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Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/83984> since 2016-09-28T17:33:08Z

Published version:

DOI:10.1111/j.1574-6968.2011.02248.x

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This is the author's final version of the contribution published as:

C. Murat; E. Zampieri; M. Vallino; S. Daghino; S. Perotto; P. Bonfante.
Genomic suppression subtractive hybridization as a tool to identify
differences in mycorrhizal fungal genomes. FEMS MICROBIOLOGY
LETTERS. 318(2) pp: 115-122.
DOI: 10.1111/j.1574-6968.2011.02248.x

The publisher's version is available at:

<http://femsle.oxfordjournals.org/cgi/doi/10.1111/j.1574-6968.2011.02248.x>

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Genomic suppression subtractive hybridization as a tool to identify differences in mycorrhizal fungal genomes

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DOI: <http://dx.doi.org/10.1111/j.1574-6968.2011.02248.x> 115-122 First published online: 1 May 2011

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Abstract

Characterization of genomic variation among different microbial species, or different strains of the same species, is a field of significant interest with a wide range of potential applications. We have investigated the genomic variation in mycorrhizal fungal genomes through genomic suppressive subtractive hybridization. The comparison was between phylogenetically distant and close truffle species (*Tuber* spp.), and between isolates of the ericoid mycorrhizal fungus *Oidiodendron maius* featuring different degrees of metal tolerance. In the interspecies experiment, almost all the sequences that were identified in the *Tuber melanosporum* genome and absent in *Tuber borchii* and *Tuber indicum* corresponded to transposable elements. In the intraspecies comparison, some specific sequences corresponded to regions coding for enzymes, among them a glutathione synthetase known to be involved in metal tolerance. This approach is a quick and rather inexpensive tool to develop molecular markers for mycorrhizal fungi tracking and barcoding, to identify functional genes and to investigate the genome plasticity, adaptation and evolution.

Keywords

- genomic suppressive subtractive hybridization
- mycorrhizal fungi
- genomic variation
- *Tuber*
- *Oidiodendron maius*

Introduction

Comparative genomics, by revealing genome variations in closely related organisms, can provide valuable information both to understand the basic principles involved in diversification and to identify potentially interesting traits. For example, genome-wide approaches have provided important information on genome plasticity and have allowed the identification of species/strain-specific genes related to the exploitation of the substrate, to disease and stress tolerance ([Hepworth et al., 2007](#); [Huanget al., 2007](#)).

Despite the increasing number of fully sequenced genomes, direct comparison of genomic sequences remains expensive and time consuming and it requires bioinformatic skills especially for organisms with relatively large genomes. As an alternative approach, the genomic suppressive subtractive hybridization (gSSH) method has been developed to identify sequences present in a

genome (tester) and absent in another one (driver). The gSSH method is a modification of the one developed by [Diatchenko \(1996\)](#) for the generation of subtracted cDNA libraries and it was first applied in a study of *Helicobacter pylori* ([Akopyants et al., 1998](#)). When applied to bacterial genomes, gSSH has proved useful for the identification of species-specific markers and bacterial virulence factors, for molecular epidemiology and biodiversity studies ([Winstanley, 2002](#)). It has been used to compare the genomes of bacterial species such as *Escherichia coli*/*Salmonella typhimurium* ([Bogushet al., 1999](#)), *Yersinia pestis*/*Yersinia pseudotuberculosis* ([Radnedge et al., 2001](#)) and *Mycoplasma agalactae*/*Mycoplasma bovis* ([Marenda et al., 2004, 2005](#)). It has also been applied to metagenomic studies, in order to compare the rumen microbial communities ([Galbraith et al., 2004, 2008](#)). If gSSH has been widely used to study differences between bacterial genomes, to our knowledge there is only one report where this technique has been applied to filamentous fungi ([Harmset al., 2002](#)). Another technique, genomic subtraction hybridizations (gSH), has been used in some filamentous fungi, where several rounds of gSH were applied to *Magnaporthe grisea* to isolate the mating genes ([Kanget al., 1994](#)) and to *Verticillium dahliae* to investigate intraspecies variation ([Patterson & Dobinson, 1998](#)).

