RNA extraction from decaying wood for (Meta)transcriptomic analyses

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ABSTRACT

Wood decomposition is a key step of the terrestrial carbon cycle and of economic importance. It is essentially a microbiological process performed by fungi and to an unknown extent by bacteria. To gain access to the genes expressed by the diverse microbial communities participating to wood decay we developed an RNA extraction protocol from this recalcitrant material rich in polysaccharides and phenolic compounds. This protocol was implemented on 22 wood samples representing as many tree species from 11 plant families in the Angiosperms and Gymnosperms. RNA was successfully extracted from all samples and converted into cDNAs from which were amplified both fungal and bacterial protein coding genes, including genes encoding hydrolytic enzymes participating to lignocellulose hydrolysis. This protocol applicable to a wide range of decomposing wood types represents a first step towards a metatranscriptomic analysis of wood degradation under natural conditions.

RESUME

La décomposition du bois est une étape clé du cycle terrestre du carbone et revêt une importance économique. Il s'agit essentiellement d'un processus microbien réalisé par les champignons et de façon plus incertaine par les bactéries. Pour accéder aux gènes exprimés par les communautés microbiennes participant à la dégradation du bois nous avons développé un protocole d'extraction d'ARN à partir de ce matériel récalcitrant riche en polysaccharides et composés phénoliques. Ce protocole a été mis en œuvre sur 22 échantillons de bois représentant autant d'espèces d'arbres Angiospermes ou Gymnospermes. L'ARN a été extrait avec succès de tous les échantillons et converti en ADNc à partir duquel ont été amplifiés aussi bien des gènes fongiques que bactériens dont des gènes codant des enzymes hydrolytiques participant à l'hydrolyse de la lignocellulose. Ce protocole applicable à une

grande diversité de bois en décomposition représente une première étape vers une analyse métatranscriptomique de la dégradation du bois en conditions naturelles.

Key words

Wood degradation, RNA extraction, metatranscriptomics

Introduction

Wood is a structural component of perennial tree plants whose durability, strength, but also flexibility allows them to reach large dimensions despite being subjected to important mechanical constraints. Because of these characteristics, wood is extensively used as a construction material and for the manufacturing of numerous goods such as furniture.

Wood durability is synonymous of delayed and slow decomposition by microorganisms. These characteristics can, in part, be explained by the specific chemical composition of this material. Wood is essentially composed of the secondary cell walls of dead plant cells, rich in cellulose, hemicellulose and impregnated by lignin, a polyphenolic polymer recalcitrant to (bio)chemical attack and that restricts the diffusion of microbial hydrolytic enzymes (Pereira et al. 2003). Many woods are also impregnated by large quantities of phenolics or other plant secondary metabolites that act as antimicrobial agents (Valette et al. 2017). Finally, most woods have an unbalanced chemical composition, being extremely poor in macronutrients such as nitrogen (Cornwell et al. 2009; Rinne et al. 2016), thus representing a harsh environment to microbial life.

Despite these characteristics, no wood is truly recalcitrant to microbial attack largely performed by guilds of specifically adapted saprotrophic/pathogenic fungi whose actions contribute, from an ecological point of view, to the recycling of the organic carbon (Lonsdale et al. 2008; Arnstadt et al. 2015) trapped in this dead plant biomass (estimated in the range of 50-200 $m^3.ha^{-1}$ in temperate unmanaged forests (Albrecht 1991)).

From a mechanistic point of view, wood degradation is largely the result of the action of extracellular hydrolytic enzymes, but also of low molecular weight agents (Mn^{3+}, reactive oxygen species) of microbial origin (Blanchette 1995; Arantes et al. 2012). Noticeably, several fungal taxa (essentially in the Basidiomycota) secrete complex enzyme cocktails capable of hydrolyzing all structural components of wood, cellulose (by cellulases),

