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Distinguishing homokaryons and heterokaryons in *Phellinus sulphurascens* using pairing tests and ITS polymorphisms

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Abstract *Phellinus sulphurascens* Pilát causes laminated root rot of coniferous species in both western North America (WNA) and Asia. Accurate somatic incompatibility tests for mapping population structures have been difficult to conduct for *P. sulphurascens* because no single, unambiguous criterion has allowed differentiation of homokaryotic and heterokaryotic isolates. In a population study of *P. sulphurascens* in WNA, two types of ITS sequences were found in the single spore and vegetative isolates. All single spore isolates (SSIs) had either ITS type-1 or type-2 whereas some vegetative isolates had both ITS types. The segregation pattern for inheritance of ITS, which we observed in SSIs from eight basidiocarps, suggested that each ITS type occurred in a different nucleus and that each basidiospore inherited only one ITS type. In four SSIs from Russia and eight heterokaryotic isolates from Japan, nine different ITS types, referred to as type-3 to -11, were detected. A variety of pairing tests conducted between known Asian and WNA homokaryon and heterokaryon isolates did not always give consistent results with respect to fungal mat morphologies and formation of demarcation lines. However, the ITS types that occurred after pairing tests did follow consistent patterns. Thus, using ITS polymorphisms and pairing tests between Asian tester isolates and 49 vegetative isolates from WNA, we were able to accurately distinguish between homokaryotic and heterokaryotic isolates.

Keywords ITS polymorphism · Laminated root rot · Pairing test · *Phellinus sulphurascens*

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Introduction

Phellinus sulphurascens Pilát of the Hymenochaetales (basidiomycota) is an important pathogen causing laminated root rot in Douglas-fir (*Pseudotsuga menziesii*) and other conifers in western North America (Buckland et al. 1954; Wallis and Reynolds 1965; Thies and Sturrock 1995). Since about 1940, mycologists have recognized two forms of *Phellinus weirii* (Murr.) Gilb.: a non-cedar form and a cedar form. Currently, the non-cedar form is reported as

P. sulphurascens and separated from *P. weirii* as distinct species (Kotlaba and Pouzar 1970; Larsen and Cobb-Poulle 1990; Banik et al. 1993; Larsen et al. 1994; Lim et al. 2005b). The geographical region of *P. sulphurascens* infection stretches from the Klamath Mountains of northern California to the northern limits of Douglas-fir in British Columbia; and east to Idaho (Hansen and Goheen 2000). The fungus has also been reported in Japan and Siberia (Aoshima 1953; Parmasto and Parmasto 1979). In infected stands, laminated root rot spreads mainly by growth and transfer of vegetative mycelia from infected roots contacting uninfected roots. The brownish, resupinate polyporoid basidiocarps form rarely in the forest; although they discharge considerable numbers of basidiospores when present, these inocula are apparently not important in initiating new infections (Nelson 1976). After the death of infected trees, *P. sulphurascens* can live as a saprobe for 50 years or more in infected stumps and large roots (Childs 1963; Hansen 1979c). Effective management of forest disease requires that we understand the population structure and life cycle of the causal agents. While the epidemiology of *P. sulphurascens* is relatively well described, little is known about the genetic variation of its population.

The distribution of clonally related mycelia (individuals or genets) has traditionally been mapped by somatic incompatibility (SI) tests (Worrall 1997); these are rooted in the concept of a genetic mating system that restricts the fusion of mycelia of the same species that have different mating type genes (Buller 1931). SI tests permit tracing fungal movement, distribution, and dispersal mechanisms within forest stands (Wilson 1991). However, accurate SI tests have been difficult to conduct for *P. sulphurascens*. In most Basidiomycota, one of the morphological characteristics that identify heterokaryotic mycelia is clamp connection resulting from the fusion of two compatible homokaryotic hyphae (Chase and Ullrich 1983; Alexopoulus et al. 1996). Like other members of the Hymenochaetales, heterokaryotic *P. sulphurascens* does not form clamp connections. As well, in this species both homokaryons and heterokaryons have multinucleate cells (Hansen 1979b; Rizzo et al. 1995; Kauserud and Schumacher 2001). Distinguishing heterokaryons (*he*) from homokaryons (*ho*) for population and other genetic studies of *P. sulphurascens* is thus a challenge.