The gSSH method is based on a suppression PCR effect and combines normalization and subtraction in a single procedure to exclude genomic sequences that are common to the populations being compared. This dramatically increases, as compared with gSH, the probability of obtaining low-abundance genomic DNA fragments, and simplifies analysis of the subtracted library. [Rebrikov \(2004\)](#) found an enrichment of over 1000-fold for rare sequences in a single round of gSSH.

In the present study, gSSH was used to investigate genomic variability in some mycorrhizal fungi belonging to the *Ascomycota*. Mycorrhizal fungi play a pivotal role in terrestrial ecosystems because of their beneficial associations with land plants ([Smith & Read, 2008](#)), and comprehension of genome variation in these fungi is fundamental to better understand the evolution and adaptation of this symbiosis. In particular, we have tested the resolution power of the gSSH method to reveal genomic differences in species that are phylogenetically distant (*Tuber melanosporum* Vittad. and *Tuber borchii* Vittad.) and close (*T. melanosporum* and *Tuber indicum* Cooke & Masee) ([Jeandrozet et al., 2008](#)), and between two isolates of the same species (*Oidiodendron maius* OmMa3 and OmMa2), featuring different degrees of metal tolerance ([Vallino et al., 2011](#)).

Materials and methods

Fungal material

For interspecies gSSH, fruiting bodies of *T. melanosporum* M105 ([Murat et al., 2004](#)) and of *T. borchii* F9 ([Zampieriet al., 2009](#)), both harvested in Piedmont (Italy), as well as fruiting bodies of *T. indicum* 080110-1 ([Zampieriet al., 2009](#)) and mycelium of *T. melanosporum* Mel28 ([Martinet et al., 2010](#)) were used. *Tuber melanosporum* and *T. borchii* are phylogenetically distant species, whereas *T. melanosporum* and *T. indicum* are phylogenetically closely related ([Jeandrozet et al., 2008](#)). *Tuber melanosporum* Tmel28 mycelium was grown for a month in a dark room at 25°C in flasks containing 50mL of 1% liquid malt. For intraspecific gSSH, mycelium of *O. maius* OmMa3 and *O. maius* OmMa2 were used. These two strains were isolated in the Mont Avic park (Piedmont, Italy) and featured different metal tolerances on nickel and chrome ([Vallino et al., 2011](#)). Fungal cultures of *O. maius* were grown in Czapek dox liquid medium (Oxoid) for 30 days under shaking conditions (120r.p.m. in conical flasks) at 25°C.

gSSH protocol

Genomic DNA was extracted with the DNeasy Plant Mini Kit (Qiagen SA, Courtaboeuf, France), from 20mg of ascocarp or from 100mg of mycelium, following the manufacturer's instructions. Genomic DNA was quantified by NanoDrop (Celbio).

The gSSH method was performed according to the protocol described by [Marenda \(2004\)](#). *Tuber melanosporum* was used as tester and *T. borchii* and *T. indicum* were used as drivers in two separate gSSH experiments. For the *O. maius* gSSH experiment, OmMa3 was used as the tester and OmMa2 as the driver. About 1µg of tester and driver DNA were digested with 30U of MboI. For MboI-digested tester DNA, adapters A1-bam (RJ48+Jbam12) and A2-bam (RN48+Nbam12) ([Marenda et al., 2004](#)) were used. After two hybridizations and a nested PCR, the product of the nested PCR was checked on agarose gel, where it yielded a smear ranging from about 100 to 700bp (data not shown).

Approximately 10ng of the nested PCR products were directly ligated into the pGEM-T vector (Promega, Madison, WI) and transformed into competent *E. coli* XL2-Blue cells (Stratagene). Bacterial colonies were screened by PCR, using primers N24 and J24 ([Marenda et al., 2004](#)). All amplified products were run on agarose gel to select amplicons longer than 100bp, which were purified with the Qiaquick PCR purification kit (Qiagen) and quantified by NanoDrop (Celbio).