hemicellulose (by hemicellulases), but also lignin (by so-called class II peroxidases) (Eastwood et al. 2011; Talbot et al. 2015). Lignivorous fungal taxa however differ from each other by their exact mode of deconstructing wood components (Cragg et al. 2015). Schematically, so-called "white-rot fungi" secrete first large amounts of peroxidases that remove lignin, leaving behind a white fibrous material enriched in cellulose (Otjen and Blachette 1987). As for "brown-rot fungi", they presumably produce large amounts of diffusible low molecular weight oxidants that degrade polysaccharides, thus leaving behind a brownish brittle material enriched in chemically modified lignin (Arantes et al. 2012). This functional classification scheme has received experimental support from biochemical studies carried out on simplified lignocellulosic substrates (Ruiz-Duenas et al. 2009; Fernandez-Fuejo et al. 2012) and from comparative genomic studies that revealed a differential distribution of genes encoding enzymes active on polysaccharides or lignin between brown-rot and white-rot species (Eastwood et al. 2011; Floudas et al. 2012; Koheler et al. 2015). However, extensive sampling of basidiomycete genomes has revealed a high diversity of fungal wood decay mechanisms (Riley et al. 2014).

Despite this apparent wealth of knowledge on wood degradation by individual species of fungi, the exact mode and pace of wood degradation in the field need to be clarified (Shwarze et al. 2013). Indeed, under natural conditions, wood degradation is accomplished not by one, but by several fungal species that act either simultaneously or successively on the same substrate (Rajala et al. 2011; Yamashita et al. 2015; Purahong et al. 2016). Additional points that need to be addressed in wood degradation regard the microbial response to potentially toxic resins and phenolics that accumulate in wood (Valette et al. 2017) and adaptive responses to macronutrient limitation (Rajala et al. 2011). Finally, a controversial point regards the role of bacteria in this process (Bugg et al. 2011; Hoppe et al. 2014; Johnston et al. 2016). Molecular surveys of bacterial communities present in decomposing

wood samples demonstrate that they are diversified and differ between plant species and decomposition stages (Hoppe et al. 2014) although their actual contribution to wood lignocellulose degradation remains uncertain (Johnston et al. 2016).

Metatranscriptomics, *i.e.* the qualitative and quantitative analysis of the pool of genes expressed by a microbial community, constitutes an experimental approach to access the functions expressed by this community. The functional annotation of metatranscriptomic messenger RNA (mRNA) sequences gives information on the relative contribution of different pathways to a specific biogeochemical process (Damon et al. 2012; Baldrian and López-Mondéjar 2014), while the taxonomic annotation of the same mRNAs indicates which microbial taxa contribute to this process and suggests potential synergies and/or functional complementarities between taxa.

Thus far, metatranscriptomics has not been implemented on decaying wood. One challenge resides in the development of a reliable protocol to extract pure RNA from such a low living biomass substrate rich in polysaccharides and phenolics, known to be potent inhibitors of many enzymes used in molecular biology, such as reverse transcriptase or DNA polymerases. While several protocols have been published for the extraction of RNAs from woody tissues of living plants (Chang et al. 1993; Moser et al. 2004; Le Provost et al. 2007; Gambino et al. 2008; Lorenz et al. 2010), none of them seems to have been implemented on decaying dead wood. In the present study, we evaluate a new protocol to extract pure, undegraded RNA from wood samples representative of several of the major botanical families that dominate in Northern hemisphere forests.

Materials and methods

Plant material

Decomposing wood from 22 tree species were collected in different geographic sites in France and Italy (Fig. 1, Table 1). For each plant species, a variable number of wood pieces of different size classes (from twigs to trunk fragments) and at different stages of decomposition were randomly sampled. In addition to "tree-specific" wood samples we also collected five "forest-specific" samples in five geographic sites in France and Italy (Table 1). Forests in these sites, and therefore the corresponding composite wood samples, were either monospecific (e.g. pure *Larix decidua* stands at Colle della Lombarda) or mixed species forests (up to four angiosperm dominant tree species at the Mandria Natural Park (Manpa) site). In each forest we collected about 100 decomposing wood fragments along two 20 m-long transects.

Wood fragments were brought to the laboratory and after removing bark fragments, wood was reduced to sawdust using a stainless steel grater. For each plant species or forest, all individual samples were mixed together in equal quantities and 0.1 g (fresh weight) aliquots of the resulting composite samples were placed in 2 ml microcentrifuge tubes, frozen in liquid nitrogen and stored at -70 °C until RNA extraction. Wood water content was estimated after drying the samples for 24 h at 70 °C (Table 1).

RNA extraction

Pestles, mortars, all glass and plastic-ware and aqueous solutions were autoclaved before use. Molecular biology grade chemicals were dissolved in water treated overnight with 0.1 % (v:v) of diethyl pyrocarbonate (DEPC) and then autoclaved.

