

Frequent recovery of *Sydowia polyspora* from brown spot needle blight-associated loblolly pine (*Pinus taeda*) needles in Alabama

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Abstract

Sydowia polyspora is a widespread conifer-associated fungus that can function as an endophyte, saprophyte, or opportunistic pathogen, but its occurrence in southeastern pine systems remains poorly characterized. During surveys of symptomatic loblolly pine (*Pinus taeda*) in Washington and Cullman counties, Alabama, fungal isolates morphologically consistent with *Sydowia spp.* were recovered from needles exhibiting foliar disease symptoms. Isolates were characterized using colony morphology, microscopy, ITS sequencing, translation elongation factor 1-alpha (EF1- α) sequencing, species-specific EF1- α primers, and maximum-likelihood phylogenetic analyses. Colonies exhibited morphological features consistent with *Sydowia spp.*, including melanized septate hyphae, chlamydospore-like structures, and catenate conidia. ITS and EF1- α sequence analyses supported assignment of the isolates to *S. polyspora*, with phylogenetic analyses placing Alabama isolates within the broader *S. polyspora* lineage alongside reference sequences from North America, Europe, and Asia. Species-specific amplification using the EF1- α primers SpolEF4F and SpolEF3R provided additional support for species identification. In a

broader 2019–2021 survey across nine counties in Alabama, *S. polyspora* was recovered from 60% of surveyed stands. To our knowledge, this study represents the first peer-reviewed documentation of *S. polyspora* in Alabama. These findings identify *S. polyspora* as a widespread and common component of fungal communities associated with symptomatic loblolly pine needles in Alabama.

Keywords: brown spot needle blight, fungal phylogenetics, endophyte, Southeastern United States

Introduction

Sydowia polyspora (Bref. & Tavel E. Müller anamorph: *Hormonema dematioides* Lagerb. & Melin) is a cosmopolitan conifer-associated fungus exhibiting remarkable ecological plasticity, functioning variously as an endophyte, saprophyte, latent pathogen, and opportunistic pathogen across a broad range of hosts and environmental conditions (Pan et al., 2018; Ridout & Newcombe, 2018). *Sydowia polyspora* has been associated with shoot dieback in *Pinus pinea* (Silva et al., 2020), and bark beetles and associated damage in *P. yunnanensis* (Pan et al., 2018). *Sydowia polyspora* has also been shown to increase the severity of *Dothistroma* needle blight in ponderosa pine, suggesting that it may function also as a disease modifier within conifer foliar microbiomes (Ridout & Newcombe, 2015). Despite increasing recognition of its ecological importance, relatively little is known about the occurrence of *S. polyspora* in southeastern pine systems. Here, we document the association of *S. polyspora* with symptomatic loblolly pine foliage in Alabama using morphological characterization, locus-specific sequencing, species-specific amplification, and phylogenetic analyses. To our knowledge, this represents the first

peer-reviewed morphological and molecular documentation of *S. polyspora* associated with symptomatic *P. taeda* foliage in Alabama.

Materials and Methods

From March 2023 to August 2023, pine needle samples exhibiting symptoms of BSNB were collected from loblolly pine (*P. taeda*) in Washington and Cullman counties in Alabama, targeting a total of 336 trees across nine and five sampling plots, respectively. At each sampling unit, 4 needle segments (1-2cm) per tree were processed. We used a structured sampling design consisting of a central subplot with a radius of 2.07 m and three surrounding subplots with radii of 7.32 m, positioned at azimuths of 0°, 120°, and 240° relative to the central subplot. The complete four-subplot plot encompassed 2.23 acres. Symptomatic needle segments were surface-sterilized by immersion in a 7.5% sodium hypochlorite (bleach) solution for 30 seconds, rinsed with distilled water, washed with 70% ethanol for 30 seconds, and subjected to a final rinse with distilled water. Surface-sterilized needle segments were plated on sabouraud dextrose agar and colony morphology was characterized on pine needle agar and malt extract agar following Silva et al. (2020). A total of 10 fungal isolates displaying olive-green to dark-green or brownish-black colonies and the presence of acervuli were selected for morphological characterization. Fungal structures were mounted and stained with lactophenol cotton blue to facilitate the observation of hyphae, conidia, and associated reproductive structures. Sporulation was examined using a compound microscope at 10x magnification, and photomicrographs were captured at 100x magnification.