Reverse dot blot and clone analysis

The specificity of the identified genomic regions was verified by reverse dot blot hybridization. About 20ng of the purified PCR products and 50ng of driver and tester genomic DNA (as positive controls) were heat denatured (10min at 100°C), spotted on two Hybond-N+ membranes (Amersham) and UV cross-linked to the membrane. About 1µg of driver and tester genomic DNA were labelled using Biotin DecaLabel DNA Labeling kit (Fermentas) and used to hybridize one of the two membranes with the Biotin Chromogenic Detection Kit (Fermentas), following the manufacturer's instructions. The clones that hybridized only with the tester DNA were considered as positive clones and were sequenced by Genelab (Rome, Italy) or by DiNAMYCODE s.r.l. (Turin, Italy), using the J24 primer. All sequences were edited with sequencer software 4.2.2 (Gene codes corporation, Ann Arbor, MI). Similarity searches were performed using NCBI online standard blastn and blastx (basic local alignment search tool) algorithm ([Altschulet al., 1997](#)) and the blastn tool on *Tuber* genome TE database in the Mycor website (<http://mycor.nancy.inra.fr/IMGC/TuberGenome/>).

Technique validation

To further verify the specificity of the technique, the primers G13177f (CATACCACAATATAYGCATC) and G13177r (GTATGGGTGCCGATGTTAG) were designed on the clones gSSHmb-2 and gSSHmb-46 and on the bases of blastn results at the NCBI and *Tuber* genome database.

The primers were used in PCR reactions on the following samples: *Tuber brumale* 080130-1, *T. indicum* 080110-1, *T. borchii* F9, *Tuber aestivum*, *Tuber mesentericum* 1, *Tuber magnatum* F8, *Tuber rufum* 2773 and four samples of *T. melanosporum* collected in Italy, Spain and France. The PCR mix was as follows: 10 × buffer (2.5µL), 2.5mM dNTPs (2µL), 10µM primer f (1µL), 10µM primer r (1µL), water (15.2µL), Red Taq 1UµL-1 (Sigma) (0.7µL) and 1/10 diluted DNA (2µL) in a final volume of 25µL. The PCR was carried out on a Gene Amp PCR System 2700 (Applied Biosystems, Milan, Italy) thermocycler with denaturation at 94°C for 3min, followed by 25 cycles

of 94°C for 30s, 61°C for 20s and 72°C for 20s and an extension at 72°C for 5min. All amplified products were checked on agarose gel.

Results and discussion

After subtraction of *T. melanosporum* M105 with the *T. borchii* genomic DNA and reverse dot blot analysis, the interspecies gSSH experiment yielded 16 specific sequences (Table 1; accession numbers [HN262670–HN262685](#)). Eleven sequences out of 16 (69%) showed similarity to transposable elements (TEs), one sequence showed similarity to a chromosome segregation ATPase-like protein, one sequence matched a hypothetical protein and three sequences (gSSHmb-22, gSSHmb-26 and gSSHmb-40) showed no similarity to sequences in the databases.

Table 1 Specific sequences obtained in *Tuber melanosporum* vs. *Tuber borchii*.

