Eur J Plant Pathol (2016) 144:411–418
DOI 10.1007/s10658-015-0777-6

Differentiation of *Stemphylium vesicarium* from *Stemphylium botryosum* as causal agent of the purple spot disease on asparagus in Germany

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Accepted: 24 September 2015 / Published online: 1 October 2015

Abstract The purple spot disease of asparagus is the most important disease in German asparagus growing regions. Two different *Stemphylium* species, *S. vesicarium* and the closely related species *S. botryosum,* are described as causal pathogens. Because of the strong phenotypical similarities the morphological differentiation is very difficult. Therefore the development of a suitable alternative to distinguish these species is an important need. The aim of this study was to develop a molecular and genetic based differentiation method for *S. vesicarium* and *S. botryosum*, and to analyze asparagus samples from Germany with this method to identify the prevalent causal agent of the purple spot disease in Germany. The sequences of three different DNA-markers were compared to get the most appropriate basis. Additionally to the commonly used ITS regions, parts of the protein-coding genes *gapdh* (glyceraldehyde-3-phosphate-dehydrogenase) and cytochrome *b* were analysed. The most significant difference between the two species was a 3 kb intron present in the *S. botryosum* cytochrome *b* region but not in *S. vesicarium*. This difference showed to be suitable for the distinction of these two *Stemphylium* species by a simple PCR-reaction. In addition to the qualitative analysis, the frequencies of these species were detected directly from asparagus field samples with the help of qPCR. In all german samples collected in 2010, 2011, 2013 and 2014 only *S. vesicarium* could be identified.

Keywords *Stemphylium vesicarium* · *Stemphylium botryosum* · Purple spot disease · Asparagus · Species identification

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Introduction

The purple spot disease or *Stemphylium* leaf spot of asparagus (*Asparagus officinalis*) has become a significant problem in all asparagus growing regions worldwide (Suzui 1973; Menzies 1980; Lacy 1982; Blancard et al. 1984; Falloon et al. 1984; Gindrat et al. 1984), and become a serious problem in Germany in the late 80s (Menzinger and Weber 1990). Due to the premature defoliation of fern in autumn and the resulting reduced photosynthetic potential, up to 52 % yield loss can be caused (Bansal et al. 1992).

Stemphylium Wallr. is a genus of filamentous ascomycets with *S. botryosum* Wallr. (Teleomorph: *Pleospora tarda* E.G. Simmons) as the type species. The most important morphological characteristic to distinguish *Stemphylium* from the closely related genera *Ulocladium* and *Alternaria* is the pre-currently proliferating conidiophore (Simmons 1969). There are more than 30 recognizable species known for the genus *Stemphylium* (Câmara et al. 2002).

S. vesicarium (Wallr.) E.G. Simmons (Teleomorph: *Pleospora allii*), which is also known to cause the brown spot disease of pears, was identified also as causal agent of the purple spot disease of asparagus in the USA (Michigan (Hausbeck et al. 1997; Meyer et al. 2000), California (Falloon et al. 1987) and Washington (Johnson and Lunden 1984)), Australia (Cunnington and Irvine 2005), South Africa (Thompson and Uys 1992) and New Zealand (Bansal et al. 1992). The closely related species *S. botryosum* was detected in asparagus samples from Germany (Leuprecht 1988; Neubauer 1998), Japan (Suzui 1973) and Greece (Elena 1996). For many years, the identification of *Stemphylium* species was mainly based on morphological characteristics, such as conidial shape, size, length/width ratio, colour, ornamentation and septation (Simmons 1967, 1969, 1985). Many of these characters overlap among related species, making determinations on species level difficult and in some cases incorrect (Cunnington and Irvine 2005; Shenoy et al. 2007; Wang et al. 2010). However, *S. botryosum* seems to be highly variable on plant material and in pure culture. *Stemphylium* species are also known to show high conidial variability at different temperatures and on different substrates (Leach and Aragaki 1970; Leuprecht 1990). With durations of 3 months to form fertile ascospores for *S. vesicarium* and 8 months for *S. botryosum* (Simmons 1985; Chaisrisook et al. 1995), this morphological characteristic is not suitable for rapid species identification. As the two species may differ in etiology, the identification of the causal agent is crucial for the development of a suitable pest management.