One hundred mg of each frozen wood sample were ground to powder under liquid nitrogen in a mortar. Four ml of water-equilibrated (acidic) phenol and 4 mL of autoclaved extraction buffer (0.1 mol Tris-HCl, pH 8.0; 0.1 mol NaCl; 20 mmol Na_2EDTA; 1 % w/v

polyvinylpyrrolidone-40; with or without 2 % w/v of cetyl trimethylammonium bromide (CTAB)) were then added to the mortar immediately after nitrogen evaporation. Once the suspension was completely thawed, it was transferred as *ca* 1.5 mL aliquots in 6-8 2 mL centrifuge tubes. After a centrifugation at 7000 *g* for 10 min at 4 °C, the upper aqueous phase was transferred to new 2 mL tubes and mixed thoroughly by inversion for ca 1 mim to an equal volume of phenol:chloroform:isoamyl alcohol (24:25:1 vol:vol:vol, pH 8.0). After a second identical centrifugation step, the upper aqueous phase was re-extracted one or three times with an equal volume of chloroform and re-centrifuged as before. Following transfer of the aqueous phase to a new 1.5 mL tube, the nucleic acids were precipitated by adding an equal volume of 2-propanol. After mixing by inversion and an incubation at -20 °C for at least for 2 h, the nucleic acids were pelleted by centrifugation at 18,000 *g* for 30 min at 4 °C. Pellets were dissolved in 500 µL of water to which was added an equal volume of 6 mol LiCl for the selective precipitation of RNA. After an overnight incubation at 4 °C, RNA was recovered by centrifugation at 18,000 *g* for 30 min at 4 °C. Residual salts were removed by washing the pellet with 200 µL of 70 % ethanol. After a last centrifugation at 18,000 *g* for 5 min at 4 °C, the pellets were dried 10 min on ice and dissolved in 50 µL of water.

RNA post extraction treatments

For each RNA sample in 50 µL of water, residual DNA was removed using 2 U of RNase-free *DNase 1* according to the manufacturer instructions (DNA-free™ Kit, Ambion, Waltham, MA USA). In case RNA preparations did not satisfy purity criteria, as judged by the color of the extract and low OD_{260}:OD_{280} and OD_{260}:OD_{230} values, RNA was further purified using the "RNA Clean & Concentrator™-5 kit" from Zymo Research, Irvine, CA USA to remove potential reverse transcriptase (RT) and PCR inhibitors.

RNA quality

RNA integrity was evaluated following either non-denaturing agarose gel electrophoresis (1.2 % agarose in 0.5X TBE buffer) and ethidium bromide staining or by capillary electrophoresis using a Bionalyzer 2100 (Agilent, Santa Clara, CA USA) and a RNA Pico Chip (Agilent). RNA purity was assessed by spectrophotometry (spectrophotometer Nanodrop ND-1000; Thermo Fisher Scientific, Waltham, MA USA) at wavelengths 230, 260, 270 and 280 nm. RNA concentration was evaluated by fluorimetry using the Qubit RNA HS Assay kit and Qubit Fluorometer 2.0 (Thermo Fisher Scientific).

cDNA synthesis

500 ng of total "tree specific" RNA were used for cDNA synthesis in the presence of 4 µmol of random hexamers (10 µL final volume). The mixture was heated 5 min at 70°C and kept on ice for at least two minutes before adding 10 µL of reaction mix (4 µL of 5x buffer (Thermo Fisher Scientific); 2 µL dNTPs, 10 µmol each; 1.5 µL RNAsine at 40 $U.\mu L^{-1}$; 2 µl of 5 % Bovine Serum Albumin (BSA); 1 µL of M-MLV Reverse Transcriptase at 200 $U.\mu L^{-1}$ (Thermo Fisher Scientific); 0.5 µL RNA grade water). After 1 h at 42 °C, the enzyme was inactivated by incubating 10 min at 70°C.