Clone ID (accession number)	bp	blastn	blastx (nr)	blastn <i>Tuber</i> TE
gSSHmb-1 (HN262670)	128	–	–	Yes
gSSHmb-2 (HN262671)	392	EF112489.1 <i>Tuber melanosporum</i> clone Tmt1d retrotransposon Tmt1 Score: 403 <i>E</i> -value: 3e-109	BAB91415.1 marY1-like reverse transcriptase [<i>Tricholoma ustale</i>] <i>E</i> -value: 2e-17	Yes
gSSHmb-3 (HN262672)	138	–	–	Yes
gSSHmb-7 (HN262673)	136	–	–	Yes
gSSHmb-22 (HN262674)	158	–	–	–
gSSHmb-26 (HN262675)	300	–	/	–
gSSHmb-28 (HN262676)	141	–	/	Yes
gSSHmb-30 (HN262677)	168	XM_002836857.1 <i>Tuber melanosporum</i> Mel28 hypothetical protein Score: 283 <i>E</i> -value: 5e-73	XP_002836903.1 hypothetical protein [<i>Tuber melanosporum</i> Mel28] <i>E</i> -value: 1e-16	–
gSSHmb-33 (HN262678)	268	XM_002838776.1 <i>Tuber melanosporum</i> Mel28 hypothetical protein Score: 200 <i>E</i> -value: 5e-48	XP_002838822.1 hypothetical protein [<i>Tuber melanosporum</i> Mel28] <i>E</i> -value: 7e-15	Yes
gSSHmb-34 (HN262679)	340	–	YP_002458508.1 chromosome segregation ATPase-like protein [<i>Desulfitobacterium hafniense</i> DCB-2] <i>E</i> -value: 3e-08	–
gSSHmb-36 (HN262680)	59	–	–	Yes
gSSHmb-40 (HN262681)	87	–	–	–

Clone ID (accession number)	bp	blastn	blastx (nr)	blastn <i>Tuber</i> TE
gSSHmb-43 (HN262682)	316	–	/	Yes
gSSHmb-44 (HN262683)	137	–	–	Yes
gSSHmb-46 (HN262684)	382	EF112489.1 <i>Tuber melanosporum</i> clone Tmt1d retrotransposon Tmt1 Score: 381 <i>E</i> -value: 2e-102	XP_571377.1 retrotransposon nucleocapsid protein [<i>Cryptococcus neoformans</i> var. <i>neoformans</i> JEC21] <i>E</i> -value: 9e-24	Yes
gSSHmb-48 (HN262685)	135	–	–	Yes

- For each clone the result of different blast searches is shown.
- [↵*](#) No hit.
- [↵†](#) Not significant similarity (*E*-value >10⁻³).

After subtraction of *T. melanosporum* Mel28 with the *T. indicum* genomic DNA and reverse dot blot analysis, 34 specific sequences (32 single independent sequences and two forming a contig) were obtained (Table 2;). All sequences, except one, shared similarity with the TE. Clone gSSHmi-18 showed no similarity to any sequence in the databases.

Table 2 Specific sequences obtained in *Tuber melanosporum* vs. *Tuber indicum*.

Clone ID (accession number)	bp	blastn	blasttx (nr)	blastn <i>Tuber</i> TE
gSSHmi-1 (HN262686)	125	/	–	Yes
gSSHmi-5 (HN262687)	126	/	–	Yes
gSSHmi-6 (HN262688)	139	/	–	Yes
gSSHmi-7 (HN262689)	166	/	/	Yes
gSSHmi-9 (HN262690)	90	/	–	Yes
gSSHmi-12 (HN262691)	51	XM_002836396.1 <i>Tuber melanosporum</i> Mel28 hypothetical protein Score: 69.9 <i>E</i> -value: 3e-09	–	Yes
gSSHmi-13 (HN262692)	95	XM_002836396.1 <i>Tuber melanosporum</i> Mel28 hypothetical protein Score: 69.9 <i>E</i> -value: 2e-09	–	Yes
gSSHmi-14 (HN262693)	329	/	/	Yes
gSSHmi-15 (HN262694)	124	/	–	Yes
gSSHmi-17 (HN262695)	155	EF112488.1 <i>Tuber melanosporum</i> clone Tmt1c retrotransposon Tmt1 Score: 176 <i>E</i> -value: 3e-41	/	Yes
gSSHmi-18 (HN262696)	134	/	–	–
gSSHmi-20 (HN262697)	67	EF112488.1 <i>Tuber melanosporum</i> clone Tmt1c retrotransposon Tmt1 Score: 124 <i>E</i> -value: 1e-25	/	Yes