We extracted DNA from harvested mycelium using a modified cetyltrimethylammonium bromide protocol (Folorunso et al., 2025). We then used the DNA as template for PCR amplification and sequencing of two loci: internal transcribed spacer (ITS) and a portion of the translation elongation factor 1-alpha (EF1- α) gene. The ITS barcode region (approximately 500 bp) of the fungal ribosomal DNA was amplified using the universal primers ITS1 and ITS4 (White et al., 1990). For 19 isolates, the resulting amplicons were subjected to unidirectional Sanger sequencing using the universal fungal primer ITS5 (White et al., 1990). In addition, a partial EF1- α gene was amplified using primers 983F and 2218R (Rehner & Buckley, 2005) and isolates were screened using *S. polyspora*-specific EF1- α primers SpolEF4F and SpolEF3R (Guertin et al., 2019).

PCR amplification conditions varied among primer sets. Amplification of the ITS region using an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s, with a final extension at 72°C for 10 min. Amplification of the EF1- α region using primers 983F and 2218R employed a touchdown PCR protocol beginning with an initial denaturation at 95°C for 3 min, followed by 11 cycles of 95°C for 45 s, annealing beginning at 68°C and decreasing by 1°C per cycle for 75 s, and extension at 68°C for 90 s. This was followed by 35 cycles consisting of 95°C for 45 s, 56°C for 75 s, and 68°C for 90 s, with a final extension at 68°C for 5 min. Species-specific amplification of *S. polyspora* using primers SpolEF4F and SpolEF3R similarly employed a touchdown protocol with an initial denaturation at 95°C for 3 min, followed by 10 cycles of 95°C for 30 s, annealing beginning at 65°C and decreasing by 1°C per cycle for 45 s, and extension at 68°C for 45 s. This was followed by 25 cycles of 95°C for 30 s, 55°C for 45 s, and 68°C for 45 s, with a final extension at 68°C for 5

min. Final Sanger sequencing was performed at the Genomics Core Lab at the University of Alabama at Birmingham. Resulting sequence chromatograms were quality-trimmed in Sangerseq program in R, and the sequences were queried against the National Center for Biotechnology Information GenBank database using the BLASTn tool (v2.15.0+).

Raw ITS sequences were trimmed to remove the ITS5 priming sequence, allowing up to two mismatches, and reads shorter than 100bp after trimming were excluded. The retained ITS sequences were aligned using MAFFT and automatically trimmed with trimAI (gappyout). An unrooted, approximately maximum-likelihood phylogeny tree was inferred from the trimmed nucleotide alignment using FastTree under its default Jukes-Cantor + CAT model. Reference ITS sequences of the *Sydowia* species were retrieved from GenBank (accession numbers: JX421712.1, MN103809.1, KY246330.1, KY246318.1, GQ412729.1, KY081694.1, MG062867.1, PQ453976.1, PP294687.1, MT663488.1, OK051294.1, and MW055438.1) and included in the analysis. Similarly, reference EF1- α sequences for *Sydowia polyspora*, *Neodothiora populina*, and *Hormonema* were downloaded from GenBank (accession numbers: PP525033.1, PP525052.1, DQ677899.2, MF034403.1, MW173127.1, and PP525056.1) and incorporated into a EF1- α gene tree.

Additional samples from a broader survey of symptomatic loblolly pine across Alabama (2019 to 2021, Washington, Elmore, Butler, Madison, Greene, Lamar, Cullman, Wilcox, and Pickens counties) were also examined to assess the geographic occurrence of *S. polyspora*. Symptomatic needles were collected from 282 trees and were cut into 1–2 cm segments, surface sterilized in 10% NaOCl for 1 min followed by 70% ethanol for 1 min, rinsed twice with distilled water, and

plated on fungal growth media. Following fungal growth, hyphal tips and conidial heads were transferred to 2% malt extract agar to obtain pure cultures. DNA was extracted from fungal cultures, and the ITS region was amplified and Sanger sequenced. Resulting sequences were compared with reference sequences in the NCBI GenBank database using BLASTn to identify isolates as *S. polyspora*.

ChatGPT (OpenAI) was used during manuscript preparation to assist with revising the organization and wording of the text. It was not used to generate data, conduct analyses, or draw scientific conclusions. All AI-assisted text was critically reviewed and revised by the authors, who verified the accuracy of the scientific content and take full responsibility for the final manuscript.