Until about a decade ago, homokaryotic and heterokaryotic isolates of *P. sulphurascens* could only be potentially differentiated by qualitative assessments that combined counts of nuclei in individual cells with the characteristics of cultures in petri dishes (Hansen 1979a). In 1993, Angwin and Hansen carried out pairing tests between *he* and *ho* and back-pairing tests to assess the mating compatibility of single-spore isolates (SSIs) of *P. weirii sensu lato*. The pairing test between *he* and *ho*, also reported as the "Buller phenomenon", was originally described by Buller (1931), who showed that heterokaryons can donate a nucleus to a homokaryon. Angwin and Hansen (1993) reported that *he–ho* pairings always produced primary demarcation lines that formed at the interface between two vegetative isolates. Often the primary line was accompanied by a more lightly pigmented secondary line. This secondary line formed as the homokaryon became heterokaryotized by the donating heterokaryon; the secondary line indicated somatic incompatibility between the newly established heterokaryon and the donating heterokaryon (Rayner and Todd 1979; Angwin and Hansen 1993). However, Angwin and Hansen acknowledged that, even with these tests, it was difficult to directly identify compatibility reactions in culture. This is because there is an imperfect association between the formation of demarcation lines and mating compatibility in *P. sulphurascens* pairings (Angwin and Hansen 1993). An alternative method, like molecular markers, for accurately assessing mating compatibility and differentiating homokaryons and heterokaryons was therefore still needed for *P. sulphurascens*.

In exploring different molecular markers for *P. sulphurascens*, we discovered that the species has several types of internal transcribed spacer (ITS) regions. Hansen et al. (1998), who also investigated ITS sequences in *P. weirii sensu lato*, found no significant differences between ITS 1 region sequences occurring in North American, Japanese, and Siberian isolates; however, the authors were unable to align ITS 2 region sequences because of an anomaly in the region. Intra-specific ITS polymorphisms have been reported in other basidiomycetes (Aanen et al. 2001; Hughes and Peterson 2001; Ko and Jung 2002). In this work we established that different ITS types were located in separate nuclei by studying ITS sequences occurring in single spore

isolates. We then determined if ITS typing would be complementary to plate pairing as a means of distinguishing western North American (WNA) homokaryons and heterokaryons of *P. sulphurascens*.

Materials and methods

Fungal isolates and single spore isolates from WNA and Asia

One hundred and ten *P. sulphurascens* isolates were isolated from decayed host trees or tissues of basidiocarps collected in four biogeoclimatic zones in British Columbia, Canada. Twelve isolates originating in the western United States were also obtained. Nine of these isolates were provided by the Center for Forest Mycology Research (CMFR, Forest Products Laboratory, Madison, WI); the other three were provided by Dr. E.M. Hansen (Oregon State University, Corvallis, OR, USA) (Table 1). Eight vegetative isolates were collected in Hokkaido, Japan (Table 2). SSIs from eight different basidiocarps collected in North American were obtained. Five of these eight basidiocarps were produced on autoclaved blocks of red alder (*Alnus rubra* Bong.) inoculated with *P. sulphurascens* mycelium (Fig. 1, Table 3). To obtain SSIs, sections of the hymenium of the basidiocarp were attached to the lid of a petri dish containing 1% malt extract agar (MEA) and discharged basidiospores were collected on the surface of the media. The other SSIs were provided by Dr. E.M. Hansen (Oregon State University, Corvallis, OR, USA) (Table 3). An additional four SSIs originating in Russia from two different parental basidiocarps were provided by the CMFR (Forest Products Laboratory, Madison, WI) (Table 2); the parental isolates were not available for analysis. Prior to DNA extraction all isolates were grown on 1% MEA at room temperature (approximately 20°C).

DNA extraction, PCR amplification and ITS region sequencing

Total DNA was extracted from mycelia grown on 1% MEA plates as described by Lim et al. (2005a). PCR amplification of the ITS region was carried out using the ITS 5 and ITS 4 primer set (White et al. 1990). PCRs were performed in a PTC-100 thermal cycler (MJ research, MA, USA) with the following cycling parameters: initial denaturation at 94°C for 4 min, then 30 cycles of denaturation at 94°C for 50 s,

Table 1 ITS types occurring in isolates of *P. sulphurascens* isolated from coniferous hosts in western North America

Collection site		ITS type of *P. sulphurascens* isolates[a]		
		Type-1	Type-2	Types-1 and -2
Canada (110)	BC	501[g], 502, 511, 512, 513, 514, 515, 520, 522, 523, 538[d], 543, 544, 555, 560, 561, 570, 572, 573, 575, 581, 602, 603, 606, 607, 610, 614, 616, 621, 622	505, 507, 517, 519, 546, 547, 548, 551, 552, 554, 567, 568, 577, 578[d], 584, 592, 593, 604, 608, 609, 611, 628, 629	503[e], 508, 509[g], 510, 514, 516, 518, 521, 524, 525, 526, 527, 529, 530, 531, 532, 533, 534, 535, 536, 537, 539[g], 540, 541, 542, 553, 556, 557, 558, 565, 566, 569, 571, 574[c], 579, 580, 583, 585, 588, 590[g], 591, 600, 601[g], 605, 612, 613, 615, 617, 623[b], 624[g], 630, 631, 632, 633, 634, 635, WL12
USA (12)	WA	Mat-3-1,	Mat-9-2	T-124
	Oregon	PW-G-7312, MP8, MP13, MP16	PW-DF-A3-10, PW-WRC-Rhod-KF[f], PW-Cedar A3[f]	GR-762-4
	Idaho		FP-134847-T	

