Mycorrhiza
DOI 10.1007/s00572-011-0403-1

ORIGINAL PAPER

High consistency between replicate 454 pyrosequencing analyses of ectomycorrhizal plant root samples

Håvard Kauserud · Surendra Kumar · Anne K. Brysting · Jenni Nordén · Tor Carlsen

Received: 29 April 2011 / Accepted: 5 July 2011

Abstract In this methodological study, we compare 454 sequencing and a conventional cloning and Sanger sequencing approach in their ability to characterize fungal communities PCR amplified from four root systems of the ectomycorrhizal plant *Bistorta vivipara*. To examine variation introduced by stochastic processes during the laboratory work, we replicated all analyses using two independently obtained DNA extractions from the same root systems. The ITS1 region was used as DNA barcode and the sequences were clustered into OTUs as proxies for species using single linkage clustering (BLASTClust) and 97% sequence similarity cut-off. A relatively low overlap in fungal OTUs was observed between the 454 and the clone library datasets — even among the most abundant OTUs. In a non-metric multidimensional scaling analysis, the samples grouped more according to methodology compared to plant. Some OTUs frequently detected by 454, most notably those OTUs with taxonomic affinity to Glomales, were not detected in the Sanger dataset. Likewise, a few OTUs, including *Cenococcum* sp., only appeared in the clone libraries. Surprisingly, we observed a significant relationship between GC/AT content of the OTUs and their proportional abundances in the 454 versus the clone library datasets. Reassuringly, a very good consistency in OTU recovery was observed between replicate runs of both sequencing methods. This indicates that stochastic processes had little impact when applying the same sequencing technique on replicate samples.

Keywords Ectomycorrhiza · Community ecology · 454 pyrosequencing · Sanger sequencing · *Bistorta vivipara*

Electronic supplementary material The online version of this article (doi:10.1007/s00572-011-0403-1) contains supplementary material, which is available to authorized users.

H. Kauserud (✉) · S. Kumar · J. Nordén · T. Carlsen
Department of Biology, Microbial Evolution Research Group (MERG), University of Oslo,
P.O. Box 1066, Blindern,
0316 Oslo, Norway
e-mail: haavarka@bio.uio.no

A. K. Brysting
Department of Biology, Centre for Ecological and Evolutionary Synthesis (CEES),
P.O. Box 1066, Blindern,
0316 Oslo, Norway

Introduction

The knowledge about ectomycorrhizal (ECM) community ecology has increased dramatically since molecular methods were introduced in this research field some 20 years ago (Pace 1997; Horton and Bruns 2001). Cloning and Sanger sequencing or direct sequencing of the nuclear ribosomal internal transcribed spacer (ITS) region from root tips have been used in numerous studies to characterize ECM communities (e.g., Tedersoo et al. 2010). In contrary to animals and plants (Chase and Fay 2009), the ITS region is now the preferred barcoding region for fungi. The implementation of next generation sequencing (NGS) technologies (e.g., 454 pyrosequencing) initiates a new era, enabling large-scale analyses of these complex and normally species-rich fungal communities (e.g., Buée et al. 2009; Tedersoo et al. 2010; Wallander et al. 2010). In addition to providing qualitative information about the mycorrhizal community composition, NGS may have the capacity to provide quantitative information about fungal abundance in the mycorrhizosphere. However, little is still known about potential biases involved during NGS analyses. Regarding

Published online: 16 July 2011

fungi, the number of studies comparing NGS and the more traditional well-established methods is limited. In a study where ECM root tips were analyzed by both Sanger sequencing and 454 pyrosequencing, the two methods yielded qualitatively similar results, but there were slight, but significant, differences that affected the taxonomic view of the fungal communities (Tedersoo et al. 2010). By molecular analyses of fungi occurring in house dust samples, Amend et al. (2010a) observed that, at the order level, the composition of the fungal communities determined from pyrosequencing data was comparable with that derived from clone libraries. Regarding the analyses of 16S diversity in bacterial communities, a low degree of reproducibility was observed by Zhou et al. (2011) in replicate 454 runs. Engelbrektson et al. (2010) showed that both amplicon length and primer pair markedly influenced estimates of bacterial richness and evenness.