Results

Morphological and molecular characterization was conducted using fungal isolates recovered from symptomatic loblolly pine needles collected in Washington and Cullman counties, Alabama. Colonies evaluated exhibited morphological characteristics consistent with *Sydowia* species, producing olive to dark olive colonies similar to those described previously (Guertin et al., 2019). Colony morphology varied with culture medium and age. One-week-old cultures on pine needle agar produced sparse, radiating, pale-brown mycelium, whereas mature cultures on malt extract agar developed dense, dark olive to black colonies, occasionally with whitish-grey aerial mycelium (Figure 1). Growth was evident within one week of isolation and continued progressively thereafter. Microscopic examination revealed structures consistent with both vegetative and anamorphic stages of *Sydowia* spp. Vegetative hyphae were heavily melanized,

thick-walled, septate, and branched, and mature cultures developed dense stromatic aggregations composed of clustered, swollen, thick-walled cells characteristic of chlamydospores (Figure 1). Asexual reproduction was observed directly on vegetative hyphae, with small globose to ellipsoidal conidia produced in chains (catenate) or arising directly from hyphal structures (Figure 1).

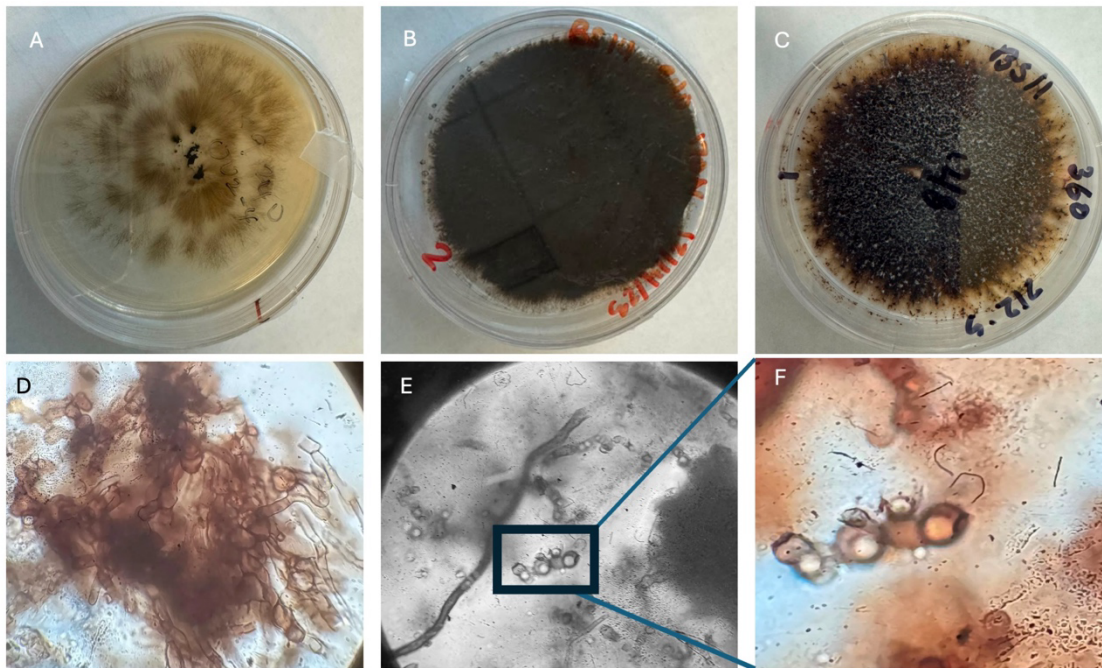


Figure 1. Colony morphology of *Sydowia* sp. on different media; (A) One-week-old culture on Pine Needle Agar showing sparse, radiating growth. (B, C) Mature cultures on Malt Extract Agar displaying dense, dark olive-black pigmentation with variable whitish-grey aerial mycelium. Microscopic morphology of *Sydowia* sp. stained with lactophenol cotton blue; (D) Dense stromatic aggregation of heavily melanized, thick-walled, swollen cells characteristic of chlamydospores in mature culture, (E) Melanized, septate, and branched vegetative hyphae with associated catenate conidia, and (F) Magnified region in (E), showing globose to ellipsoidal conidia arranged in chains arising directly from the vegetative mycelium.