[a] WL12—Williams Lake, BC, Canada; MP—Mary's Peak, Corvallis Oregon, USA. All isolates collected from hosts in BC were given three-digit representation numbers in the 500s and 600s. All BC isolates can be found in the culture collection of the Pacific Forestry Centre (PFC), 506 West Burnside Road, Victoria, BC, Canada

[b–g] Host trees that isolates of *P. sulphurascens* were isolated from; [b]*Larix occidentalis*, [c]*Picea engelmannii*, [d]*Pinus contorta*, [e]*Pinus monticola*, [f]*Thuja plicata*, [g]*Tsuga heterophylla*. All other isolates were isolated from Douglas-fir (*Pseudotsuga menziesii*)

Table 2 ITS types occurring in isolates of *P. sulphurascens* isolated from coniferous hosts in Asia

Isolates	ITS type	Host	Locality
3305-ss1	3	*Larix*	Russia, Primorsk, Maise
3305-ss2	4	*Larix*	Russia, Primorsk, Maise
3228-ss1	5	*Larix*	Russia, Primorsk, Maise
3228-ss2	5	*Larix*	Russia, Primorsk, Maise
FH-1230	4/5	*Abies sachalinensis*	Japan, Hokkaido, Kamikawa
FH-1246	9/10	*Larix gmelini* var. *japonica*	Japan, Hokkaido, Kamikawa
FH-1127	4/8	*Picea jezoensis*	Japan, Hokkaido, Kamikawa
FH-1301	4/7	*Picea jezoensis*	Japan, Hokkaido, Kamikawa
FH-1313	4/7	*Picea jezoensis*	Japan, Hokkaido, Kamikawa
FH-1241	4/6	*Picea jezoensis*	Japan, Hokkaido, Oketo
FH-1506	8/11	*Abies sachalinensis*	Japan, Hokkaido, Oketo
FH-1507	8/11	*Larix gmelini* var. *Olgensis*	Japan, Hokkaido, Oketo

Fig. 1 A *Phellinus sulphurascens* basidiocarp (isolate PFC 541) produced on autoclaved blocks of red alder. Inoculated blocks were incubated in a growth chamber for about 10 months prior to being placed over moistened peat moss in an outdoor shadehouse (Pellow et al., unpublished data). The basidiocarp pictured here and the others induced on alder blocks and used in this study formed 1–2 months after placement in a shadehouse. The diameter of red alder blocks ranged from 9 to 12 cm

annealing at 52°C for 40 s, extension at 72°C for 50 s, with a final extension at 72°C for 10 min. About 3 µl of each PCR product was electrophoresed on 0.5% agarose gel containing ethydium bromide in Tris–acetate EDTA (TAE) buffer. The PCR product sizes were determined by comparison to 100 bp DNA marker (GIBCO BRL, MD, USA). PCR was performed five times independently to reduce the PCR bias occurring from two ITS type fragments. Each amplified PCR product was pooled into one microtube and purified using Qiaquick PCR Purification Kit (Qiagen, ON, Canada). Sequencing was performed on an ABI 3700 automated sequencer (PerkinElmer Inc., MA, USA) at the DNA synthesis and Sequencing Facility, MACROGEN (Seoul, Korea). The sequences were proofread, edited and compared using the PHYDIT program version 3.2 (http://plasza.snu.ac.kr/~jchun/phydit/). When double peaks were detected from sequence-chromatograms, their PCR products were sequenced after cloning using TOPO TA cloning kit (Invitrogen Inc. Burlington, ON, Canada) according to the instructions of the manufacturer. The sequences have been deposited in GenBank (Wheeler et al. 2007). Accession numbers for ITS type-1 sequences are: DQ137390 (PFC583), DQ137391 (PFC583-ss3), DQ137392 (WL12) and for ITS type-2 sequences are: DQ137394 (PFC583), DQ137395 (PFC583-ss2), DQ137396 (WL12). Accession numbers for Asian ITS types isolates are: EF527208 (type-3, 3305-ss1), EF527209 (type-4, 3305-ss2), EF527210 (type-5, 3228-ss1), EF527211 (type-6, FH-1241), EF527212 (type-7, FH-1301), EF527213 (type-8, FH-1127), EF527214 (type-9, FH-1246), EF527215 (type-10, FH-1246), and EF527216 (type-11, FH-1507).