In this methodological study, we evaluate the ability of 454 pyrosequencing to analyze the fungal communities associated with whole root systems of the ectomycorrhizal plant *Bistorta vivipara* (L.) Delarbre, and we compare this new technology to the well-established approach of cloning and Sanger sequencing. *B. vivipara* is one of the few herbaceous plants that form both ectomycorrhizal and arbuscular mycorrhizal relationships (see e.g. Hesselmann 1900; Massicotte et al. 1998; Eriksen et al. 2002; Mühlmann et al. 2008). *B. vivipara* produces a small and condensed root system which potentially makes it easy to analyze the entire root system simultaneously through PCR with fungal specific primers. It has a wide circumpolar distribution in arctic–alpine habitats, but in Northern Europe it also occurs less frequently in lowland grassland and mire habitats. DNA was extracted from four different root systems of *B. vivipara*, and the fungal ITS1 region was targeted with each sequencing approach.

Materials and methods

Sampling in field

The *B. vivipara* plants used for this study were collected from a lowland population in southern Norway (Oslo, Blankvann, N 60°01′34″, E 10°40′14″). The area is characterized by Cambrian–Silurian bedrocks and calcareous soil. The locality was a species-rich meadow with a high number of forbs (25 species were recorded within 6×6 m^2), bordered by coniferous wood. For this particular study, two plants from the sample plot A (A1 and A2) and two plants from plot B (B1 and B2) were analyzed. Sample plots A and B were located 3 m from each other. The plants were brought to the laboratory where the root systems were carefully washed the same day, first in spring water and then in distilled water. We then transferred 80–90% of the entire root systems to CTAB buffer and stored them at −20°C for later DNA extraction. The approximate weights of the rinsed root systems were 0.09 g.

DNA extraction

The roots were crushed in CTAB buffer for 4 min at 20 Hz using a Retsch TissueLyser. After crushing and CTAB lysis, each sample was divided into two replicates which were analyzed in parallel through all the following steps. The DNA extraction procedure combined a standard CTAB extraction procedure (Murray and Thompson 1980) with the cleaning steps of E.Z.N.A. blood DNA kit (Omega Bio-Tek, Norcross, GA, USA). The resulting DNA extracts were diluted 10× and subjected to two different PCR amplification and sequencing techniques: Sanger sequencing of clone libraries and 454 pyrosequencing.

Cloning and Sanger sequencing

Samples were prepared for cloning and Sanger sequencing by performing PCR amplification using the fungal specific primers ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993). PCR was performed on an Eppendorf thermocycler (Eppendorf AG, Hamburg, Germany) in 25-μL reactions containing 1 μL template DNA and 24 μL reaction mix. Final concentrations were 0.2 mM dNTP mix, 0.4 μM of each primer, and 0.5 U DyNAzyme EXT (Finnzymes Oy, Espoo, Finland). The PCR amplification program was as follows: 4 min at 94°C, followed by 35 cycles of 25 s at 94°C, 30 s at 50°C, 2 min at 72°C, and a final extension step at 72°C for 10 min before storage at 4°C.

The ITS fragments were cloned with the TOPO TA Cloning kit (Invitrogen, Paisley, UK) using blue/white screening according to the manufacturer's manual, except that half reaction volumes were used. A total of 48 positive colonies from each replicate of all four samples (96 from each plant root system) was subjected to direct PCR with the M13R/T7 primers using the following PCR conditions: 10 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C, followed by a final extension at 72°C for 10 min. All PCR products were sequenced using the ABI BigDye Terminator sequencing buffer, v3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, USA), and M13R as sequencing primer, and visualized on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems).

All sequence chromatograms were controlled manually in the program BioEdit Sequence Alignment Editor version 5.0.9 (Hall 1999). The obtained Sanger sequences are accessioned in GenBank with accession nos. JF894430–JF894787.

