.000	0.013	0.090	0.015	0.046

Wilcoxon rank sum test of sequence variation

Wilcoxon rank sum tests were conducted on the intraspecific and interspecific genetic distances of *EF-1 α* , ITS and *β -tubulin* to assess their degree of genetic variation. The test values of different gene fragments were compared (Tables 5, 6) as the basis for selecting a DNA fragment for barcoding.

Table 5 Wilcoxon rank sum test of the intraspecific genetic distance of different sequences.

Positive rank/W ⁺	Negative rank/W ⁻	Positive rank sum, negative rank sum and significant probability P	Result
ITS	<i>EF-1α</i>	W ⁺ =1, W ⁻ =51, P=0.000	<i>EF-1α</i> > ITS
ITS	<i>β-tubulin</i>	W ⁺ =6, W ⁻ =79, P=0.000	<i>β-tubulin</i> > ITS
<i>EF-1α</i>	<i>β-tubulin</i>	W ⁺ =33, W ⁻ =21, P=0.004	<i>EF-1α</i> > <i>β-tubulin</i>

Table 6 Wilcoxon rank sum test of the interspecific genetic distance of different sequences.

Positive rank/W ⁺	Negative rank /W ⁻	Positive rank sum, negative rank sum and significant probability P	Result
ITS	<i>EF-1α</i>	W ⁺ =0, W ⁻ =1221, P=0.000	<i>EF-1α</i> > ITS
ITS	<i>β-tubulin</i>	W ⁺ =293, W ⁻ =1352, P=0.000	<i>β-tubulin</i> > ITS
<i>EF-1α</i>	<i>β-tubulin</i>	W ⁺ =1221, W ⁻ =0, P=0.000	<i>EF-1α</i> > <i>β-tubulin</i>

The analysis of interspecific and intraspecific genetic distances showed that the ranking of the effectiveness of the three tested genes for distinguishing intraspecific genetic distance was *EF-1 α* > *β -tubulin* > ITS ($p < 0.05$). Taken together, the analyses of barcoding intervals and interspecific/intraspecific differences indicate that, among the tested gene fragments, the *EF-1 α* fragment is most suitable for DNA barcoding for rapid identification of *B. stevensii*.

Detection of *B. stevensii* by DNA barcoding**PCR amplification and Blast search of the sequencing results**

The success rate of amplification and sequencing for *EF-1 α* , ITS and *β -tubulin* sequences that had been isolated and morphologically verified in the laboratory was 100% (Table 2). The *EF-1 α* , ITS and *β -tubulin* sequences were 271–274 bp, 487–567 bp and 512–591 bp in length, respectively. According to Blast searches of the NCBI database, the homology between the isolates and *B. stevensii* was 100% after proofreading and stitching of the sequencing results. Sequences were submitted to the GenBank database. The sequence numbers are shown in Table 2.

Verification of the DNA barcoding fragments of the isolated strains

The experiments described above show that *EF-1 α* is a suitable fragment for DNA barcoding of *B. stevensii*. During detection and identification of isolated strains iso50624, iso2953 and