ITS-RFLP marker

To confirm the sequencing data for differentiating ITS types of both vegetative and SSIs of *P. sulphurascens* from WNA and to reduce further sequencing cost, we

Table 3 Segregation of the ITS types among homokaryons derived from SSIs of parental basidiocarps originating in BC and Oregon

Parental type	ITS type-1	ITS type-2
MP8 (type-1)	ss3, ss5	
MP13 (type-1)	ss8	
MP16 (type-1)	ss1, ss2, ss3, ss9	
PFC603 (type-1)	ss1-ss20	
PFC547 (type-2)		ss1, ss2, ss3, ss4, ss5
PFC568 (type-2)		ss1-ss19
PFC583 (both)	ss3, ss6, ss9, ss11, ss12, ss14, ss15, ss17	ss2, ss4, ss5, ss7, ss8, ss10, ss16, ss18, ss20
PFC541 (both)	ss2, ss4, ss6, ss8, ss9, ss15, ss19, ss20	ss1, ss5, ss7, ss10, ss11, ss12, ss13, ss14, ss18

searched restriction enzymes using Gene Tool V2.0 (http://tools.neb.com/NEBcutter2/index.php) and carried out restriction fragment length polymorphism (RFLP). For each strain, 10 µl of the pooled ITS amplified products were digested in 15 µl volumes, containing 1.5 µl buffer, 3.4 µl distilled water, and 0.1 µl restriction enzyme as described by the manufacturers instructions. The restriction fragments were separated on a 2% agarose gel stained with ethydium bromide and the sizes of the fragments were compared to a 100 bp DNA molecular marker.

Pairing tests between WNA and Asian homokaryons and heterokaryons according to ITS types

Pairing tests were conducted as described by Angwin and Hansen (1993). For this test, 3 mm diameter plugs were removed from the edges of actively growing colonies of *ho* or *he* isolates and placed 1 cm apart in 9 cm Petri-dishes containing 1% MEA. The inoculated plates were incubated at 20°C for 8 weeks in the dark to allow fungal growth and establishment of a reaction zone between the two isolates. Using this design a variety of pairing tests were conducted between *ho* and *he* using two WNA SSIs and their parental heterokaryons, three of the four Russian SSIs, and five of the eight Japanese heterokaryons (Table 4). These isolates were chosen as being representative of the 11 different ITS types identified in the three populations of *P. sulphurascens*; sibling pairings were excluded. Each pairing test was replicated three times. When fungal mat morphology changed or darkened, or when primary and secondary demarcation lines were observed, a small plug of mycelia was taken from the changed mat mycelia, on

Table 4 ITS sequence results for pairings between WNA and Asian isolates of *P. sulphurascens* with different ITS types

Isolates	PFC583-ss6	PFC583-ss7	3305-ss1	3305-ss2	3228-ss1	PFC583	FH-1241	FH-1301	FH-1127	FH-1246	FH-1507
ITS type	1	2	3	4	5	1/2	4/6	4/7	4/8	9/10	8/11
1	–	ND[a]	1/3	1/4	1/5	ND	1/4, 1/6	1/4, 1/7	1/8	1/9	1/8
2		–	2/3	2/4	2/5	ND	2/4, 2/6	2/4, 2/7	2/8	2/9	2/11
3			–	ND	3/5	3/1, 3/2	3/4, 3/6	3/4	3/4	3/4, 3/10	3/8, 3/11
4				–	4/5	4/1	ND	ND	ND	4/9, 4/10	4/8, 4/11
5					–	5/1, 5/2	5/4, 5/6	5/4	5/4, 5/8	5/10	5/11
1/2						–	1/2, 4/6	1/2, 4/7	1/2, 4/8	1/2, 9/10	1/2, 8/11
4/6							–	4/6, 4/7	4/6, 4/8	4/6, 9/10	4/6, 8/11
4/7								–	4/7, 4/8	4/7, 9/10	4/7, 8/11
4/8									–	4/8, 9/10	4/8, 8/11
9/10										–	9/10, 8/11
8/11											–

[a] ND—not determined. The area of the table highlighted in gray represents ITS results from *he–ho* pairings; results to the left of the highlighting are ITS results from *ho–ho* pairings and results below the highlighting are ITS results from *he–he* pairings

the demarcation line, and/or in the zone between the primary and secondary lines. Each mycelia plug was transferred onto a new Petri dish containing 1% MEA. After several days, edge mycelia from these growing inocula were subcultured again onto fresh 1% MEA. DNA was then extracted from the mycelia growing onto the second set of plates. ITS regions were amplified, cloned and sequenced to determine if a new heterokaryon had been formed. About five selected clones from each new heterokaryon were used for sequencing. The stability of each new heterokaryon was examined by determining if its initial ITS profile matched with that found after the isolate had been subcultured at least five times.

Determining karyotic status of WNA isolates

Two out of 12 of the Asian isolates of *P. sulphurascens* used (3305-ss1 = type-3 *ho* and FH-1507 = type-8/11 *he*) were selected as testers for pairing tests with 27 ITS type-1 isolates and 22 ITS type-2 isolates of *P. sulphurascens* from British Columbia. These two Asian isolates were chosen because of the consistency and clarity of the morphological changes and demarcation lines that occurred when they were paired with other isolates of *P. sulphurascens* (Table 5). The objective of the pairing tests was to determine the karyotic status of these WNA isolates. All pairings were performed as described above. Each pairing test was replicated three times.

