كشف وتعريف أنواع الفطر *Neotyphodium* من الحمض الريبي النووي منقوص الأوكسجين (DNA) المستخلص من بذور مفردة محفوظة في الكحول الاتيلي

Detection and Identification of *Neotyphodium* Species from DNA Extracted from Single Seeds Stored in Ethanol.

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المُلَخَّص

تم الحصول على عينات بذور من ثلاثة أنواع من الزيوان *Lolium* من سورية، ونوعين من الزيوان وأربعة أنواع من الهشيمية *Festuca* ونوع من الجنس *Melica* من فرنسا، وأدخلت البذور إلى اليابان محفوظة في الإيثانول. كما تم استخدام بذور حيّة من ثلاثة أنواع من الزيوان ونوع من الهشيمية من اليابان والولايات المتحدة الأمريكية للمقارنة. أظهرت الفحوص المجهرية وجود مشيجة نموذجية للفطور الداخلية من الجنس *Neotyphodium* في 90 إلى 100 % من البذور. تم استخلاص الحمض الريبي النووي منقوص الأوكسجين (DNA) من كل بذرة على حدة، كما تم استخلاص الحمض الريبي النووي منقوص الأوكسجين من البذور الحية بالطريقة نفسها للمقارنة. لقد مكّن تفاعل البوليميراز التسلسلي (PCR) باستخدام مرنسات عامة لمنطقة الفاصل الداخلي المستنسخ من الدنا الريبوزومي (ITS-rDNA)، ومرنسات متخصصة بأنواع الفطر *Neotyphodium* (Noc1 و / أو Noc2) من الحصول على تضخيمات من مجين الفطور الداخلية في معظم الحالات بغض النظر عن الطريقة التي تم فيها حفظ البذور. وبالاعتماد على واسمات السلاسل البسيطة المتكررة (SSR) لأنواع *Neotyphodium*، تم الحصول على تضخيمات بأحجام متوقعة باستخدام مرنسات متخصصة للموقعين B9 و B11. تُظهر هذه الدراسة إمكانية كشف وتعريف الفطور الداخلية من الجنس *Neotyphodium* من بذرة واحدة محفوظة في الكحول الأيتيلي، ما يُسهم في حل مشكلات نقل العينات النباتية الحية بين الدول.

الكلمات المفتاحية: فطور داخلية، *Neotyphodium*، نجيليات، بذور، حجر زراعي.

Abstract

Seed samples of three *Lolium* species from Syria, as well as two *Lolium,* four *Festuca* and one of *Melica* sp. from France were submerged in ethanol and shipped to Japan for analysis. Living seeds of three *Lolium* and one *Festuca* cultivar from Japan and USA were also used for comparison. Microscopic

observations showed the presence of typical mycelium of *Neotyphodium* endophytes in 90 - 100 % of the seeds. Genomic DNA from individual seeds in ethanol, and from living seeds were extracted with the same protocol. PCR amplification of all DNA samples , using universal primers for rDNA-ITS region and specific primers for *Neotyphodium* species (Noc1 and / or Noc2), have successfully generated amplicons from the genome of endophytes in most cases, regardless of storage conditions of the seeds. Among SSR markers for *Neotyphodium* species, primers for loci B9 and B11 generated amplicons of expected size. This study demonstrated the possibility to detect and identify the *Neotyphodium* endophytes from single seeds stored in ethanol which can make shipping living samples between countries easy.

Keywords: *Neotyphodium*, Endophytic fungi, Grasses, Seeds, Plant quarantine.

Introduction

Endophytic fungi belonging to the genus *Neotyphodium* Glenn, Bacon and Hanlin are seed transmitted symbionts forming mutualistic associations with grasses of the subfamily Poaideae (Clay, 1988). They are ecologically and agriculturally important fungi producing a range of alkaloids as mycotoxins which can affect grazing animals. On the other hand, they can enhance the resistance of host grasses against insect pests and nematodes (Schardl and Phillips, 1997; Clay and Schardl, 2002). Some *Neotyphodium*- grass combinations contain an insect toxin (N-formyl loline), while no mammalian toxins were detected (Bouton and Easton, 2004; Sugawara *et al.*, 2006).

The *Neotyphodium* endophytes are indigenous to temperate regions of Asia and Europe (Jauhar, 1993), but have been introduced to North Africa, Americas, Australia and pacific islands including New Zealand and Japan. Very few studies on endophytes were carried out in the Middle East. Some endophytic fungi were observed and isolated for the first time in Syrian grasses in 2005 (Naffaa, 2005).