KY465922 *B. stevensii* iso2593 GGC AAA CTG GCC GCA CTT GGT TTT T— — GCG ACC GGT GTG AGG CCG
 KY465923 *B. stevensii* iso8438 — — — — — — — — — —
 KY465924 *B. stevensii* iso50624... .. — — — — — — — — — —
 AY573219 *D. mutila* CBS112553 — — — — — — — — — —
 DQ458871 *D. mutila* JL375 — — — — — — — — — —
 JQ512121 *D. mutila* UCROK1429 — — — — — — — — — —
 EU012413 *B. stevensii* UCD1965S... .. — — — — — — — — — —
 AY236904 *B. stevensii* CMW7060 — — — — — — — — — —
 DQ458870 *D. mutila* CBS112554 — — — — — — — — — — C
 JX515787 *D. mutila* UCD147 — — — — — — — — — — A
 JQ517318 *D. mutila* UCROK1425 — — — — — — — — — —
 DQ458874 *D. seriata* CBS119049 T— — — — — — — — — — C C T...
 AY339266 *B. lutea* CMW10309 A C T... TC... T— — — — — C ... C A ...
 GU251233 *B. dothidea* PD313 A C T... TC... A ... TT — — T... C ... C A ...
 GU251260 *B. ribis* PD289 A... T... TC... TT — — ... C ... C A ...
 AY236888 *B. parva* CMW9081 A... T... TC... TT — — ... C ... C A ...
 GU251301 *B. sarmentorum* PD78 A C T... A... T... A... CC TTT ... C... C CC ...
 GU251300 *B. iberica* PD257 A C T... A... T... A... CC TTT ... C... C CC ...
 EU017541 *B. australis* CBS11904... .. A C T... TC... TT — — ... C ... C A ...
 AY236901 *B. rhodina* CMW9074 A... A... TT — — ... C ... C T...
 KU221233 *B. sinensis* 3.17723 A C T... TC... A ... TT — — T... C ... C A ...
 KY465922 *B. stevensii* iso2593 ATG CGC CCC CTC ACT AGC GAA AAA TGC CCC GAC CAC TCA TGT GCT CTC
 KY465923 *B. stevensii* iso8438 — — — — — — — — — —
 KY465924 *B. stevensii* iso50624... .. — — — — — — — — — —
 AY573219 *D. mutila* CBS112553 — — — — — — — — — —
 DQ458871 *D. mutila* JL375 — — — — — — — — — —
 JQ512121 *D. mutila* UCROK1429 — — — — — — — — — —
 EU012413 *B. stevensii* UCD1965S... .. — — — — — — — — — — A
 AY236904 *B. stevensii* CMW7060 — — — — — — — — — —
 DQ458870 *D. mutila* CBS112554 — — — — — — — — — —
 JX515787 *D. mutila* UCD147 — — — — — — — — — —
 JQ517318 *D. mutila* UCROK1425 — — — — — — — — — —
 DQ458874 *D. seriata* CBS119049 — — — — — — — — — — G C...
 AY339266 *B. lutea* CMW10309 C... A... — — — — — — — — — — CT... G... AGA T... G... GCC A A —
 GU251233 *B. dothidea* PD313 C... A A A C... — — CA... C... CGC — — T... AG... — — C... C G TCT
 GU251260 *B. ribis* PD289 A... G... — — — — — — — — — — CT... G... AGA... G— — CC A G AT
 AY236888 *B. parva* CMW9081 A... G... — — — — — — — — — — CT... G... AGA... G— — CC A G AT
 GU251301 *B. sarmentorum* PD78 C... G A G — — C... CG TCC CT... A... A A A... T— — A... C ACT
 GU251300 *B. iberica* PD257 — — — — — — — — — — CG TCC CT... A... A A A... — — A... C ACT
 EU017541 *B. australis* CBS11904... .. A... G... — — — — — — — — — — CT... G... AGA T... G... GCC ATA AT
 AY236901 *B. rhodina* CMW9074 C... T... C... — — — — — — — — — — T T... — — A C G...
 KU221233 *B. sinensis* 3.17723 C... A A A C... — — CA... C... CGC — — T... AG... — — C... T G TCT
 KY465922 *B. stevensii* iso2593 GTA CGA CCA CAG GCT AAC GCG CGC CAC A
 KY465923 *B. stevensii* iso8438 — — — — — — — — — —
 KY465924 *B. stevensii* iso50624... .. — — — — — — — — — —
 AY573219 *D. mutila* CBS112553 — — — — — — — — — —
 DQ458871 *D. mutila* JL375 — — — — — — — — — — C...
 JQ512121 *D. mutila* UCROK1429 — — — — — — — — — — G
 EU012413 *B. stevensii* UCD1965S... .. TT... — — — — — — — — — —
 AY236904 *B. stevensii* CMW7060 — — — — — — — — — —
 DQ458870 *D. mutila* CBS112554 — — — — — — — — — — C G...
 JX515787 *D. mutila* UCD147 — — — — — — — — — —
 JQ517318 *D. mutila* UCROK1425 — — — — — — — — — — G
 DQ458874 *D. seriata* CBS119049 — — — — — — — — — — A
 AY339266 *B. lutea* CMW10309 G... T G G C ... AC T — —
 GU251233 *B. dothidea* PD313 G... T T ... CAC ...
 GU251260 *B. ribis* PD289 G... G C ... GC AT — —
 AY236888 *B. parva* CMW9081 G... G C ... AGC AT — —
 GU251301 *B. sarmentorum* PD78 CAG ... T... GCT ... T ... C T... T— —
 GU251300 *B. iberica* PD257 CAG ... T... GCT ... T ... C T... T— —
 EU017541 *B. australis* CBS11904... .. G... T G G C ... AC T — —
 AY236901 *B. rhodina* CMW9074 G... A... — — — — — C... T
 KU221233 *B. sinensis* 3.17723 A G ... T T ... CAC ...