Results

ITS polymorphisms in WNA and Asian isolates of *P. sulphurascens*

We observed 11 types of ITS sequences, which we referred to as type-1 to type-11. Among 212 *P. sulphurascens* isolates, which included 200 from WNA (122 vegetative isolates and 78 SSIs) and 12 from Asia (8 vegetative isolates and 4 SSIs), sequence comparison of the ITS regions showed polymorphisms at seven sites. There were insertion/deletion events at three sites in the ITS 1 region and at two sites in the ITS 2 region. Two C/T transition events were also found, but only in the ITS 2 region (Fig. 2). Isolates of *P. sulphurascens* from WNA had only two ITS types: some isolates were either only type-1 or only type-2, while the remainder had both ITS types (Table 1). To verify potential sequencing biases during the experiments, we searched for unique enzyme cutting sites to carry out RFLP. We found that the *Bsl*I enzyme recognizes the site CCNNNNN/NNGG and produces different banding patterns, thereby allowing differentiation of the ITS type-1 and type-2 sequences in WNA isolates. For ITS type-1, three recognition sites were digested by *Bsl*I, resulting in four distinct bands of approximately 43, 208, 239 and 241 bp. The ITS type-2 PCR product was digested at three locations and produced four bands of 43, 112, 129 and 451 bp (Fig. 3A). Banding patterns for the two ITS types were thus easily distinguished by the largest two bands on the agarose gel (Fig. 3B); the small bands were not visible. The RFLP results were consistent with the sequence data. These results indicated that there were no sequencing biases during the experiments. Isolates containing only one ITS type, type-1 or type-2, had only one RFLP pattern whereas isolates with both ITS types had a mixed RFLP pattern (Fig. 3B). Analysis of ITS sequence and RFLP results from a total of 78 WNA SSIs showed that each SSI had only one of the two ITS types; the two types never occurred together in SSIs (Table 3).

Despite the low number of Asian isolates of *P. sulphurascens* that we analyzed, we found that they contained a high number of ITS polymorphisms. The Russian SSIs each had only one ITS type; they were type-3, type-4, or type-5. Each of the eight Japanese isolates possessed two different ITS types (Table 2). However, because we could not find a restriction enzyme to cut the ITS amplicons from the Japanese isolates, we confirmed these ITS types by cloning and sequencing. Clones from each Japanese isolate had two different types of ITS sequences (Table 2).

We also examined the inheritance of ITS types in single spore progeny obtained from two different parental basidiocarps (PFC541 and PFC583) from WNA. Eight single-spore progeny were type-1 and nine were type-2 (Table 3), thus confirming a simple Mendelian segregation pattern. However, many of our WNA parental isolates had only one ITS type and these produced progeny with the same single ITS type (Table 3).

Table 5 ITS and karyotic status results for 25 of the vegetative isolates of *P. sulphurascens* isolated in BC that had only one ITS type

Isolates	Tester homokaryon and heterokaryon				Determination of karyotic status
	3305-ss1 (type-3)		FH-1507 (type-8/11)		
	LD[a]	ITS type[b]	LD	ITS type	
Type-1 vegetative isolates					
PFC502	C	3/1	D	1/1, 8/11	Heterokaryon
PFC512	C	3/1	D	1/1, 8/11	Heterokaryon
PFC513	B	3/1	D	1/1, 8/11	Heterokaryon
PFC514	B	3/1	D	1/1, 8/11	Heterokaryon
PFC515	B	3/1	D	1/1, 8/11	Heterokaryon
PFC522	C	3/1	D	1/1, 8/11	Heterokaryon
PFC543	C	3/1	D	1/1, 8/11	Heterokaryon
PFC544	C	3/1	D	1/1, 8/11	Heterokaryon
PFC555	C	3/1	D	1/1, 8/11	Heterokaryon
PFC560	B	3/1	D	1/1, 8/11	Heterokaryon
PFC603	C	3/1	D	1/1, 8/11	Heterokaryon
PFC607	B	3/1	D	1/1, 8/11	Heterokaryon
PFC614	C	3/1	D	1/1, 8/11	Heterokaryon
Type-2 vegetative isolates					
PFC505	B	3/2	D	2/2, 8/11	Heterokaryon
PFC519	B	3/2	D	2/2, 8/11	Heterokaryon
PFC546	B	3/2	D	2/2, 8/11	Heterokaryon
PFC567	C	3/2	D	2/2, 8/11	Heterokaryon
PFC577	C	3/2	D	2/2, 8/11	Heterokaryon
PFC604	B	3/2	D	2/2, 8/11	Heterokaryon
PFC608	C	3/2	D	2/2, 8/11	Heterokaryon
PFC609	C	3/2	D	2/2, 8/11	Heterokaryon
PFC611	C	3/2	D	2/2, 8/11	Heterokaryon
PFC629	B	3/2	D	2/2, 8/11	Heterokaryon
PW-WRC-Rhod-KF	B	3/2	D	2/2, 8/11	Heterokaryon
PW-Cedar A3	B	3/2	D	2/2, 8/11	Heterokaryon

A total of 49 single ITS type isolates from BC were paired in culture with two Asian tester isolates (3305-ss1 = type-3 *ho* and FH-1507 = type-8/11 *he*); all 49 were determined to be heterokaryons