Although immunological (Hill *et al.*, 2002) and DNA-based methods (Groppe and Boller, 1997; Moon *et al.* 2000) are used to detect endophytes in host grasses, microscopic observations of leaf sheaths, stems, or seeds are still commonly used due to the simplicity of the procedure (Ohkubo *et al.*, 2000). Observation of chemically cleared flowers of infected grasses using differential interference contrast microscopy allowed also the detection of the endophytes within immature ovaries of host plants (Sugawara *et al.*, 2004).

Research on the fungi is not yet spurred well in developing countries, since molecular biology studies are required for identification due to their simple morphological traits, and their habitat within host plants. International cooperation may accelerate such studies, but stricter quarantine measures rises difficulties for shipping of living samples. Therefore, to circumvent such difficulties, detection and identification of the fungi from a single seed stored in ethanol was tested.

Materials and methods

• Plant samples

Seed samples of three *Lolium* species from Syria, as well as two *Lolium,* four *Festuca* and one of *Melica* species from France were submerged in ethanol and

shipped to the National Institute of Livestock and Grassland Science in Japan. Living seeds of *Lolium* and *Festuca* cultivars / experimental lines from Japan and USA were also used for comparison (Table 1).

Table 1. Seed sample names, plant species and their origin.

Sample names	Plant species	Origin
Lp-1	*Lolium perenne*	Syria
Lr-8	*L. remotum*	Syria
Lt-10	*L. temulentum*	Syria
10178	*L. perenne*	France
11314	*L. perenne*	France
Clarine	*Festuca arundinacea*	France
660002	*F. arundinacea*	France
650005	*F. pratensis*	France
760001	*F. rubra*	France
R1-4	*Melica ciliata*	France
Harusakae	*F. pratensis*	Japan
Sachiaoba	*L. multiflorum*	Japan
53-10-4-4	*L. multiflorum*	Japan
Bright star II	*L. perenne*	USA

• Detection of endophyte mycelium in seeds

Seeds were soaked overnight in 5 % sodium hydroxide, rinsed in water, stained with Aniline blue, and 10 seeds of each sample were examined with a microscope as recommended by Latch (1987) to confirm the infection status of the seeds.

• DNA extraction

Single seeds were used for DNA extraction. Each seed was dried and placed in a polypropylene tube and crushed into powder by a small steel ball using a Multi-beads shocker (MB400U, Yasui Kikai K.K., Osaka) being frozen in liquid nitrogen. Total DNAs, from the samples transferred in ethanol (70 %) or from living seeds, were extracted by the DNeasy Plant Mini Kit (QIAGEN K.K., Tokyo) following the supplier's instructions (with modification concerning the sample amounts).

• Amplification and sequencing of the rDNA-ITS region

Different primer pairs were used to analyse different regions of the DNA (Table 2). Polymerase chain reaction (PCR) amplification of the nuclear ribosomal DNA region as described by Moon *et al.*(2000), using forward primers designed for analysis of plant rDNA in general (ITS1P), for fungi in general (ITS1F) and *Neotyphodium occultans* Moon, Scott & Christensen (Noc1) combined with an universal reverse primer (ITS4) or a primer designed for *N. occultans* (Noc2). Noc1 and Noc2 were used to avoid generation of amplicons from saprophytic, or parasitic fungi in/ and around the seeds. The resulted amplicons were sequenced directly as described by White *et al.* (1990) using Noc1 and ITS4 primers. The sequence data were compared with data from known *Neotyphodium* species by using GenBank data and aligned with each other by Clustal X program (ver. 1.83, National Center for Biotechnology Information, Bethesda, MD, USA).

• Microsatellite profiling

Microsatellite profiles were amplified from DNA preparations as previously described (Moon *et al.*, 1999). The sequences of the primers used to amplify each microsatellite locus (B9.1 & B9.4) and (B11.1 & B11.2) are shown in the table 2. The amplicons generated from B9 and B11 were used to produce a phylogenetic tree.

Table 2. Names, sequences and references of primers used in this study.