Sequences of the internal transcribed spacers (ITS) of the nuclear ribosomal DNA and protein coding genes, for example *gapdh*, are important molecular markers in phylogenetic analyses of fungi (White et al. 1990; Begerow et al. 2010; Schoch et al. 2012). Besides the monophyly of the genus *Stemphylium*, taxonomic relations could be verified using ITS regions and *gapdh* gene sequences (Câmara et al. 2002; Inderbitzin et al. 2009; Köhl et al. 2009; Wang et al. 2010).

Furthermore, considerably large amounts of sequence data are available of the mitochondrial coded cytochrome *b* gene (*cyt b*), which was frequently analysed in regard to QoI resistance (Grasso et al. 2006; Sierotzki et al. 2007; Stammler et al. 2013). Due to this fact, the resolving power of this region as taxonomic marker could be verified for many agronomic important fungi. Besides the relatedness within the order *Uredinales* (Grasso et al. 2006), species identifications in the genera *Monilinia* and *Phyllosticta* (Miessner and Stammler 2010; Stammler et al. 2013) were done using *cyt b* sequences.

The aim of this study was: (i) to develop a molecular and genetic based differentiation method for *S. vesicarium* from *S. botryosum*, and (ii) to analyze asparagus samples from Germany with this method to identify the prevalent causal agent of the purple spot disease in Germany. The sequences of three molecular markers (ITS, *gapdh* and *cyt b*) were used to distinguish the two described causal agents (*S. vesicarium* and *S. botryosum*) of the purple spot disease of asparagus. In addition, we developed a method based on sequence aberrations in the *cyt b* gene, suitable for quantification of both pathogens in field samples.

Materials and methods

Origin of isolates

Four *S. vesicarium* and five *S. botryosum* isolates were obtained from international culture collections. Strain 224 is the type of *S. botryosum*, which was also used in the analyses of Köhl et al. (2009). Another eight *Stemphylium* isolates provided by Zapf et al. (2011) and two *Stemphylium* isolates provided by A. Wichura / R. Weber (Agricultural Chamber of Lower Saxony), which were all isolated from asparagus, were also analysed. Details of the used isolates are given in Table 1. Isolates of related *Stemphylium* species, like *S. herbarum*, *S. alfalfa, S. sedicola and S. tomatonis*, were not included as these species are from different hosts and assumed to be identical (synonymous) to *S. vesicarium* (Câmara et al. 2002; Inderbitzin et al. 2009; Köhl et al. 2009).

Analyses of the DNA marker regions

Fungal mycelium (~25 mg) from 14 days old cultures grown on potato dextrose agar (PDA) was used for DNA and RNA extraction using the Nucleospin® Plant II Kit and Nucleospin® RNA Kit (Machery and Nagel, Düren, Germany), respectively. According to the manufacturer's intructions, reverse transcription of the RNA for the cDNA synthesis was made with the Verso cDNA kit (Thermo, Ulm, Germany). PCR reactions were carried out for sequence analyses. Primers KES 1968 (5′-GCACCGACCACAAAAATC-3′) and KES 1969 (5′-GGGCCGTCAACGACCTTC-3′) were used