Fig. 4 – Arrangement of *Botryosphaeria stevensii* and closely related species based on *EF-1a*.

Discussion

Classification and identification of *B. stevensii* are mainly performed using morphological methods, in which the characteristics of colonies and asexual and sexual organs are assessed (Aldwinckle & Jones 1990). However, we have found that it is difficult to induce *B. stevensii* to produce conidia and ascospores. Many types of medium have been assessed as methods of inducing sporulation, including PDA, 50% PDA supplemented with alfalfa, PDA supplemented with pine needles, OA, OA supplemented with pine needles, water agar, water agar supplemented with pine needles, and apple medium (200 g apple steamed with water, 20 g water agar, 1000 mL water). *B. stevensii* spores were observed on water agar medium supplemented with pine needles after one month of culture. Because of the difficulty of inducing sporulation, identification of *B. stevensii* is time consuming, strenuous, and complicated. However, DNA barcoding based on the interspecific and intraspecific specificity of DNA fragments allows rapid and automatic identification of species and overcomes many limitations of traditional taxonomy methods, and this method has the potential to greatly simplify the process of identifying *B. stevensii* (Hebert et al. 2003, Hebert & Gregory 2005).

As a new technique for species identification, DNA barcoding has enormous value in identification of fungi. Gao et al. (2013) employed DNA barcoding in the classification and identification of quarantined *Phytophthora* spp. DNA barcoding can be used to identify single spores, old samples, and other difficult samples, such as those containing little DNA, without requiring isolation and culture of pathogenic fungi. Each species has its own unique, verified DNA barcoding sequence. Although DNA barcoding in animals and plants has become an increasingly mature technology in recent years, the technique has been applied less frequently to plant pathogenic fungi. In addition, few studies have assessed DNA barcoding as a method of identifying genera or other higher taxonomic groups. The BOLD SYSTEMS database (<http://boldsystems.org>) is an authoritative database of DNA barcodes that can be used to identify a large number of species.

In this study, we utilized DNA barcoding to identify *B. stevensii* isolates from apples imported from North America and New Zealand. We selected 400 DNA barcoding sequences for three genes from 139 strains of 12 species in the genus *Botryosphaeria* for assessment of intraspecific and interspecific genetic variation, which revealed that *EF-1 α* is a suitable DNA barcoding fragment that should allow *B. stevensii* to be distinguished from closely related species capable of infecting *M. pumila*. Three *B. stevensii* strains isolated from imported apples from the United States and New Zealand were identified to verify the accuracy of the DNA barcoding method used in this study. Sequence information for *EF-1 α* was obtained, and an *EF-1 α* -based DNA barcode was established for *B. stevensii*. In addition, conserved and mutated *EF-1 α* sites in *B. stevensii* and closely related species were identified. These findings provide a basis for specific primer and probe design for *EF-1 α* and establishment of specific detection methods and corresponding technical standards. With a database of the biological information of target species established, and the capability to identify pathogenic fungi using a small amount of mycelium, DNA barcoding will sharply reduce the time required for detection and identification of *B. stevensii* and improve the accuracy of identification. The use of DNA barcoding to determine which fruit should be quarantined could facilitate efficient operation of customs offices at frontier ports.

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