Primer names	Description of primers, designed for:	Primer sequences	References
ITS1P	Plant in general	5`-CCTTATCATTTAGAGGAAGGAG-3`	Gardes and Bruns, 1993
ITS1F	Fungi in general	5`-CTTGGTCATTTAGGAAGTAA-3`	Gardes and bruns, 1993
ITS4	Reverse primer	5`-TCCTCCGCTTATTGATATGC-3`	White et *al.*, 1990
Noc1	Neotyphodium occultans	5′- TCACTCCCAAACC-CCTGTGGACTTAT-3′	Sugawara et *al.*, 2006
Noc2	Neotyphodium occultans	5′- CGCGACGAGACCGCCAA-3′	Sugawara et *al.*, 2006
B9	SSR primer	(B9.1) 5`- AATCGTTGTGCGAGCCATTCTGGC-3` (B9.4) 5`-GCCCCTCATGCATTATCTCCTTG-3`	Moon et *al.*, 1999
B11	SSR primer	(B11.1) 5`-CATGGATGGACAAGAGATTGCACG-3` (B11.2) 5`- TTCACTGCTACAATTCTGTCCAGC - 3`	Moon et *al.*, 1999

• Statistical analyses

A phylogentic relationship of 14 isolates was performed using SSR data. Distance analyses and the rooted phylogentic tree at Bright starII-4 were done with the help of the Treecon software by the method of Neigbor-Joining. 100 bootstrap replicates were carried out for this method to indicate the significace of each branch (only values greater than 50% are shown).

Results and Discussion

Microscopic observations showed the presence of endophytic mycelium in 90 - 100 % of observed seeds with different morphological aspects (Fig.1). The mycelia were convoluted as previously described by Latch *et al.* (1988) and Naffaa *et al.* (1998).

The results showed that all DNA samples produced PCR products confirming that the plant DNA template was correctly extracted from all plant species studied with the primer pair (ITS1P – ITS4) (Fig.2, gel A). Fig. 2 (gel B) shows also that the DNA template of fungi was correctly extracted from seeds of all plant species studied using the primers (ITS1F – ITS4) for the fungi in general. Specific Primers for *Neotyphodium* species (Noc1/ ITS4 and Noc1/ Noc2) successfully generated amplicons from the genome of the endophytes in many cases, regardless of storage conditions of the seeds (Fig.2, gels C and F).

The SSR markers for *Neotyphodium* species, primers for loci B9 and B11, generated microsatellite amplicons of expected size in seed DNA preparations, except the samples from the endophyte of *Melica ciliata* seeds at locus B9 (Fig 2, gels D and E). Six bands of sizes from 300 to 2000 bp, and 8 bands of sizes from 100 – 1000 bp were obtained at loci B11 and B9 respectively. The amplicons generated from loci B9 and B11 were used to produce a phylogenetic tree (Fig 3). As seen in the phenogram, all isolates from seeds of the same plant species were similar (92 – 100 % similarity), except the isolates from *Festuca pratensis* (65005 from France) which were divided into two different groups. Isolates from the same plant species were regrouped together regardless of the geographical origin of the seeds, except for *Neotyphodium* isolates (Bright-star II)