Table 1 *Stemphylium* Isolates investigated in this study

Isolate ID	Country	Host	Origin	*Stemphylium* spp		
				Primary	After sequencing	
143	BE	*Allium cepa*	MUCL 3822	*S. vesicarium*	*S. vesicarium*	
144	US	*Seaweed*	MUCL 41719	*S. botryosum*	*S. vesicarium*	
145	BE	*Barley*	MUCL 20440	*S. botryosum*	*S. vesicarium*	
146	DE	*Lupinus* spp.	DSM 62928	*S. botryosum*	*S. vesicarium*	
147	BE	*Beta* spp.	MUCL 51851	*S. botryosum*	*S. vesicarium*	
148	NL	*Allium cepa*	CBS 486.92	*S. vesicarium*	*S. vesicarium*	
149	NL	*Allium cepa*	CBS 311.92	*S. vesicarium*	*S. vesicarium*	
221	DE	*Asparagus*	Stem 12–21	*Stemphylium* sp.	*S. vesicarium*	
222	DE	*Asparagus*	Stem 12–29	*Stemphylium* sp.	*S. vesicarium*	
223	JP	*Asparagus*	NBRC 31381	*S. vesicarium*	*S. vesicarium*	Species identification
224 (T)	CA	*Medicago sativa*	MUCL 11717	*S. botryosum*	*S. botryosum*	
110	DE	*Asparagus*	Stembo 1	*S. botryosum*	*S. vesicarium*	
111	DE	*Asparagus*	Stembo 2	*S. botryosum*	*S. vesicarium*	
112	DE	*Asparagus*	Stembo 3	*S. botryosum*	*S. vesicarium*	
113	DE	*Asparagus*	Stembo 4	*S. botryosum*	*S. vesicarium*	
114	DE	*Asparagus*	Stembo 5	*S. botryosum*	*S. vesicarium*	
115	DE	*Asparagus*	Stembo 6	*S. botryosum*	*S. vesicarium*	
116	DE	*Asparagus*	Stembo 7	*S. botryosum*	*S. vesicarium*	
117	DE	*Asparagus*	Stembo 9	*S. botryosum*	*S. vesicarium*	
150	DE	*Pear*	St.sp.24.7.13.P4-K1	*S. vesicarium*	*S. vesicarium*	Validation of differentiation methods
152	DE	*Asparagus*	St.sp.25.9.13.D	*S. vesicarium*	*S. vesicarium*	
154	DE	*Asparagus*	St.sp.25.9.13.C	*S. vesicarium*	*S. vesicarium*	
155	DE	*Allium cepa*	St.sp.3.9.13.ZW	*S. vesicarium*	*S. vesicarium*	
156	DE	*Asparagus*	St.sp.25.9.13.F	*S. vesicarium*	*S. vesicarium*	
159	DE	*Asparagus*	St.sp.25.9.13.A	*S. vesicarium*	*S. vesicarium*	
160	IT	*Pear*	BASF SE	*S. vesicarium*	*S. vesicarium*	
161	IT	*Pear*	BASF SE	*S. vesicarium*	*S. vesicarium*	
162	IT	*Pear*	BASF SE	*S. vesicarium*	*S. vesicarium*	
163	IT	*Pear*	BASF SE	*S. vesicarium*	*S. vesicarium*	
164	IT	*Pear*	BASF SE	*S. vesicarium*	*S. vesicarium*	
165	IT	*Pear*	BASF SE	*S. vesicarium*	*S. vesicarium*	

Stembo 1–9 Isolate provided by Zapf et al. 2011; Stem 12–21 and Stem 12–29 provided by A. Wichura / R. Weber

MUCL BCCM/MUCL fungi and yeasts catalogue, *CBS* Centraalbureau voor Schimmelcultures, *DSM* Deutsche Sammlung von Mikroorganismen und Zellkulturen, *NBRC* Nite Biological Resource Center

for amplification of *gapdh* according to Câmara et al. (2002). For the ITS region primer sequences of White et al. (1990) ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and modified ITS4 (5′-TCCTCCGCTTATTGATATGCTT AA-3′), were used. The following reaction conditions were used for both the *gapdh* and ITS regions. Phusion MasterMix 2 × (Thermo, Ulm, Germany) with an initial heating step for 30 s at 98 °C followed by 35 cycles of 5 s at 98 °C, 5 s at 64 °C, 10 s at 72 °C and 1 min at 72 °C for final elongation. With the help of the Sanger method, the PCR products were sequenced. Alignments of these