454 Pyrosequencing

Samples were prepared for 454 pyrosequencing by performing nested PCR amplification using the fungal specific primers ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993) in the first step, and fusion primers including ITS5 and ITS2 (White et al. 1990) in the nested step. Fusion primers were constructed by adding eight different unique 6-bp tags and 454 pyrosequencing adaptors A and B to ITS5 and ITS2, respectively. Tags were added to both primers and were at least 3 bp different from each other in both directions. Tags were selected so that a single error would not result in a sequence being added to the data for another sample. PCR was performed on an Eppendorf thermocycler in 25-μL reactions containing 2 μL template DNA and 24 μL reaction mix. Final concentrations were 0.16 mM dNTP mix, 0.2 μM of each primer, and 0.4 U polymerase (Phusion, Finnzymes, Vantaa). The PCR amplification program was as follows: 30 s at 98°C, followed by 30 cycles of 10 s at 98°C, 20 s at 55°C, 20 s at 72°C, and a final extension step at 72°C for 7 min before storage at 4°C. PCR products were cleaned with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), quantified using Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany), and pooled into equimolar amplicon libraries. GS FLX sequencing of the tagged amplicons was performed at the Norwegian High Throughput Sequencing Centre (http://www.sequencing.uio.no) using one out of 16 lanes of a 454 plate. Moreover, 10,839 454 sequences were retrieved and submitted to NCBI SRA under accession number SRP004613. These 454 sequences have also been used in evaluating our recently published bioinformatics pipeline CLOTU (Kumar et al. 2011).

Bioinformatics and statistical analyses

We analyzed the sequence data using CLOTU (Kumar et al. 2011), which is available at http://www.bioportal.uio.no. A fasta file was created including both the clone-based Sanger sequences and 454 sequences and uploaded to CLOTU. Sequences with any mismatch against tags, and sequences lacking ITS2 primer site, or shorter than 150 bp were removed prior to trimming of adapters and tags. The dataset was analyzed both excluding and including all sequences with Ns. Since all sequences including Ns clustered into frequent OTUs including a high number of sequences (also including sequences without Ns), we kept also the sequences with Ns in the further analyses. Notably, none of the singleton sequences included Ns. Sequences were clustered with BLASTClust (single linkage clustering) at 97% similarity and 75% coverage of the longest sequence. The longest sequence from each cluster was used for BLASTn searches (Altschul et al. 1997). A downloaded version of NCBI/GenBank was used as the reference library.

One of the singleton OTUs recovered by 454 sequencing had 100% match and 100% coverage in ITS1 against ITS GenBank accessions of the wood decay polypore *Gloeoporus taxicola*. Molecular analyses on this taxon were concurrently conducted in the laboratory and, hence, this singleton OTU was considered a result of contamination and omitted from further analyses.

OTU community composition was analyzed with two-dimensional non-metric multidimensional scaling (NMDS; Venables and Ripley 2002; Oksanen et al. 2008), using square-root transformed OTU abundance data. The 16 samples (four root systems, two replicates of each, analyzed with two sequencing methods) were located in the ordination space according to the pair-wise dissimilarities (Jaccard index; Chao et al. 2005) of community composition. The effect of sequencing method was tested with a permutation test (Oksanen et al. 2008).

Results

In the 454 dataset, 222 sequences were removed because of tag-match failure and 186 because of failure to identify primer ITS2 at the beginning of the sequence. Only one sequence was filtered out for being too short (<150 bp). A total of 10,430 454 and 358 clone-based Sanger sequences were retained for further analyses after quality assessment. Using a 97% cut-off, the sequences clustered into 57 OTUs, of which 22 represented singletons. All OTUs along with their best BLAST hits and distribution on the plant root systems/replicates are shown in Table S1.

In total, 52 OTUs were recovered by 454 sequencing and 17 by cloning/Sanger sequencing. Eighteen (34.6%) and six (35.3%) of these appeared as singletons in the two datasets, respectively. In Fig. 1, results from an NMDS ordination of the 16 samples are depicted, which shows that the composition of fungal OTUs in replicate runs using the same sequencing method are very similar to each other. Regarding the most frequent OTUs (≥5 reads), all OTUs were recovered in all pairs of 454 replicates, except one (Table S1). A high consistency was also observed between the clone library replicates when it comes to the most frequent clone library OTUs (≥3 reads) (Table S1). Moreover, the quantitative data showed a high consistency in recovering the abundances of the most frequent OTUs (Fig. 2). As can be seen in Fig. 2a, b, there was a high correlation between the replicate runs when it comes to the OTUs' number of reads.