[a] Line demarcation—A, no line; B, weak line and different mat morphology; C, strong line and secondary line; D, thick line

[b] 3/1 and 3/2 represent the ITS types of new heterokaryons formed from pairing vegetative isolates and *ho* tester

Pairing tests between WNA and Asian homokaryons and heterokaryons and selection of testers

When WNA type-1 and type-2 SSIs were paired with Russian SSIs (*ho–ho*), we initially observed formation of either no line or a very faint line where the two mycelia came into contact (Fig. 4A). However, a few weeks after pairing, the mycelium color of the interacting isolates darkened at the contact area. ITS sequencing of the darkened mycelia confirmed that most of these pairing resulted in new heterokaryons (Table 4). We obtained similar results when we paired WNA type-1 SSIs with WNA type-2 SSIs. These data confirmed that, despite their variation in ITS sequences, the WNA and Asian isolates we examined in this study represent the same biological species, namely *P. sulphurascens*.

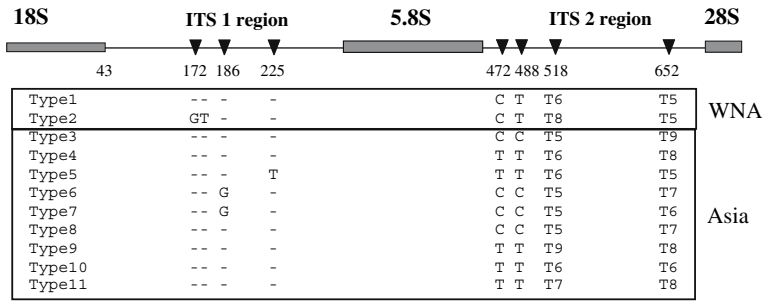

Fig. 2 The position of polymorphic sites in the ITS region of *Phellinus sulphurascens* which were amplified and sequenced by ITS 5/ITS 4 primers. Five insertion/deletion events and two C/T transition events were found from WNA and Asian isolates. Tn in the ITS 2 region represents the number of thymines

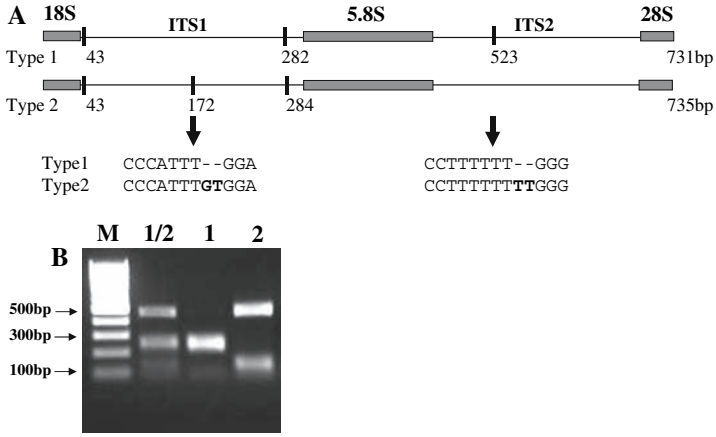

Fig. 3 Restriction map and RFLP of *Bsl*I for ITS types found in WNA isolates of *P. sulphurascens*. (**A**) Sequences are for type-1 and type-2 single spore isolates derived from the parental heterokaryon of *P. sulphurascens* isolate PFC583. (**B**) Banding pattern of ITS type-1 and type-2 PCR products digested by *Bsl*I. M, marker; 1/2, both ITS types from *P. sulphurascens* PFC583; 1, ITS type-1 from single spore progeny of PFC583; 2, ITS type-2 from single spore progeny of PFC583

When WNA homokaryon and Asian heterokaryon (*ho–he*) isolates were paired, one of two patterns was observed in pairing plates after 8 weeks. In some plates, fungal mat morphologies differed from parental isolates (see Fig. 4B). In other plates, fungal mat morphologies were very similar to parental isolates, but there was the formation of both strong primary demarcation lines and secondary lines (Fig. 4C and D). Unlike the results of Angwin and Hansen (1993), these secondary lines were not limited to the SSI portions of plates, but occurred in either the SSI (*ho*) portion or in the portion occupied by the heterokaryon. ITS sequencing of mycelia from both patterns resulting from these *ho–he* pairings indicated that two new heterokaryons had formed in most cases (Table 4). Comparison of ITS data before and after subculturing these new heterokaryons at least five times showed that they were stable, i.e., none of the new heterokaryons had reverted to homokaryons.

When WNA heterokaryons were paired with different Asian heterokaryons (*he–he*), single strong demarcation lines that formed initially had always widened after a few weeks of growth (Fig. 4E). Mycelia cultured from the zone of the demarcation line had ITS sequences that consistently matched one of the two parental isolates, thus confirming that no new heterokaryons had formed (Table 4).