DNA sequences and database sequences were done to identify the different *Stemphylium* spp. The partial *cyt b* gene was amplified using the primers KES 183 (5′-CGATAGCTGCAGGAGTTTGC-3′) and KES 184 (5′-GCTTCAGCATTTTTCTTCATAGTT-3′). PCR was performed using 2 × HotStart-IT FideliTaq Mastermix (USB, Staufen, Germany) with an initial heating step of 1 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 30 s at 52 °C, 10 min at 68 °C and the final elongation for 5 min at 68 °C. PCR products were sequenced by primer walking sequencing. Alignments of DNA and corresponding cDNA sequences were used for the identification of the exon/intron structure of the amplified *cyt b* gene.

Molecular genetic species identification

The pre-assigned species assignment were checked using sequences of the ITS region and the partial *gapdh* gene. Sequences were aligned by using the Lasergene Programms (DNASTAR, Madison, USA). ITS and *gapdh* sequences of strains identified as *S. vesicarium* (ITS: AF442803; AF229484; *gapdh*: AF443902, AY278821.1) and *S. botryosum* (ITS: AF442782, KC584238; *gapdh*: AF443879; AF443881), obtained from Genbank, were used in this analysis in addition to the 19 *Stemphylium* isolates sequenced in this study.

Species-specific identification of *Stemphylium* spp.

With the sequence information from the study above, a primer pair for the qualitative differentiation of *S. vesicarium* and *S. botryosum* KES 1999 (5′-GACCGTCGGCCATATAAAGGGTCG-3′) and 2000 (5′-AACCGTCTCCGTCTATCAATCCT GCT-3′) was selected, which amplify the *cyt b* gene of the two species but generated products of different, species-specific length. Reaction conditions with an initial heating step for 30 s at 98 °C followed by 35 cycles of 10 s at 98 °C, 5 s at 72 °C and 1 min at 72 °C with 2 × Phusion Mastermix were used.

qPCR based differentiation of *S. vesicarium* and *S. botryosum*

Quantitative detection of *S. vesicarium* and *S. botryosum* was achieved by real-time PCR by coupling allele-specific primers with a 5′Nuclease Assay. A 214 bp fragment specific for *S. vesicarium* was amplified with the primers KES 1995 (5′-AGGGTCGCTACAGA CTGGGTCACT-3′), KES 1997 (5′-GCACTCATAAGGTTAGTAA TAACTGTAGC-3′) and the double-dye probe 5′-FAM-CTGCTTAATGTACAGGCGAAAC-BHQ1–3′. For the amplification of *S. botryosum* (215 bp), the primers KES 1995 and KES 1998 (5′-CAGCTATTACTTCGCCTTTTTAACTGTAGCA-3′) and the previous mentioned double-dye probe were used. The reactions were performed on a Rotor-Gene Q (Qiagen, Hilden, Germany) with Takyon qPCR MasterMix Plus dTTP No Rox reagents (Eurogentec, Köln, Germany) under the following conditions: 3 min at 95 °C and 40 cycles at 95 °C for 10 s and 60 °C for 45 s. The received Ct-values of the *S. vesicarium* reaction and the *S. botryosum* reaction were used to calculate the relative allele frequencies based on the method described by Germer et al. (2000).

Validation of the developed differentiation methods

To validate the developed differentiation methods (qualitative PCR and quantitative qPCR) six *S. vesicarium* strains isolated from pears and six isolates provided by J. Bohlen-Janßen (Agricultural Chamber of Lower Saxony) were used. Details of these isolates are given in Table 1.

Monitoring of Asparagus fields

Samples of infested asparagus fields were taken all over Germany in the years 2010, 2011, 2013 and 2014 at different stages of epidemiological development.