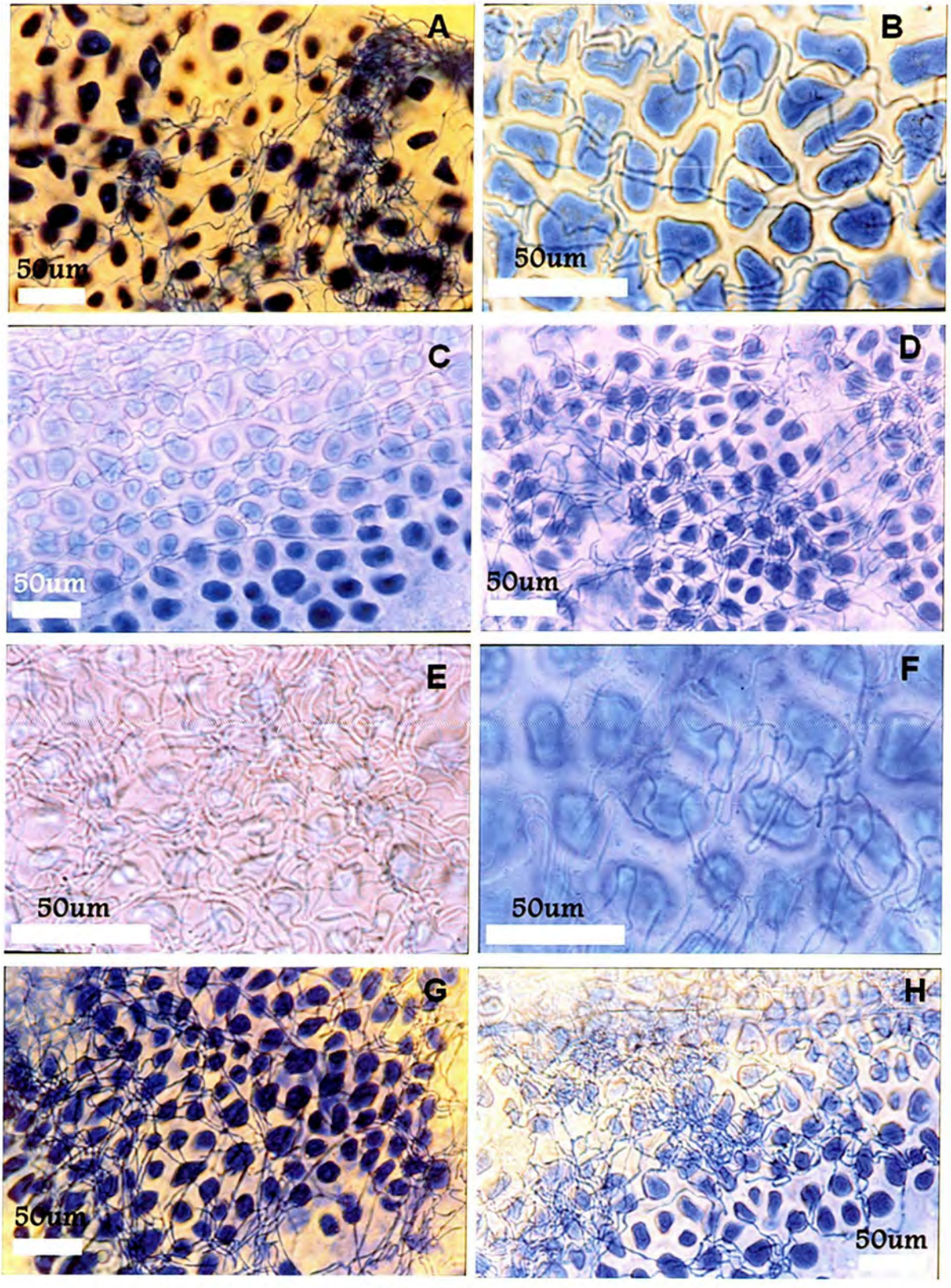

Figure 1. Mycelium observed in *Festuca* and *Lolium* seeds by microscope.

A: *L. remotum* (Lr-8) from Syria (X20). **B**: *F. arundinacea* (660002) from France (X40). **C**: *L. perenne* (10178) from France (X20). **D**: *L. perenne* (Lp-1) from Syria (X20). **E**: *L. perenne* (11314) from France (X40). **F**: *F. arundinacea* (Clarine) from France (X40). **G**: *L. perenne* from USA (X20). **H**: *L. multiflorum* (52-10-4-4) from Japan (X20).

from *L. perenne* from USA as they were genetically different) 90 % difference) from other isolates of the same species from France and Syria. Bright-star II-4 was completely different (100 %) from other isolates, it seems that no endophyte was present in this seed, and this result was confirmed by PCR. All endophyte isolates (Lr-8 and Lt-10 from Syria, Sachiaoba and 53-10-4-4 from Japan) obtained from