Clone ID (accession number)	bp	blastn	blasttx (nr)	blastn <i>Tuber</i> TE
gSSHmi-21 (HN262698)	142	/	–	Yes
gSSHmi-1 II (HN262699)	53	/	–	Yes
gSSHmi-2 II (HN262700)	157	/	/	Yes
gSSHmi-4 II (HN262701)	95	/	–	Yes
gSSHmi-5 II (HN262702)	236	XM_002836396.1 <i>Tuber melanosporum</i> Mel28 hypothetical protein Score: 77.8 <i>E</i> -value: 1e-11	/	Yes
gSSHmi-6 II (HN262703)	103	EF112489.1 <i>Tuber melanosporum</i> clone Tmt1d retrotransposon Tmt1 Score: 129 <i>E</i> -value: 4e-27	–	Yes
gSSHmi-10 II (HN262704)	81	EF112489.1 <i>Tuber melanosporum</i> clone Tmt1d retrotransposon Tmt1 Score: 128 <i>E</i> -value: 8e-27	–	Yes
gSSHmi-11 II (HN262705)	102	XM_002836396.1 <i>Tuber melanosporum</i> Mel28 hypothetical protein Score: 121 <i>E</i> -value: 1e-11	/	Yes
gSSHmi-12 II (HN262706)	126	/	/	Yes
gSSHmi-13 II (HN262707)	12097	XM_002836396.1 <i>Tuber melanosporum</i> Mel28 hypothetical protein Score: 81.8 <i>E</i> -value: 8e-13	–	Yes
gSSHmi-18 II				
gSSHmi-14 II (HN262708)	180	/	/	Yes
gSSHmi-17 II (HN262709)	95	/	–	Yes
gSSHmi-19 II (HN262710)	138	/	/	Yes
gSSHmi-22 II (HN262711)	128	/	/	Yes
gSSHmi-23 II (HN262712)	164	XM_002836396.1 <i>Tuber melanosporum</i> Mel28 hypothetical protein Score: 79.8 <i>E</i> -value: 3e-12	/	Yes
gSSHmi-25 II (HN262713)	127	XM_002836396.1 <i>Tuber melanosporum</i> Mel28 hypothetical protein Score: 52 <i>E</i> -value: 7e-4	/	Yes
gSSHmi-26 II (HN262714)	177	/	/	Yes
gSSHmi-28 II (HN262715)	123	EF112487.1 <i>Tuber melanosporum</i> clone Tmt1b retrotransposon Tmt1 Score: 77.8 <i>E</i> -value: 1e-11	–	Yes
gSSHmi-29 II (HN262716)	86	/	–	Yes
gSSHmi-30 II (HN262717)	121	/	–	Yes
gSSHmi-33 II (HN262718)	172	/	–	Yes

- For each clone the result of different blast searches is shown.
- \leq * Not significant similarity (*E*-value>10⁻³).
- \leq † †No hit.
- \leq ‡ ‡For the contig only one sequence was submitted.

To further validate the specificity of our technical approach, primers G13177f and G13177r were designed on two gSSH clones (gSSHmb-2 and gSSHmb-46) and used to amplify genomic DNA from different *Tuber* species (Fig. 1). Only the four *T. melanosporum* samples yielded an amplified band of the expected size. This band was sequenced and analyzed, finding high nucleotide similarity (96%) to the *T. melanosporum*-specific gypsy element, identified by [Riccioni \(2008\)](#).