Results

Species identification

PCR with primer pair KES 1968 and KES 1969 for the *gapdh* gene resulted in an amplification product of 850 bp. Amplification of the ITS region with primer pair KES 1806 and KES 1816 resulted in a product of 558 bp. For the identification at species level, the *gapdh* gene and the ITS region of the reference strains, ordered from different international culture collections or received from Zapf et al. (2011), were sequenced and compared to database sequences of *S. vesicarium* (ITS: AF442803 (T); AF229484; gapdh: AF443902 (T), AY278821.1) and *S. botryosum* (ITS: AF442782 (T),

KC584238; gapdh: AF443879; AF443881 (T)). The sequences were compared with sequences from type material (T). All strains except strain 224 were found to be identical with the *S. vesicarium* sequences in both regions, even though four of them (Table 1) were preliminary classified as *S. botryosum*. Strain 224 and the published *S. botryosum* sequences were identical in their *gapdh* and ITS sequence. Comparing *S. vesicarium* and *S. botryosum,* six single substitutions in the ITS region and 24 in the amplified *gapdh* region were found. Identical sequences were found in strains of the same species originating from different hosts and different countries.

Cyt b of *S. vesicarium* and *S. botryosum*

With the primer pair KES 183 and KES 184, the *cyt b* region could be amplified with DNA and cDNA, respectively. The cDNA fragments showed identical sizes of 550 bp for both *Stemphylium* species. While the DNA fragments showed a length of 3 kb for *S. vesicarium* (KJ934233) and 6 kb for *S. botryosum* (KJ934234). The discrepancy between the DNA and cDNA fragments indicated intron regions which are removed during the splicing process. Alignments of the DNA and cDNA sequences revealed two introns (1323 bp and 1252 bp) for *S. vesicarium*. The same introns plus an additional intron of 2931 bp were found in *S. botryosum* (Fig. 1).

Differentiation of *S. vesicarium* from *S. botryosum*

Due to the large size differences in the *cyt b* gene it was possible to differentiate *S. vesicarium* and *S. botryosum* via fragment length comparisons.

A simple PCR-reaction with the primer pair KES 1999 and KES 2000 showed fragments of 420 bp for *S. vesicarium* and 3350 bp for *S. botryosum* (Fig. 2). When using a mixture (1:1) of the DNA of *S. vesicarium* and *S. botryosum* only the short fragment of *S. vesicarium* was amplified. Therefore only pure cultures can be analysed with this method. Cross reactions could be excluded using DNA extracts from other fungal pathogens such as *Alternaria alternata* and *Botrytis cinerea* (for *A. solani, A. brassicae* data not shown). Additionally, DNA of *Puccinia triticina* was used as a related species of *P. asparagi* which is causing the rust disease on asparagus.

To quantify the two *Stemphylium* species directly from asparagus samples, a specific qPCR assay was developed using the *cyt b* gene as a suitable basis. The identical forward primer KES 1995 and probe in combination with the species specific reverse primers KES 1997 and KES 1998 were used to determine the abundances of *S. vesicarium* (KES 1997) and *S. botryosum* (KES 1998). The calculation of the frequency was done with the formula based on Germer et al. (2000). Each DNA was analysed in two separate PCR reactions, each with a primer pair specific to one or the other *Stemphylium* species. Theoretically, only the fragment of the matching species would be amplified. In practice the mismatching fragment was amplified as well, but in a much less quantity. A delay of eight cycles could be observed between mismatched and correct amplification. To test the sensitivity of this assay, the following parameters were determined. With a serial dilution of the DNA of both organisms, the linearity (regression line which gives information about the proportionality of the Ct-value to the amount of DNA) and the PCR efficiency could be investigated. The efficiency of the PCR reactions was calculated and found to be very similar (*S. vesicarium*: 99 %, *S. botryosum*: 97 %), which makes the reactions comparable and suitable for the quantification of *S. vesicarium* and *S. botryosum*. The detection and the quantification limit (0.001 ng/μg) was also defined by determining the lowest amount of target DNA that the tested assay can detect and quantify, respectively. The latter was accomplished by using a serial dilution of a 1:1 mixture of *S. vesicarium* and *S. botryosum* DNA. To exclude cross reactions with other fungal pathogens, DNA of *A. alternata*, *A. solani, A. brassicae, B. cinerea* and *P. triticina* were analysed. None of these organisms could be detected with the developed qPCR assay.