For the synthesis of cDNAs from polyadenylated eukaryotic mRNAs, 500 ng of total "forest specific" RNA were used with the Mint-2 cDNA synthesis and amplification kit (Evrogen, Moscow, Russian Federation) following the manufacturer's protocol. Briefly first strand cDNA synthesis was primed using a 'CDS adapter' which anneals to the 3' poly-A tail of eukaryotic mRNA. Upon reaching the 5' end of the mRNA the Mint RT enzyme adds a few deoxycytidines (C) nucleotides at the end of all of the neo-synthesized ss-cDNAs. These extra 'Cs' are used to anchor a so-called 'PlugOligo adapter' whose complementary sequence is added by the RT enzyme at the 3'-end of all ss-cDNAs. As a result, all ss-cDNAs are bordered at both their 3' and 5' ends with a common 23 nucleotide-long M1 sequence present in both the CDS and PlugOligo adapters that is used to PCR amplify all ds-cDNAs.

PCR amplifications

Internal fragments of eight eukaryotic or bacterial protein-coding genes were amplified using published primer pairs. The EF1α primers (Rehner et al. 2005) target the housekeeping and constitutively expressed eukaryotic elongation factor 1α. The GH7 (Edwards et al. 2008) and GH5_5 (Barbi et al. 2014) primers target the two corresponding fungal families of cellulases. The GH11 primers (Barbi et al. 2014) target fungal hemicellulases (endoxylanases). The AA2 primers (Barbi et al. 2014) target fungal (basidiomycetes) class II peroxidases involved in lignin hydrolysis. *nifH* primers (Poly et al. 2001) target bacterial nitrogenase-encoding genes and the *rpoB* (Mollet et al. 1997) and *gyrB* (Barret et al. 2015) primers target the bacterial housekeeping and constitutively expressed RNA polymerase and DNA gyrase β-subunits encoding genes, respectively.

PCRs were performed in 25 µL final volume containing either 1 µL of "tree specific" single-strand cDNA or 50 ng of "forest specific" ds-cDNA, 2.5 µL of 10 x *Taq* Buffer (Thermo Fisher Scientific), 0.1 mmol dNTPs; 2 µmol of forward and reverse primers; 0.3% BSA; 1U *Taq* polymerase (Thermo Fisher Scientific); 13.4 µL water. Amplifications were performed using a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA USA) and the cycling parameters given in the publications describing each of the primer pairs. Fungal rDNA ITS region was amplified from two tree-specific samples using primers ITS1f (Gardes and Bruns 1993) and ITS4 (White et al. 1990) using the following program: initial denaturation for 4 min at 94 °C, 30 cycles of denaturation (30 s at 94 °C), annealing (45 s at 50 °C), and elongation (1 min at 72 °C), followed by a final elongation step for 5 min at 72 °C.

Sequencing and sequence analysis

PCR products were purified using the "QIAquick PCR Purification Kit" from Qiagen (Hilden, Germany) and cloned into the pCR4-TOPO vector using the "TOPO TA Cloning Kit

for Sequencing" according to the manufacturer's instructions (Invitrogen Life Technology, Karlsruhe, Germany). Where possible, five positive clones for each gene family were sequenced (Sanger sequencing; Biofidal, Vaulx en Velin, France). Nucleotide sequences were manually edited, and compared to the GenBank database using the BLASTX (protein coding sequences) or BLASTN (ITS sequences) programs. Sequences were deposited in the EMBL/GeneBank/DDJB database under accession Nos. 0000 to 0000 (submission in course).

Results and discussion

Different protocols and commercial RNA extraction kits were first tested on four decaying wood samples; two from angiosperms, *Platanus orientalis* (Plaor) and *Quercus robur* (Quero) and two from gymnosperms, *Juniperus communis* (Junco) and *Picea abies* (Picab). Two commercial kits designed to extract RNA from soil (RNA Power Soil Kit from MO BIO laboratories, Carlsbad, CA USA and ZymoBIOMICS^{TM} RNA/DNA Mini Kit (Zymo Research)) failed to extract RNA from the samples or resulted in the extraction of extremely low quantities of RNA that could not be visualized on agarose gels. The Qiagen RNeasy Plant Mini Kit (Qiagen), designed to extract RNA from live plant tissues produced inconsistent results. Although all four Qiagen RNA extracts presented satisfactory $OD_{260:280}$ ratios above 1.36, following agarose gel electrophoresis RNA extracted from Plaor and Pical appeared significantly degraded (Figure 2). We then implemented an original extraction procedure that combined steps from different protocols (Moser et al. 2004; Gambino et al. 2008), including the one by Chang et al. (1993) for lignified "live" tissues of *Pinus taeda*. This protocol was implemented with or without a detergent (2% CTAB) in the extraction buffer. Both versions resulted in the extraction of seemingly undegraded RNA from all four decaying wood samples as judged from the sharp ribosomal RNA bands resolved following both agarose (Figure 2) and capillary (Agilent bioanalyser; Supplemental Figure 1) gel electrophoresis. Both versions of the protocol differed however with respect to the final extraction yield. Unexpectedly,