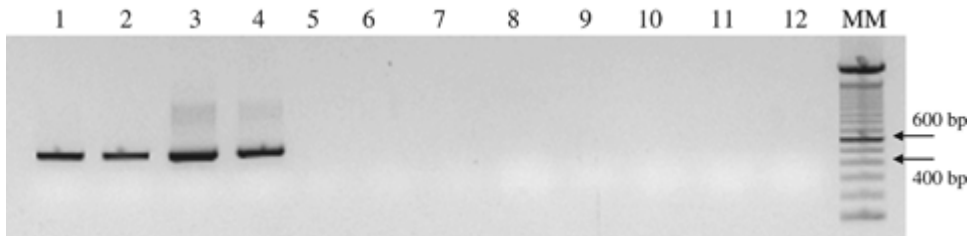


Figure 1 PCR amplification with G13177f and G13177r. 1, *Tuber melanosporum* 080107 (Italy); 2, *T. melanosporum* 080115-1 (France); 3, *T. melanosporum* 080209 (Spain); 4, *T. melanosporum* Mel28; 5, *Tuber indicum* 080110-1; 6, *Tuber brumale* 080130-1; 7, *Tuber borchii* F9; 8, *Tuber aestivum*; 9, *Tuber mesentericum* 1; 10, *Tuber magnatum* F8; 11, *Tuber rufum* 2773; 12, negative control; MM, 100bp (Invitrogen).

Tuber melanosporum is the first *Tuber* species and the second mycorrhizal fungus whose genome has been completely sequenced ([Martinet al.,2010](#)). The *T. melanosporum* genome is very large (125Mb) as compared with other filamentous fungi. Analyses of the sequencing data highlighted an extreme richness in TEs (58%) in the *T. melanosporum* genome. TEs are short DNA sequences, able to insert their own copies into new genomic positions. They were described for the first time by ([McClintock1950, 1956](#)) as ‘controlling elements’ playing a role in the evolution of genomes. The movement of TEs is responsible for genomic variation in the content of both intergenic and genic regions ([Morganteet al.,2007](#)). Interestingly, almost all the sequences we have identified in the *T. melanosporum* genome and absent in *T. borchii* and *T. indicum* corresponded to TEs, mainly belonging to the gypsy group. This may indicate either that the richness in TEs in not a common feature in species of the genus *Tuber* or that each *Tuber* species owns different kinds and distributions of TEs. The genome sequencing of other *Tuber* species could help testing these hypotheses. However, our finding supports the idea of [Martin \(2010\)](#) that *T. melanosporum* has a peculiar genome organization when compared with other fungal genomes.

Our data may be useful to develop DNA-based molecular markers for *Tuber* species' discrimination. This is particularly important for the two black truffles, which show similar morphological features and a strict neighborhood in phylogenetic analysis, but different economic value ([Genget al.,2009](#)). *Tuber indicum* has become a well-known edible fungus around the world, but sale of fruiting bodies and inoculated seedlings is forbidden in Italy to avoid fraud and ecological competition with the local, highly valuable *T. melanosporum*. Nevertheless, [Murat \(2008\)](#) demonstrated the presence of *T. indicum* in a plantation in Italy. Some molecular studies were carried out to discriminate *T. melanosporum* and *T. indicum* ([Paolocci et al.,1999](#); [Mabruet al.,2001](#); [Wanget al.,2006](#)), but until now the molecular differences between these species as well as their potential capacity of inbreeding are largely unknown. Therefore, tools for *Tuber* species' discrimination are still needed to avoid frauds in the truffle market.

The intraspecies gSSH experiment in *O. maius* yielded, after subtraction of *O. maius* OmMa3 with *O. maius* OmMa2 genomic DNA and reverse dot blot analysis, 16 specific sequences: five were single independent sequences, whereas 11 formed three contigs ([Table 3](#); accession numbers [HN262662–HN262669](#)). Of the singletons, one showed similarity to an l-galactonate dehydratase, one to a short-chain dehydrogenase/reductase family protein and three found no similarity in databases. Of the contigs, one showed similarity to glutathione synthetase, one to acetoacetyl-