We also demonstrated from these pairings that ITS types were distributed following consistent patterns. For example, when WNA SSIs (ITS type-1 or

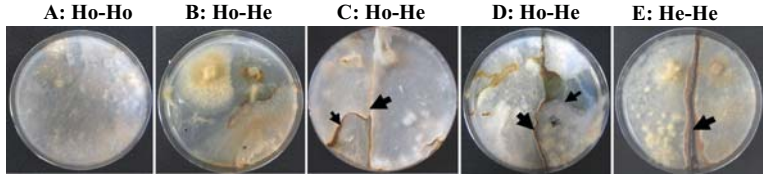

Fig. 4 Examples of plate pairing results for *ho–ho*, *ho–he*, and *he–he* combinations. Eight weeks after pairings were initiated, mycelium morphologies ranged from (**A**) complete intermingling of hyphae for *ho–ho* pairings, to (**B**) distinct mat morphologies, (**C**) strong primary demarcation lines and secondary lines in SSI portions of plates, and (**D**) strong primary demarcation lines and secondary lines in heterokaryon portions of plates for *ho–he* pairings, to (**E**) single wide demarcation lines for *he–he* pairings. Thick and thin arrows indicate primary and secondary lines, respectively

type-2) and heterokaryons (ITS types-1/2) were paired with Asian homokaryons (ITS type-3 or type-5), new heterokaryons were produced that had ITS types-1/3, -1/5, -2/3, and -2/5 (Table 4). When WNA SSIs were paired with the Asian ITS type-8/11 heterokaryon, new heterokayons were formed with ITS type-1/8, -1/11, -2/8 and -2/11. In general, this same pattern of ITS distribution was seen for the other four Asian heterokaryons assessed (Table 4). Finally, when WNA vegetative isolates with only one ITS type (e.g., -1/1) were paired with an Asian heterokaryon (e.g., ITS type-8/11), the only two ITS types found in the plate after 8 weeks were the same as the parental heterokaryons, i.e., either type-1/1 or type-8/11. These results indicate that nuclei were not exchanged between these kinds of paired isolates. Results from all of these pairings between WNA and Asian isolates of *P. sulphurascens* can be summarized as (1) all SSIs possess only one ITS type, (2) all isolates possessing two different ITS types are heterokaryons, and (3) isolates with only one ITS type may be homokaryons or heterokaryons.

Determining karyotic status of WNA isolates using testers

To determine if WNA isolates of *P. sulphurascens* collected from British Columbia and possessing only one ITS type were either homokaryons or heterokaryons we paired them with the two Asian tester isolates (3305-ss1 = type-3 *ho* and FH-1507 = type-8/11 *he*) that we showed gave consistent pairing results and which we called testers. Using pairing test and ITS typing, we provide substantial evidence that all 49 of these isolates are heterokaryons (Table 5).

Discussion

To enable population and other genetic studies of *P. sulphurascens*, a molecular-based method for accurately distinguishing homokaryons from heterokaryons was required. Nuclear ribosomal DNA (rDNA) is frequently used for taxonomic and phylogenetic studies of many different species. In some cases, species delineation is based on inter-specific sequence variation of the ITS regions but ITS polymorphisms have been reported within species for filamentous fungi belonging to zygomycetes (Sanders et al. 1995; Hosny et al. 1999; Pawlowska and Taylor 2004), ascomycetes (O'Donnell and Cigelnik 1997; Fatehi and Bridge 1998; Aviram et al. 2004) and basidiomycetes (Harlton et al. 1995; Gomes et al. 2000; Hughes and Peterson 2001; Kauserud and Schumacher 2003; Ko and Jung 2002). To maintain sequence integrity between rDNA repeat units, sequence homogenization and repair mechanisms may require recombination events between the rDNA repeat units within or between chromosomes (Arnheim et al. 1980). Thus, within an inter-breeding population these sequences are either homogenized or are in the process of being homogenized. Disruption of concerted evolution may give rise to intra-specific rDNA polymorphisms.

In our work, we observed 11 ITS types in *P. sulphurascens*: nine types in Asia and only two types in WNA. Two coexisting ITS types were detected in isolates from WNA and Japan. Two models have been suggested for how ITS polymorphisms are maintained through cellular divisions: (i) all nuclear rDNA variants are present within each nucleus, and (ii) ITS variants are distributed between different nuclei (Nelson 1996; Selosse et al. 1996; Coppin et al. 1997; Wu et al. 1998; Shiu and Glass