inclusion of CTAB in the extraction buffer, which was expected to improve cell lysis, resulted in consistently lower RNA quantities (down to *ca* 10 folds in the case of the Junco sample) compared to the protocol from which CTAB was omitted (Supplemental Table 1). In term of purity, the RNA extracts had OD_{260}:OD_{280} and OD_{260}:OD_{230} ratios below the 2.0 ratio expected for pure RNA preparations (Table 1). These low ratios suggest the presence of contaminating molecules such as proteins, phenolics and polysaccharides. To exclude contamination by phenol used for protein denaturation and removal, we measured the OD_{260}:OD_{270} ratio after one or three successive chloroform extractions. Three successive extractions did not significantly changed this ratio close to one, thus ruling out phenol contamination during extraction.

The protocol, without CTAB in the extraction buffer and with a single chloroform extraction to remove phenol, was then tested on decaying wood samples from additional 18 gymnosperm and angiosperm tree species. Besides being representative of several of the major northern hemisphere temperate plant families with tree species (e.g., Pinaceae, Salicaceae, Fagaceae, Betulaceae), these plant species differ from each other with respect to their wood structure and chemistry (Schwarze 2007). Wood samples were collected at different stages of decomposition and several sampled were clearly affected by either white or brown rot fungal species.

Total RNA was successfully extracted from all of the 22 (18+4) decaying wood samples, as well as from composite wood samples from five forests, with yields ranging from 2.52 ± 2.4 $\mu g.g^{-1}$ (*Platanus orientalis*) to 76.33 ± 7.7 $\mu g.g^{-1}$ of wood (*Populus tremula*) for individual tree species. Variation in yield could not be related to the plant taxonomic origin nor to the type of wood (Table 1) and may simply reflect the –uncontrollable- degree of microbial colonization of the samples and the global activity of the microbial communities. Following agarose gel electrophoresis, all RNA extracts presented clear bands of ribosomal

RNA (rRNA) indicative of an absence of degradation during extraction. On agarose gels, most extracts presented an unusual and distinctive electrophoretic profile characterized by up to four rRNA bands of, sometime, similar intensities (Figure 2 and Supplemental Figures 2 and 3). When run side by side with RNA extracted from a bacterium and a fungus, these four bands co-migrated with the prokaryotic (16S and 23S) and eukaryotic (18S and 28S) cytoplasmic rRNA molecules (Figure 2) suggesting that most wood-extracted RNA are composed of equal parts of bacterial and eukaryotic RNA. Such a situation contrasts with most preparations of environmental RNA usually dominated by bacterial RNA (Bailly et al. 2007; Yadav et al. 2016).

In term of purity, most wood RNA extracts had OD_{260}:OD_{280} and OD_{260}:OD_{230} ratios below the 2.0 ratio expected for pure RNA preparations (Table 1). In cases where the RNA extract was colored and/or was characterized by an OD_{260}:OD_{280} ratio below 1.0. RNA was further purified using an RNA binding resin ("RNA Clean & Concentrator™-5 kit"). Use of this kit usually did not affect the OD_{230}:OD_{260} ratio but increased the OD_{260}:OD_{280} one.

Despite the putative presence of these contaminants, RNA samples extracted from all 22 tree species were successfully converted into single stranded cDNAs from which were amplified different gene fragments of either fungal or bacterial origin. Sequences from genes constitutively expressed by all eukaryotic (EF1α) or bacterial (*rpoB* and *gyrB*) species were amplified from all or almost all (the *gyrB* sequence could not be amplified from 4 samples) cDNA samples. Sequences from genes absent from many fungal (GH5_5, GH7, GH11 and AA2 genes) or bacterial (*nifH*) species were amplified from between 27% (GH11 and *nifH*) to 59% (AA2 peroxidases) of the samples (Fig. 1). Only in the cases of *Platanus orientalis and Castanea sativa* it was possible to amplify all five fungal genes (Fig. 1). In the case of *Picea abies* and *Platanus orientalis* cDNA from which AA2, GH11, rpoB gene fragments were amplified (Figure 1), sequencing of PCR products confirmed that the amplified sequences