BLASTn analysis of ITS sequences against the NCBI GenBank database produced matches to *Sydowia* (Supplementary Table S1). Seven isolates had $\geq 97\%$ identity across alignments of 552–

738 bp, providing strong support for assignment to *Sydowia*. Four additional isolates had 100% identity, but their short alignment lengths of 160–162 bp limited the confidence of these assignments. The remaining eight isolates had 88.7–96.9% identity across alignments of 236–583 bp and were therefore treated as provisional genus-level matches rather than definitive species-level identifications. ITS results were consequently evaluated together with EF1- α sequence data, species-specific amplification, morphology, and phylogenetic placement when assessing the identity of the recovered isolates.

BLASTn analysis of partial EF1- α sequences from five isolates provided additional support for their identification as *Sydowia spp.* Sequence identity to the best-matching reference accessions ranged from 99.37% to 99.68% across alignments of 855–947 bp (Supplementary Table S2). In addition, species-specific amplification using the EF1- α primers SpolEF4F and SpolEF3R yielded products of the expected size for all isolates tested, further supporting their identification as *S. polyspora* (Supplementary Figure S1). Together with the ITS results, morphological characteristics, and phylogenetic placement, these findings support the recovery of *S. polyspora* from symptomatic *P. taeda* needles collected in Alabama.

Maximum likelihood analysis of ITS sequences placed all Alabama isolates within the broader *S. polyspora* lineage alongside reference sequences from North America, Europe, and Asia (Figure 2). Although a small subset of isolates formed a moderately divergent cluster, most grouped closely with previously described *S. polyspora* accessions, indicating only limited genetic differentiation among isolates recovered from symptomatic loblolly pine needles in Alabama. Phylogenetic analysis of EF1- α sequences provided support for these findings (Figure 2). Most

isolates clustered with published *S. polyspora* reference sequences from North America and Europe, while a small subset grouped strongly with a reference sequence identified as *Hormonema carpetanum* (accession number PP525056.1), a related but different species from the historically anamorphic state of *S. polyspora* (*Hormonema dematioides*). Overall, the ITS and EF1- α phylogenies corroborated BLAST results, supporting identification of the recovered isolates as *S. polyspora*.