Analysed asparagus samples

With the qPCR method above, 15 samples from 2014, 87 samples from 2013, 90 isolates from 2011 and 40 samples from 2010 were analysed. All tested samples and isolates were from asparagus growing regions in Germany (Fig. 3).

S. vesicarium could be identified with a frequency of 98–100 %. There was no evidence for *S. botryosum* to be the causal agent of the purple spot disease in Germany in the years of sampling.

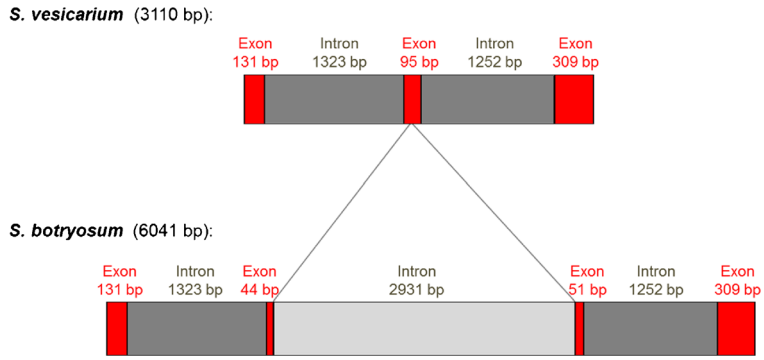

Fig. 1 Intron-exon organization of the *cyt b* gene (partial) of *S. vesicarium* and *S. botryosum*

Discussion

In this study a cost-efficient alternative, with which large quantities of asparagus samples can be analysed, was used and the causal agent of the purple spot disease was developed based on differences in the intron-exon structure of the *cyt b* gene. Characteristic substitutions in the ITS region and the *gapdh* gene, which were preliminary defined by Câmara et al. 2002, could also be verified. Based on these two loci *S. vesicarium* and *S. botryosum* could be placed into two distinct clusters, in which *S. vesicarium* showed identical sequences with *S. herbarum* and *S. alfalfa*. Even a four locus phylogeny made by Inderbitzin et al. (2009), could not differentiate between these three *Stemphylium* species as well as *S. tomatonis* and *S. sedicola*. Therefore, the separating into single species could not be supported by phylogenetic analyses and should be regarded as synonymous (Câmara et al. 2002; Inderbitzin et al. 2009; Köhl et al. 2009). So far, *cyt b* was not sequenced for *S. herbarum*, *S. alfalfa*, *S. tomatonis* and *S. sedicola* and therefore it is not known if there are differences in the sequence. Further analyses regarding *cyt b* sequences of these five closely related or even synonymous *Stemphylium* species could provide a further step on the clarification of the taxonomic relationship.

All used reference strains, except strain 224 were identified as *S. vesicarium* even though some of them were initially classified as *S. botryosum*.

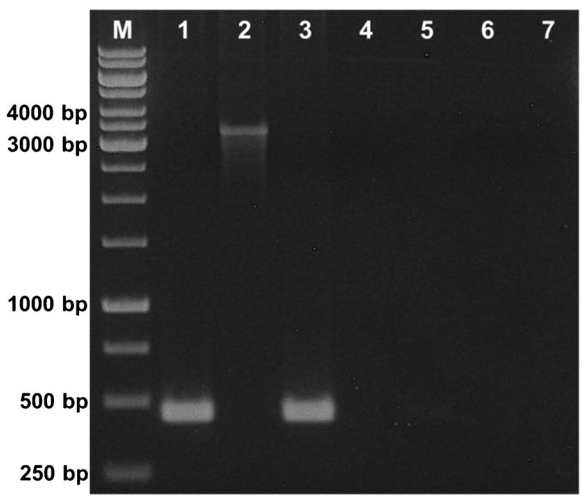

Fig. 2 Principle of the qualitative differentiation of the *Stemphylium* spp.. 1: *S. vesicarium*, 2: *S. botryosum*, 3: 1:1 Mixture of *S. vesicarium* and *S. botryosum*, 4: *A. alternata*, 5: *B. cinerea*, 6: *P. triticina*, 7: NTC, M: 1 kb DNA Ladder