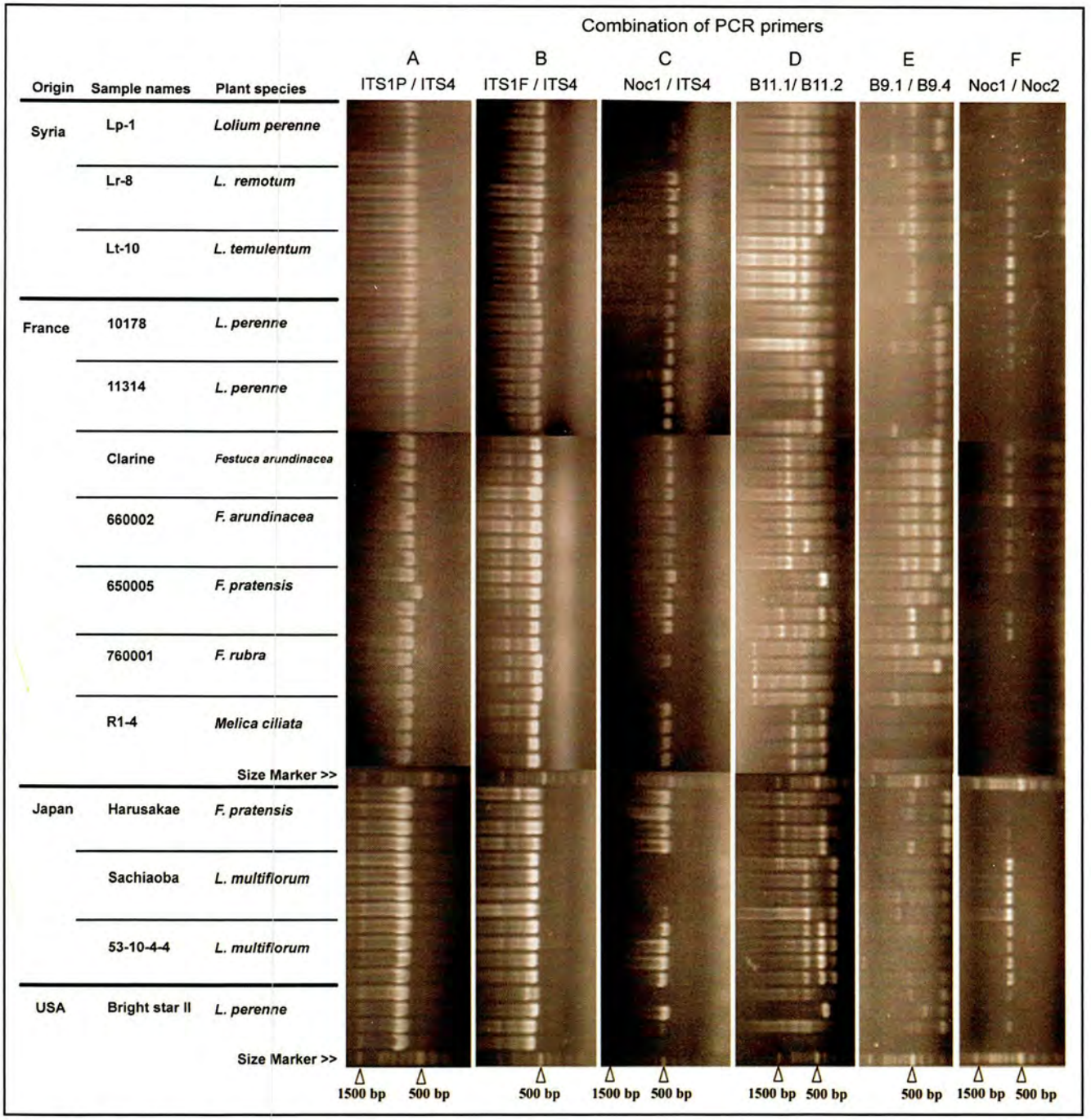

Figure 2. Amplicons generated from DNA extracted from a single grass seed, separated on 1 % agarose gel.

Gels A, B, C, F: from PCR using forward primers designed for rDNA of: (A) plant in general (ITS1P), (B) fungi in general (ITS1F) and (C) *Neotyphodium* sp. (Noc1) combined with an universal reverse primer (ITS4) or (F) a primer designed for *N. occultans* (Noc2) . Gels D and E: from PCR using primers designed for microsatellite (SSR) loci (B9.1/B9.4 and B11.1/B11.2).

annual rye-grasses (*L. remotum, L. temulentum* and *L. multiflorum*) gave similar profiles (more than 89% of similarity). However, isolates from the same country were closer in comparison with others from different countries (Fig 3), where these endophytes correspond to *Neotyphodium occultans* (Moon *et al.*, 2000). Endophytes from *Lolium perenne* (Lp-1 from Syria, 10178 and 11314 from France) gave also similar profiles, and are classified as *N. lolii,* except the endophytes of 11314 sample which corresponds to LpTG-2 group because *L. perenne* is known to lodge these two endophytes. Bright star-II isolated also from *L. perenne* is probably *N. lolii*, but is genetically different from other isolates of *N. lolii*