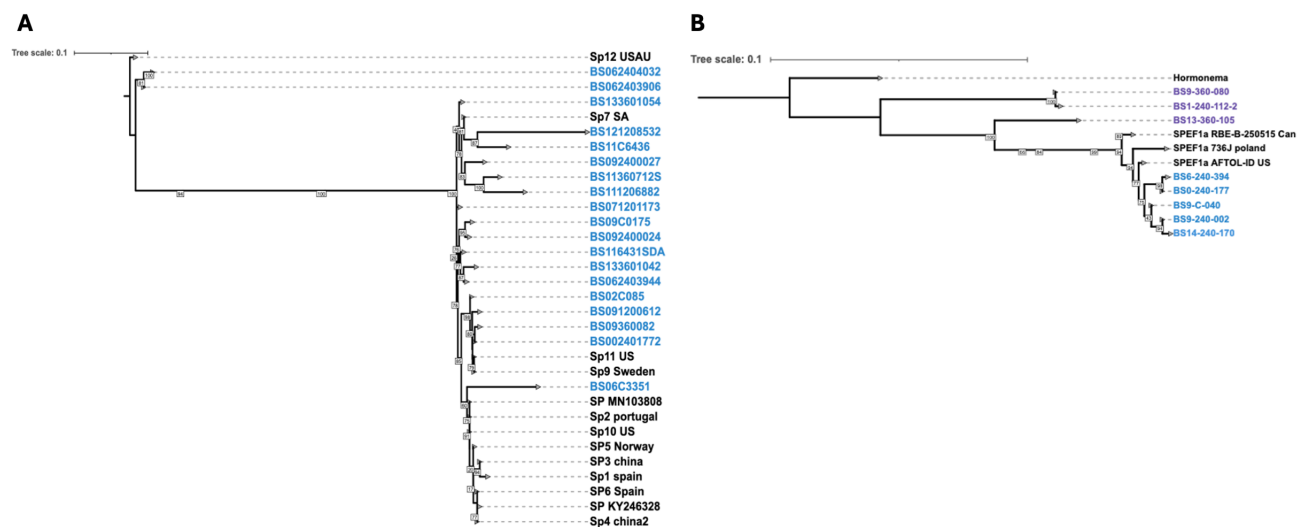


Figure 2. Maximum-likelihood phylogenetic tree based on (A) internal transcribed spacer (ITS) and (B) partial translation elongation factor 1- α (EF1- α) sequences, illustrating the evolutionary relationships of *Sydowia* isolates recovered from symptomatic *P. taeda* needles in Alabama. Isolates obtained in this study are highlighted in blue, while *S. polyspora* reference sequences retrieved from GenBank are shown in black with their geographic origins. The ITS tree was rooted using *Sydowia* isolate from USA region and the EF1- α tree was rooted using *Hormonema*, a historically anamorphic state of *Sydowia*. Bootstrap support values are displayed at the nodes, and the scale bar represents 0.1 nucleotide substitutions per site.

In our surveys, *S. polyspora* appears to occur frequently. In our sampling efforts in Washington and Cullman counties (2023), we identified *S. polyspora* species on *P. taeda* hosts in 50% of all 22 stands. In the broader surveys (2019–2021) that included Washington,

Elmore, Butler, Madison, Greene, Lamar, Cullman, Wilcox, and Pickens counties, we used ITS sequencing and confirmed isolates as *S. polyspora*, from 60% of surveyed stands, demonstrating its widespread occurrence in symptomatic loblolly pine across Alabama.

Discussion

The ecological significance of *Sydowia polyspora* in BSNB systems remains uncertain but warrants further investigation. Experimental studies have demonstrated that *S. polyspora* can induce chlorotic and necrotic lesions in pine needles and colonize stem tissues under some conditions, although disease expression appears strongly dependent on host stress and environmental context (Pan et al., 2018). In addition, Ridout & Newcombe (2015) demonstrated that *S. polyspora* increased the severity of *Dothistroma* needle blight in *Pinus ponderosa*, functioning as a pathogen enabler rather than the primary causal agent of disease. The repeated recovery of *S. polyspora* from BSNB-associated loblolly pine needles in the present study raises the possibility that similar interactions may occur within southeastern pine systems.

This study provides the first peer-reviewed documentation of *S. polyspora* associated with symptomatic *P. taeda* foliage in Alabama and adds to growing evidence that conifer needle diseases may involve more complex fungal assemblages than previously appreciated. Although pathogenicity was not evaluated in the present study, the frequent recovery of *S. polyspora* from symptomatic needles suggests that the species may represent an overlooked component of BSNB-associated co-occurring fungal communities in the southeastern United States. Future studies incorporating asymptomatic controls, quantitative prevalence estimates, and inoculation experiments will be necessary to determine whether *S. polyspora* functions primarily as a secondary colonizer, a disease modifier, or an opportunistic pathogen in association with *Lecanosticta acicola* and BSNB disease.

Data Availability Statement

All ITS sequences have been deposited into GenBank (ITS accession numbers: PZ764585-PZ764603). The sequences of a partial coding region of EF1- α gene are available in Supplementary Table 2 (accession numbers PZ784115- PZ784117).

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Funding

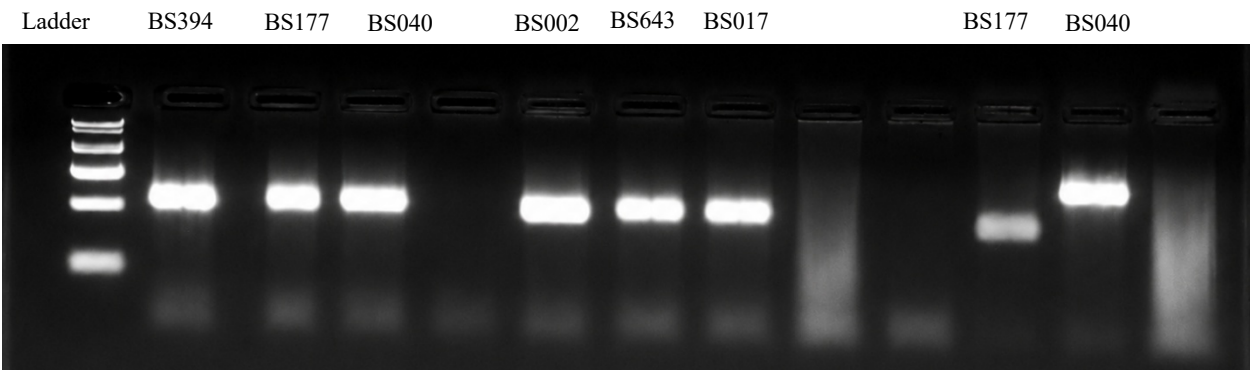
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Supplementary Materials