1999; Kauserud and Schumacher 2001, 2003; Aviram et al. 2004; Pawlowska and Taylor 2004). Pawlowska and Taylor (2004) designed two approaches to determine which model was applicable to arbuscular mycorrhizal fungi. These authors examined ITS polymorphisms directly using individual microdissected nuclei and also SSIs. In our study, we used SSIs to investigate ITS variation in *P. sulphurascens*. Basidiocarps of *P. sulphurascens* are relatively rare in nature and can be difficult to find in the forest so we developed a method for inducing basidiocarps on autoclaved blocks of red alder inoculated with the fungus (Fig. 1). Using this method we obtained two basidiocarps (PFC541 and PFC583) that produced, in an approximately 1:1 ratio, single spore progeny with either ITS type-1 or ITS type-2, suggesting a simple Mendelian segregation pattern. In the 34 homokaryotic SSIs we studied, no meiotic recombinants or progeny that included both ITS types were detected. These results suggest that the ITS variants we found in vegetative isolates of *P. sulphurascens* occurred because different ITS types reside on homologous chromosomes in different nuclei, and also that homokaryons inherit only one type of ITS. Similar segregation patterns for different types of ITS regions have been reported in the genus *Phellinus* (*P. nigrolimitatus*, Kauserud and Schumacher 2001), in other basidiomycetes (the dikaryots *Pleurotus* and *Laccaria bicolor*, Iracabal and Labarrère 1994; Selosse et al. 1996), and in other fungal species (e.g., the diploid *Saccharomyces*, Petes and Botstein 1977).

Like many other wood decay basidiomycetes, *P. sulphurascens* is heterothallic (Hansen 1979a); heterokaryotic isolates produce basidiocarps but homokaryotic isolates do not. Therefore, two different ITS types in heterokaryotic *P. sulphurascens* isolates might result from mating of either two compatible homokaryotic isolates or a homokaryon and a heterokaryon. Among the eight spore families we studied, many parental isolates had only one ITS type and all of their single spore progeny had the same parental ITS type.

Our ITS sequence and RFLP data for 122 isolates of *P. sulphurascens* revealed the occurrence of only two types of ITS (type-1 and type-2) in WNA. Surprisingly, ITS type-1 and type-2 were not found in the Asian isolates, but we only had access to a limited numbers of specimens. We found a greater diversity of ITS polymorphisms in the Asian isolates that we studied, although we had expected to find less variability in these isolates because of their limited number (12). We propose that the low ITS variation in WNA might be a result of *P. sulphurascens* having been recently introduced to WNA from an unknown locality, potentially through spore dispersion by wind or insects.

Because the *P. sulphurascens* ITS types were different in Asia and WNA, we performed pairing tests to determine whether these different isolates behaved as biological species. Results from *ho–ho* and *ho–he* pairings indicated that Asian and WNA isolates belonged to the same biological species. These results confirm those reported by Hansen et al. (1998). ITS analysis of the new heterokaryons created from these WNA × Asian pairings showed that they consistently contained all predicted combinations of the ITS types occurring in the original, paired isolates. Visual analysis of the morphologies of these same new heterokaryons frequently showed changes in fungal mat appearance and/or color and, in the case of *ho–he* pairings, the occurrence of primary demarcation lines and secondary lines. Coates and Rayner (1985) described the appearance of secondary lines as "track formation" resulting from the migration of one or the other nuclei of the heterokaryon into the homokaryon. Therefore, the secondary lines we observed when unrelated homokaryons and heterokaryons were paired represented somatic incompatibility reactions between the newly established heterokaryons. Similar nuclear migration patterns have been described for many other basidiomycetes, e.g., *Phellinus gilvus*, *Schizophyllum commune*, *Trametes versicolor*, and *Stereum gausapatum* (Ellingboe and Raper 1962; Todd and Rayner 1978; Boddy and Rayner 1982; Rizzo et al. 1995). Interestingly, we did not always observe secondary lines being formed in *ho–he* pairings shown to have resulted in the formation of a new heterokaryon by ITS analysis—in some cases only morphologically different fungal mats were formed. This result is similar to that reported by Angwin and Hansen (1993), namely that the absence of interaction lines does not necessarily indicate "compatibility" and the presence of strong interaction lines does not necessarily represent "incompatibility".

The life cycle of a typical heterothallic wood decay basidiomycete can include both homokaryotic

and heterokaryotic phases. A homokaryotic phase is generally viewed as a transient part of the life cycle of a basidiomycete (Gardes et al. 1990; Simchen 1996). Initially, a piece of wood may be colonized by basidiospores that develop into homokaryons; eventually, either two compatible homokaryons, or an homokaryon and heterokaryon, come into contact, exchange nuclei and form new heterokaryons. Hansen (1979b) suggested that most vegetative isolates of *P. sulphurascens* from decaying wood of naturally infected trees were heterokaryons. However, because the methods for distinguishing homokaryons and heterokaryons were ambiguous, it was not possible to correlate relative virulence with a particular phase of the life cycle of a basidiomycete. Now, by using pairing tests and ITS polymorphisms, we can distinguish homokaryons and heterokaryons of *P. sulphurascens* in WNA. Using this method, we proved that selected vegetative isolates with only one ITS type (type-1 or type-2) were all heterokaryons. These results support Hansen's suggestion (1979b). In future work we will compare the pathogenicity of homokaryon and heterokaryon isolates in WNA and of heterokaryons that we have generated by pairing Asian and WNA isolates. We hope that the method described here will contribute to and facilitate the genetic study of non-clamped basidiomycetes.

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