indeed belonged to these different gene families. None of the different GH11 and AA2 sequences were identical to homologous sequences deposited in databases (percentages of identity to known sequences being below 92%). Furthermore, for these two wood samples, amplification and sequencing from cDNA of the ITS fungal barcode sequence (Schoch et al. 2012) identified several taxa typically associated with decaying wood such as the Agaricomycetes *Mycena haematopoda* and *Brevicellicium olivascens*.

Failure to amplify fungal lignocellulolytic gene transcripts from single-stranded cDNAs directly synthesized from environmental (soil) RNA has been reported several times in the literature (see Weber and Kuske, 2011) Absence of amplification can result from the genuine absence of the corresponding transcripts in metatranscriptomes or to their extreme rarity. To circumvent this problem, in the literature (Luis et al. 2005; Bailly et al. 2007; Edwards et al. 2008; Damon et al. 2012), the corresponding sequences are usually amplified from "pre-amplified" double-stranded (ds) eukaryotic cDNAs obtained using the "template switching protocol" (Matz et al. 1999) as implemented by commercial kits, such as the "Mint" or "SMART" ones (from Evrogen and Clontech Laboratories, Mountain View, CA USA respectively). ds-cDNA synthesis and amplification was achieved successfully for all five RNA preparations from "forest wood samples" (Figure 3). PCR amplification of GH7, GH11, EF1α eukaryotic gene fragments was obtained for all five cDNA preparations. By contrast, amplification of these gene fragments was successful in 27%, 32% and 100% respectively of the 22 single stranded cDNA preparation obtained from the RNA extracted from single tree species. However, in the case of the GH5_5 and AA2 gene fragments amplification was only successful for 80% (4/5) and 60% (3/5) of the five ds-cDNA samples, respectively.

Conclusions

We developed a versatile RNA extraction and purification protocol from decaying wood samples that functions on a wide variety of woods, irrespective of their original physical

structure, chemical composition and state of decomposition. We therefore anticipate that this protocol could be implemented successfully on wood samples from other tree species.

All extracted RNA could be converted into cDNA using either a conventional reverse transcription approach or the template switching one. From these cDNAs a number of eukaryotic and bacterial genes were amplified, including genes important for wood degradation such as fungal genes encoding enzymes participating to lignocellulose degradation or bacterial *nifH* genes that could drive organic nitrogen entry in this nitrogen-limited ecosystem. Furthermore, the successful conversion of wood-extracted RNA into cDNA represents an essential step towards a metatranscriptomic analysis of wood degradation through either the systematic sequencing of all mRNAs after removal of rRNA (McGrath et al. 2008) or the targeted sequencing of specific enzyme-coding gene families involved in lignocellulose degradation (Kellner et al. 2014; Barbi et al. 2014; Baldrian and Lòpez-Mondéjar 2014). Finally, decaying wood cDNAs could also represent a rich source of enzymes relevant to various industrial processes such as biomass treatment, second generation biofuel production and biorefinery. These cDNAs could be screened for such enzymes following cloning and functional expression in a suitable microbial host cell (Kellner et al. 2011; Bragalini et al. 2014; Marmeisse et al. 2017).

In the course of this study we also observed that decaying wood RNA is seemingly composed of eukaryotic and bacterial RNA in similar proportions. This unusual ratio, as bacteria "over dominate" most natural microbial ecosystems such as soil (Plassart et al. 2012), could promote decaying wood as a model ecosystem to dissect the functional interactions between bacteria and fungi and their relative and complementary contributions to key ecosystem processes such as biomass degradation.