Supplementary Figure 1. Agarose gel electrophoresis of PCR amplicons for the specific detection of *S. polyspora*. Amplification was performed using the species-specific EF1- α primers SpolEF4F and SpolEF3R. Lane 1 contains a 500 bp molecular weight marker (Ladder). Subsequent lanes demonstrate successful amplification of the target gene region from the fungal isolates, indicated by the presence of distinct bands corresponding to the expected amplicon size.

Supplementary Table 1. BLAST-based identification of fungal isolates using internal transcribed spacer (ITS) sequences. The table includes the best-matching reference accession, percentage identity, alignment length, query and subject lengths, alignment coordinates, and E-value used to support fungal identification.

Sample ID	sseqid	pident	length	qlen	slen	qstart	qend	sstart	send	evalue
BS02C085-3	gi 239948756 gb GQ153122.1	100	576	586	1120	11	586	4	579	0
BS06C3351	gi 71148974 gb DQ093678.1	91.5	236	240	567	6	240	14	246	5.16e-83
BS062404032	gi 943875779 gb KT000159.1	96.6	457	576	457	109	565	1	456	0
BS062403906	gi 943875779 gb KT000159.1	96.5	458	574	457	109	566	1	457	0
BS062403944	gi 1608707477 gb MK762617.1	99.8	570	586	575	18	586	6	575	0
BS002401772	gi 2737250378 gb PP844107.1	100	162	323	515	83	244	161	322	3.39E-76
BS111206882	gi 257358076 gb GQ412724.1	92.3	583	596	595	17	593	20	594	0
BS11360712S	gi 2125415773 gb OK662610.1	95.5	576	591	584	12	584	13	583	0
BS116431SDA	gi 735997217 emb LN714608.1	99.1	577	586	730	12	586	15	591	0
BS11C6436	gi 1827551085 gb MT274432.1	95.4	371	399	521	33	399	18	388	8.18e-163
BS121208532	gi 1390038309 gb MG761051.1	88.7	532	558	1081	30	558	5	536	1.91e-180
BS071201173	gi 608793249 gb KJ180442.1	100	160	307	590	72	231	234	393	4.14e-75
BS091200612	gi 2737250378 gb PP844107.1	100	162	323	515	83	244	161	322	3.39e-76
BS09360082	gi 239948756 gb GQ153122.1	100	564	576	1120	10	573	3	566	0
BS092400024	gi 1002066125 gb KU837235.1	99.7	571	586	584	5	574	10	580	0
BS092400027	gi 735997217 emb LN714608.1	96.9	579	589	730	13	589	13	591	0
BS09C0175	gi 310915355 emb FR717223.1	97.2	738	758	850	21	757	1	733	0
BS133601054	gi 2737250378 gb PP844107.1	100	162	323	515	83	244	161	322	3.39e-76
BS133601042	gi 2007417139 gb MW764298.1	99.9	552	584	564	20	570	1	552	0

Supplementary Table 2. Blast-based identification for partial translation elongation factor 1-alpha (EF1- α) sequences. The table includes the best-matching reference accession, percentage identity, alignment length, query and subject lengths, alignment coordinates, and E-value used to support fungal identification. Note: Two of the isolates lack complete coding genes therefore NCBI could not be assigned accession number, however since these aligned with other isolates on the tree they were retained as part of recovered *S. polyspora* isolates.

SampleID	sseqid	pident	length	qlen	slen	qstart	qend	sstart	send	evalue	Accession number
BS0-240-177*	gi 2703623869 gb PP525045.1	99.366	947	979	1031	26	972	54	998	0	No CDS
BS14-240-170**	gi 2703623873 gb PP525047.1	99.532	855	976	876	25	879	22	876	0	No CDS
BS6-240-394	gi 2703623873 gb PP525047.1	99.415	855	984	876	24	878	22	876	0	PZ784115
BS9-240-002	gi 2703623873 gb PP525047.1	99.533	856	979	876	28	883	21	876	0	PZ784116
BS9-C-040	gi 2703623869 gb PP525045.1	99.676	925	978	1031	27	951	52	976	0	PZ784117

*BS0-240-177

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