The NMDS analysis further shows that the samples group more by sequencing method than by host plant and, hence, that the two methods reveal partly different

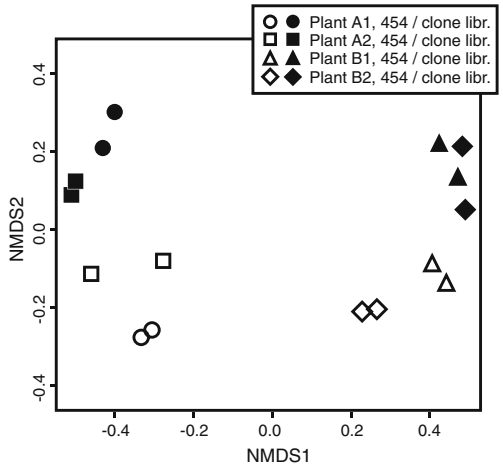

Fig. 1 NMDS ordination plot where the 16 different samples are placed according to composition of OTUs in and across the samples. The two plots A and B are separated along NMDS1 while the sequencing method falls together with NMDS2. Hence, the distribution of samples shows that there is a major effect of sequencing method (454 versus cloning/Sanger)

communities (permutation test, R^2=0.84, p<0.001). Only 12 OTUs were shared across the two datasets. Also, when it comes to the most frequently detected OTUs (defined as having ≥5 454 reads and ≥3 Sanger reads), less than half of the OTUs were recovered by both methods. However, the two most abundant OTUs, detected in two plants, were the same in both datasets. Concerning the more rare OTUs (<5 454 reads and <3 Sanger reads), only one out of 37 OTUs was detected by both methods (Table S1). In Fig. 3, the abundance of the 12 most abundant OTUs is shown across the 16 samples, also illustrating that a good consistency most often was observed between replicates involving the same technique, while the results often diverged between the 454 and the clone library approach.

Notably, no OTUs with affinity to Glomeromycota turned up in the clone library dataset although five Glomeromycota OTUs appeared among the 16 most abundant in the 454 dataset. In opposite, one OTU with good match to an accession of *Cenococcum geophilum* (GU550109, 98% similarity) was only detected in the clone libraries (six sequences obtained from one root system).

As shown in Fig. 4, a significant relationship was observed between the GC content (i.e., the GC content in the representative sequence) of the OTUs and the proportional difference in OTU abundances in the 454 and the clone libraries (linear regression, p=0.04). Typically, OTUs with high GC content were more abundant in the clone libraries while OTUs with relatively high AT content were more abundant in the 454 dataset.

Notably, all singleton OTUs recovered by 454 sequencing, except two, represented novel taxa (as judged by the BLAST matches) not recovered by the non-singleton OTUs (Table S1). Most of these singleton OTUs had high sequence coverage and BLAST matches against various GenBank accessions (Table S1) and should therefore not be considered as sequencing artifacts. No signs of sequencing errors could be traced in the alignments including the singletons and their closest GenBank matches. Moreover, the two singleton OTUs having the same BLAST hits as two non-singleton OTUs seem not to be a result of sequencing errors as these two sequences diverged by multiple polymorphisms not associated with homopolymer stretches.

Discussion

We observed several discrepancies between the 454 and the clone library datasets in OTU recovery. Although some of the differences certainly are due to the highly divergent