coenzyme A synthetase and one found no similarity. OmMa3 and OmMa2 are two isolates derived from a serpentine soil, characterized by a high content in chromium and nickel ([Vallinoet al.,2011](#)). These two isolates are genetically distinct, on the basis of genetic fingerprinting, and show different abilities to grow in the presence of heavy metals, OmMa3 growing considerably better than OmMa2 on Ni- and Cr-amended media ([Vallinoet al.,2011](#)). Heavy metal tolerance is a trait of particular interest for documenting genetic changes during adaptation, as heavy metal toxicity represents a strong directional selective pressure resulting in the substitution of tolerance alleles at some loci ([Willemset al.,2007](#)). The genetic basis of heavy metal tolerance is not fully understood, and the questions on how many genes are involved and on the dynamics of the alleles of these genes are still open. It is tempting to speculate that the sequences we have identified may represent genetic differences underlying different tolerance of the two isolates, but further investigations are needed. Interestingly, glutathione synthetase, the second enzyme in the glutathione biosynthetic pathway, is known to be involved in metal tolerance ([Pócsiet al.,2004](#); [Reisingeret al.,2008](#)). Glutathione plays a key role not only in metal detoxification but also in protecting cells from other environmental stresses, such as oxidative stress and xenobiotics ([Memon & Schröder, 2009](#)). Moreover, a recent study on *Drosophila* by [Ortiz \(2009\)](#) suggests that polymorphisms in GSH biosynthetic genes may be an important contributor to differential arsenic sensitivity. Therefore, this genomic region is a good candidate for further analyses on the genetic basis of metal tolerance in fungal isolates.

Table 3 Specific sequences obtained in *Oidiodendron maius* OmMa3 vs. *O. maius* OmMa2.

Clone ID (accession number)	Bp blastn	blastx (nr)
gSSHOM-7 II (HN262663)	209 /	–
gSSHOM-8 II	210	
gSSHOM-5 (HN262662)	361 /	XP_003069392.1 glutathione synthetase family protein [<i>Coccidioides posadasii</i> C735 delta SOWgp] <i>E</i> -value: 2e-10
gSSHOM-9 II	323	
gSSHOM-10 II	323	
gSSHOM-16	361	
gSSHOM-13 II	323	
gSSHOM-14 II	323	
gSSHOM-15 II	323	
gSSHOM-6 II (HN262664)	393 /	XP_003006148.1 acetoacetyl-coenzyme A synthetase [<i>Verticillium albo-atrum</i> VaMs.102] <i>E</i> -value: 2e-04
gSSHOM-5 III	393	
gSSHOM-2 III (HN262669)	115 /	-
gSSHOM-10 (HN262665)	281 /	/
gSSHOM-17 (HN262666)	348	XM_001941538.1 <i>Pyrenophora tritici-repentis</i> Pt-1C-BFP L-galactonate dehydratase Score: 83.8 <i>E</i> -value: 7e-13 ABA60340.1 L-galactonate dehydratase [<i>Hypocrea jecorina</i>] <i>E</i> -value: 2e-28

Clone ID (accession number)	Bp blastn	blastx (nr)
gSSHOM-4 (HN262667)	552 /	XP_002384104.1 short-chain dehydrogenase/reductase family protein, putative [<i>Aspergillus flavus</i> NRRL3357] <i>E</i> -value: 6e-22
gSSHOM-9 (HN262668)	418 /	–

For each clone the result of different blast searches is shown.

- [↵*](#) For the contigs only one sequence was submitted.
- [↵†](#) Not significant similarity (*E*-value >10⁻³).
- [↵‡](#) ‡No hit.

In conclusion, our results show that gSSH is a quick and rather inexpensive approach that allows the identification of genomic differences both among (e.g. *Tuber*) and within (e.g. *O. maius*) fungal species. The sequences obtained by gSSH may be useful to identify species or strains as well as to investigate the genome plasticity, adaptation and evolution.

Acknowledgements

Thanks are due to M. Palumbo and J. Martinis for their help in the experiments. We thank I. Gianicolo, A. Zambonelli, M. Iotti, A. Mello, B. Finot and A. de Miguel for providing fruiting bodies of *Tuber* species, and E. Martino for providing *O. maius* isolates. M.V. and C.M. acknowledge financial support by the University of Turin. E.Z. PhD fellowship was funded by the University of Turin. This research was funded by the University of Turin, by Fondazione Sanpaolo and by Regione Piemonte.

Authors' contribution

C.M. and E.Z. contributed equally to this work.

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