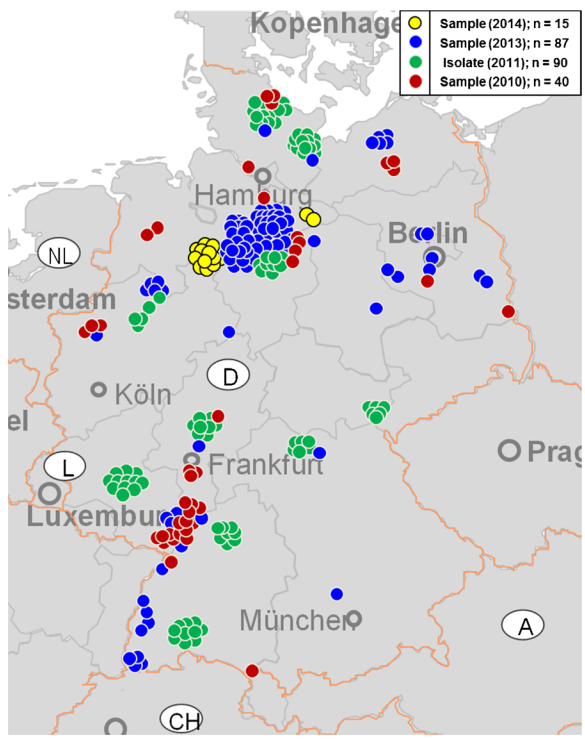

Fig. 3 Distribution of analysed asparagus samples and *Stemphylium*-isolates

The location of this gene on mitochondrial DNA (mtDNA) and the resulting high copy number gives it an advantage and reduces the detection limit for the development of reliable PCR assays (Stammler et al. 2013). Based on the development of resistances against QoI fungicides, the *cyt b* sequences of many different plant pathogenic fungi have been analysed. The *cyt b* region is intraspecific very conserved but shows interspecific differences. These differences relate to the base sequence as well as the location of non-coding regions (Grasso et al. 2006; Sierotzki et al. 2007; Stammler et al. 2013). Referring to this, the species-specific presence or absence of intron-regions could be shown for other fungal pathogens such as *Monillinia* spp. and *Phyllosticta* spp. (Miessner and Stammler 2010; Stammler et al. 2013).

The *Stemphylium* species could be qualitatively differentiated in a single PCR reaction with one primer pair. In addition to this qualitative differentiation it was possible to create a quantitative method with the help of qPCR to estimate the frequencies of the two *Stemphylium* species directly from asparagus material. With this Taqman based assay asparagus samples and *Stemphylium* isolates from Germany were analysed and the frequencies were determined. Only *S. vesicarium* could be identified in every sample. There was no hint for *S. botryosum* as causal agent of the purple spot disease.

So far, only *S. botryosum* had been described as the causal agent in Germany, a description based on morphological characteristics (Leuprecht 1988, 1990; Neubauer 1997, 1998; Zapf et al. 2011). Although a misidentification of German isolates was supposed by Falloon et al. (1987), there was no clear proof for a high abundance of *S. vesicarium* in Germany until now. Due to the results of this study the prevalent pathogen of asparagus leaf spot in Germany could be identified as *S. vesicarium*.

Acknowledgments We would like to thank all colleagues of the plant protection services and consultants providing plant samples. This work was a support for the project (313–06.01-28-1-47.025-11) funded by the German Ministry of Food and Agriculture (BMEL) and the Office for Agriculture and Food (BLE).

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