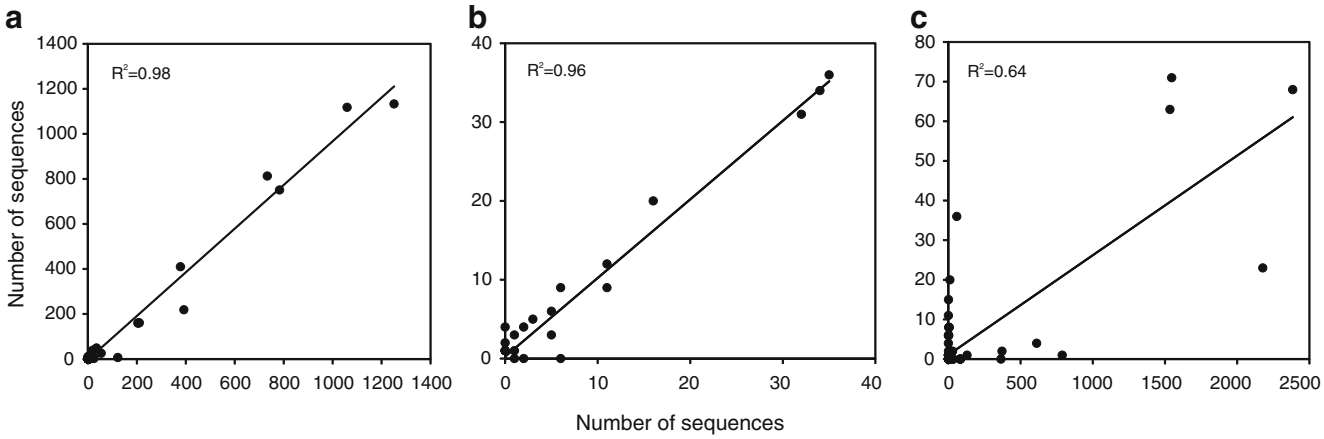

Fig. 2 Number of sequence reads in the various OTUs obtained during the replicate runs. **a** Number of reads for OTUs obtained in the 454 replicate runs. **b** Number of reads for OTUs obtained in the clone library replicates. **c** Average number of reads obtained for OTUs in the two 454 replicate runs plotted against the corresponding average number of reads obtained in the clone library replicates. All the three linear regressions were highly significant (p<0.0001)