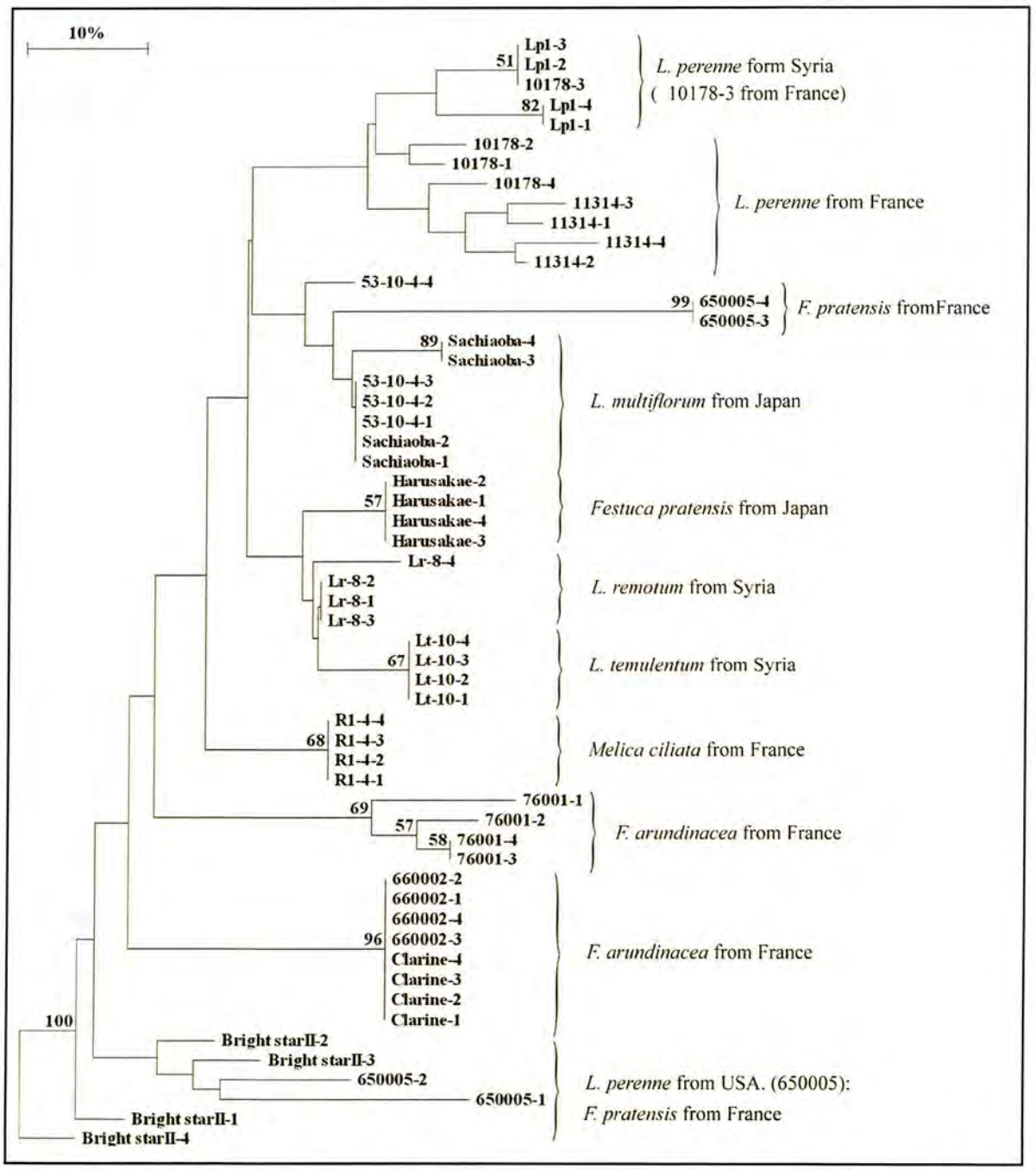

Figure 3. Phylogeny tree generated from loci B9 and B11. The rooted phylogentic tree at Bright starII-4 was built using Treecon software by the method of Neigbor- Joining.

from Syria and Japan. *Festuca arundinacea* plants contain the endophyte *N. coenophialum* (Naffaa *et al.*, 1998). Isolates from *Melica ciliata* constitute a distinct group, and correspond to *N. guerinii* according to Moon *el al.*, (2007). Isolates from *Festuca rubra* constitute also a distinct group and correspond to *Epichloe festucae*. The *Neotyphodium* isolates from *F. pratensis* from Japan, which is known as host for *N. uncinatum*, were genetically different from the USA isolates. In a previous study (Moon *et al.*, 2000), microsatellite loci were amplified from all endophytes of annual rye-grasses, in plant and pure culture DNA preparations, but not in seeds, with products observed from four of the five microsatellite loci studied except the locus B9. However, in the present study, we could obtain amplicons for all endophytes from seeds at locus B9. The molecular detection of some pathogenic fungi in living seeds was also reported in a recent study, where Hassan *et al.*, (2011) detected *Ascochyta rabiei* in infected chickpea living seeds using specific primers for ITS-rDNA region.

Results of sequencing demonstrate that all endophytes in seeds detected by PCR correspond to *Neotyphodium* spp.

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