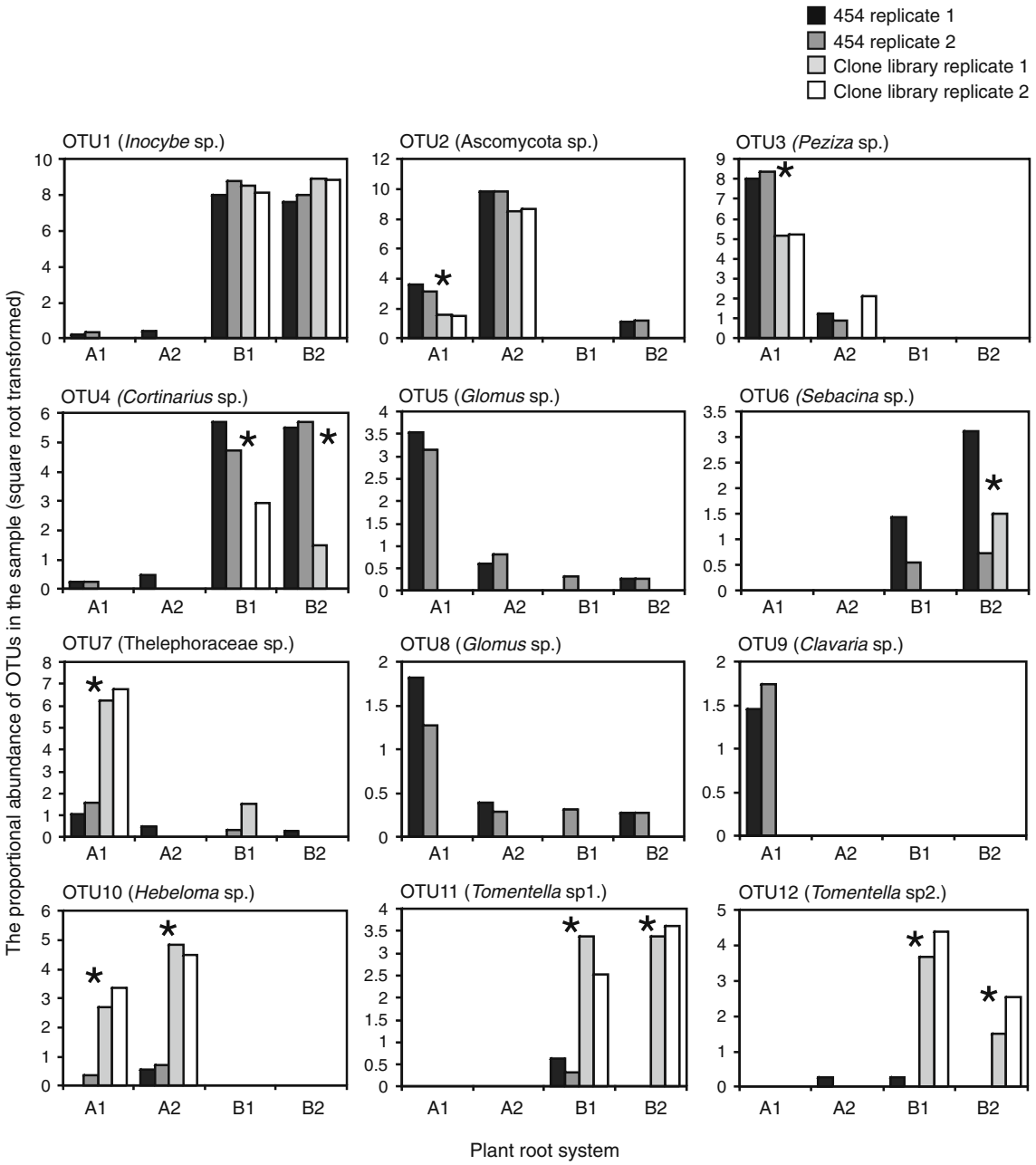

Fig. 3 Abundance of sequence reads in the 12 most abundant OTUs across the 16 samples. Square root-transformed proportional data are plotted on the *y*-axis (i.e., the proportional abundance of each OUT in each sample). *Asterisks* indicate plants where a significant deviation from even distribution was found, as judged by chi-square tests ($p<0.05$)

number of sequences obtained (10,430 454 sequences versus 358 clone-based Sanger sequences), this cannot be the sole cause, as some of the overall most abundant OTUs obtained from 454 sequencing were not found in the clone libraries. It is interesting to note that several OTUs with affinity to Glomeromycota were not recovered in the clone libraries. On the contrary, one OTU with high similarity to *C. geophilum* did not show up in the 454 dataset although it was detected in both the clone library replicates of root system A1. Also, some OTUs with affinity to Thelephoraceae had a disproportionately low number of reads in the 454 dataset compared to the clone libraries. Notably, the same primers (ITS1F and ITS4) were used in the first PCR in the 454 setup as in the PCR for the clone libraries. The 454 sequencing setup included one additional nested PCR step using the tagged primers ITS5 and ITS2. In theory, the primers ITS5 and ITS2 causes limited biases during amplification (Bellemain et al. 2010) and should amplify *Glomus*, *Cenococcum*, and Thelephoraceae. Nevertheless, we cannot rule out that the extra nested PCR step may have

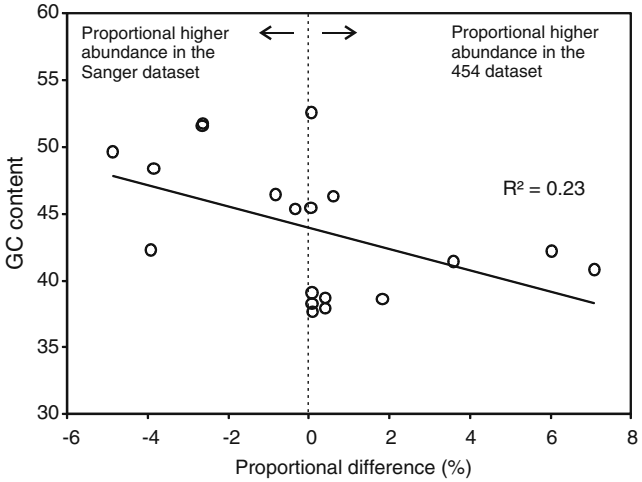

Fig. 4 Plot of GC content against the proportional difference of OTUs in the 454 dataset and the clone libraries. Only OTUs with ≥10 reads in total are plotted. Typically, OTUs with a high GC content are overrepresented in the clone libraries while OTUs with a low GC are underrepresented. A significant relationship was observed in linear regression analysis (p=0.037)

caused some of these differences. But the extra PCR step cannot explain why *Glomus* was present in the 454 data and absent in the clone libraries. Notably, the chance for not picking up *Glomus* in the clone libraries is extremely low (7.0×10^{-9}). The fact that 454 performed poorly in detecting *Cenococcum* and Thelephoraceae is quite alarming, given how widespread, abundant, and ecologically important these fungal taxa are. We have currently no other plausible explanations for the observed differences in the recovery of Glomeromycota, *Cenococcum*, and Thelephoraceae. The putative biases should preferably be analyzed more in-depth in further experiments where a nested PCR step is avoided.

Tedersoo et al. (2010) and Amend et al. (2010b) also compared the ability of 454 and cloning/Sanger sequencing in analyzing the fungal diversity in environmental samples. At least at a higher taxonomic level (order level), Amend et al. (2010b) did not observe significant differences between the fungal communities recovered from 454 versus cloning/Sanger sequencing. Tedersoo et al. (2010) observed that pyrosequencing and the traditional identification method (direct Sanger sequencing of amplicons from root tips) revealed a roughly similar phylogenetic structure in the analyzed ECM fungal community. Like in the present study, Tedersoo et al. (2010) observed that the ITS of several taxa was not captured by the traditional approach and that some taxa also were missed out using 454 pyrosequencing. Avis et al. (2010) analyzed artificial pooled fungal communities and compared the ability of the techniques T-RFLP, ARISA, and cloning/Sanger sequencing to recover the known fungi. In general, significantly distorted descriptions of the fungal communities were obtained, but when they increased the number of analyzed clones per sample (to on average of 92) they recovered on average 90% of the species.

Reassuringly, a high consistency was observed between the replicate runs of the 454 analysis and the clone-based Sanger sequencing, both in terms of which OTUs were recovered and the number of reads per OTU. Hence, little differences, both quantitatively and qualitatively, have been introduced between the replicate 454 and clone library/Sanger runs. This indicates that when using the same methodological setup, one may compare the relative abundance of a single OTU between samples. The actual number of sequences produced does not necessarily reflect the actual quantitative abundance of that OTU, but one may estimate the relative contribution of a single OTU between multiple samples. Notably, the analyzed samples in this study were probably relatively species poor compared to samples from many other fungal communities (e.g., Jumpponen and Jones 2009). We acknowledge that the consistency between replicate runs might depend on the diversity and complexity of the fungal community in question. For example, in cases where a high number of different fungi (and PCR templates) are present with approximately similar concentrations, a higher level of stochasticity during PCR might have a higher impact. Rather opposite to our findings, Zhou et al. (2011) observed a low reproducibility in replicate 454 runs of 16S amplified from bacterial communities, which might relate to a higher complexity in the bacterial communities.

It must be emphasized that with our methodological setup, we were not able to illuminate the preferential extraction/amplification/sequencing of certain taxa above others co-occurring in the samples. For example, the number of nrDNA copies, which is known to differ both between and within fungal species (see e.g. Pukkila and Skrzynia 1993), might have great influences on the number of reads recovered (Amend et al. 2010b).

Surprisingly, we observed a significant relationship between the GC content and the proportional abundances of OTUs in the 454 and the clone libraries. However, only a small part of the total variation (R^2=0.23) was accounted for by the linear relationship. It is generally acknowledged that AT-rich sequences are preferentially PCR amplified due to lower melting temperature, but this can apparently not explain our results, as both datasets are generated through PCR. It has also been hypothesized that GC-rich fragments are more difficult to clone, but this should lead to opposite trends compared to our observations. More in-depth experiments are needed to fully understand the mechanisms behind the observed trend.

Interestingly, most of the singleton OTUs recovered by 454 sequencing seemingly represented unique fungal lineages not represented among the non-singleton OTUs. In their methodological study, Tedersoo et al. (2010)

concluded that most of the singletons were caused by sequencing artifacts. However, our results indicate that it might be problematic to ascribe singletons mainly to sequencing and/or PCR errors.

In this study, we have shown that different sequencing techniques may recover different parts of the fungal communities residing in ECM root systems. A high consistency was found between replicate runs using the same sequencing techniques, both in terms of which OTUs were recovered and the number of reads per OTU. We also found indications that the AT/GC composition of the sequences influences their recovery rate. Moreover, our results indicate that singleton OTUs might represent unique fungal lineages and not just sequencing errors. Lastly, we encourage researchers to include at least a few replicates during their 454 analyses to control for experimental stochasticity. So far, this has mostly been neglected in 454 pyrosequencing studies of fungal communities.

Acknowledgments The University of Oslo is acknowledged for financial support and three anonymous reviewers for helpful comments. We thank biology students at the University of Oslo who did the field work and initiated this work as a part of a course project.

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402

Amend A, Samson R, Seifert K, Bruns T (2010a) Deep sequencing reveals diverse and geographically structured assemblages of fungi in indoor dust. PNAS 107:13748–13753

Amend AS, Seifert KA, Bruns TD (2010b) Quantifying microbial communities with 454 pyrosequencing: does read abundance count? Mol Ecol 19:5555–5565

Avis PG, Branco S, Tang Y, Mueller GM (2010) Pooled samples bias fungal community descriptions. Mol Ecol Res 10:135–141

Buée M, Reich M, Murat C, Nilsson RH, Uroz S, Martin F (2009) 454 Pyrosequencing analyses of forest soils reveal an unexpectedly high fungal diversity. New Phytol 184:449–456

Chao A, Chazdon RL, Colwell RK, Shen T (2005) A new statistical approach for assessing similarity of species composition with incidence and abundance data. Ecol Lett 8:148–159

Chase M, Fay M (2009) Barcoding of plants and fungi. Science 325:682–683

Engelbrektson A, Kunin V, Wrighton KC, Zvenigorodsky N, Chen F, Ochman H, Hugenholtz P (2010) Experimental factors affecting PCR-based estimates of microbial species richness and evenness. ISME J 4:642–647

Eriksen M, Bjureke KE, Dhillion SS (2002) Mycorrhizal plants of traditionally managed boreal grasslands in Norway. Mycorrhiza 12:117–123

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98

Hesselmann H (1900) Om mycorrhizabildningar hos artiska växter. Bihang Till K Svenska Vet Akad Handlingar 26:1–46

Horton T, Bruns T (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black box. Mol Ecol 10:1855–1871

Jumpponen A, Jones KL (2009) Massively parallel 454 sequencing indicates hyperdiverse fungal communities in temperate *Quercus macrocarpa* phylloshere. New Phytol 184:438–448

Kumar S, Carlsen T, Mevik B, Enger P, Blaalid R, Shalchian-Tabrizi K, Kauserud H (2011) CLOTU: an online pipeline for processing and clustering of 454 amplicon reads into OTUs followed by taxonomic annotation. BMC Bioinformatics 12:82

Massicotte HB, Melville LH, Peterson RL, Luoma DL (1998) Anatomical aspects of field ectomycorrhizas on *Polygonum viviparum* (Polygonaceae) and *Kobresia bellardii* (Cyperaceae). Mycorrhiza 7:287–292

Mühlmann O, Bacher M, Peintner U (2008) *Polygonum viviparum* mycobionts on an alpine primary successional glacier forefront. Mycorrhiza 18:87–95

Murray MG, Thompson WF (1980) Rapid isolation of high weight plant DNA. Nucl Acids Res 8:4321–4325

Oksanen J, Kindt R, Legendre P, O'Hara B, Simpson GL, Stevens MHH (2008) vegan: Community Ecology Package. R package

Pace NR (1997) A molecular view of microbial diversity and the biosphere. Science 276:734–740

Pukkila PJ, Skrzynia C (1993) Frequent changes in the number of reiterated ribosomal-rna genes throughout the life-cycle of the Basidiomycete *Coprinus cinereus*. Genetics 133:203–211

Tedersoo L, Nilsson RH, Abarenkov K, Jairus T, Sadam A, Saar I, Bahram M, Bechem E, Chuyong G, Kóljag U (2010) 454 Pyrosequencing and Sanger sequencing of tropical mycorrhiza fungi provide similar results but reveal substantial methodological biases. New Phytol 188:291–301

Venables WN, Ripley BD (2002) Modern applied statistics with S. Springer, New York

Wallander H, Johansson U, Sterkenburg E, Durling MB, Lindahl B (2010) Production of ectomycorrhizal mycelium peaks during canopy closure in Norway spruce. New Phytol 187:1124–1134

White TJ, Bruns T, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninski JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, pp 315–322

Zhou J, Wu L, Deng Y, Zhi X, Jiang YH, Tu Q, Xie J, Van Nostrand JD, He Z, Yang Y (2011) Reproducibility and quantitation of amplicon sequencing-based detection. ISME J 1:11