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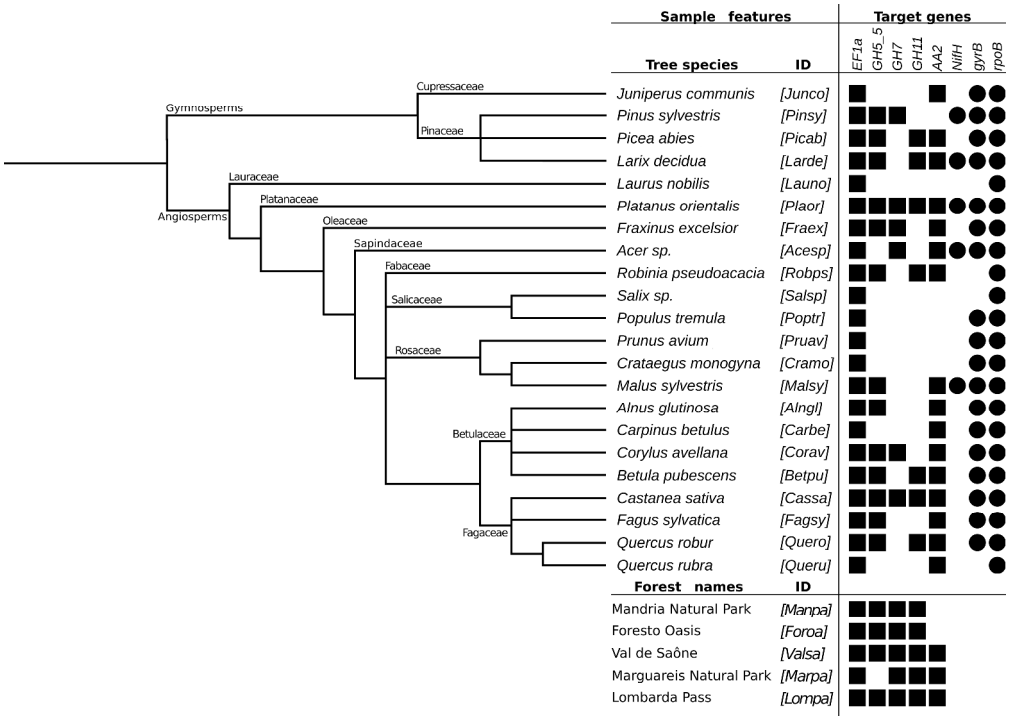
Legends of the Figures

Fig. 1. Taxonomic position of the tree species from which decaying wood RNA was extracted and outcome of the PCRs performed on single-stranded (tree-specific RNA extracts) or double-stranded cDNA (forest-specific extracts) to amplify five eukaryote-specific (EFIα, GH5_5, GH7, GH11, AA2) and three bacteria-specific (*nifH*, *gyrB*, *rpoB*) gene fragments. Black squares and dots indicate successful PCR amplifications for the eukaryote and bacteria-specific genes, respectively. Bacterial genes were not amplified from "forest-specific" cDNAs that were synthesized from eukaryotic poly-A mRNA.

Fig. 2. Specific electrophoretic profile of four selected decaying wood RNA extracts. RNA extracts were size fractionated by electrophoresis on non-denaturing agarose gels stained with ethidium bromide. Each extract is characterized by prominent ribosomal RNA fragments that co-migrate with bacterial (Bc, RNA extracted from the bacterium *Escherichia coli*) and eukaryotic (Eu, RNA extracted from the fungus *Tulasnella calospora*) ribosomal RNA. Eukaryotic 28S (a) and 18S (b) and bacterial 23S (c) and 16S (d) ribosomal RNA. For each sample a comparison is showed between RNA extracted using the "S, C and Q" protocols. (S), standard protocol described in this article. (C) Standard protocol described in this article with the addition of 2% CTAB in the extraction buffer. (Q) RNeasy Plant Mini Kit from Qiagen. The 1 Kbp DNA Ladder from EuroClone (Pero, Italy) was used as molecular weight marker (L).

Fig. 3. Conversion of eukaryotic poly-A mRNA extracted from a forest-specific decaying wood sample (A) into large quantities (µg amounts) of double-stranded cDNA (B) that is used as matrix for the PCR amplification of different eukaryote-specific gene fragments (C). (A) The Valsa decaying wood RNA extract presents the four characteristic ribosomal RNA bands

(arrowheads) observed in other wood extracts (Fig. 2). 1 Kbp Gene Ruler by Thermo Fisher Scientific was used as ladder in the first gel lane. (B) Double-stranded cDNAs synthesized using the template switching protocol (as implemented in the Mint kit) and further amplified by PCR range in size from *ca* 100 bp to more than 3 kbp. 1 Kbp DNA Ladder by EuroClone was used in (B) and Gel Pilot 100 bp Plus Ladder by Qiagen was used in (C).



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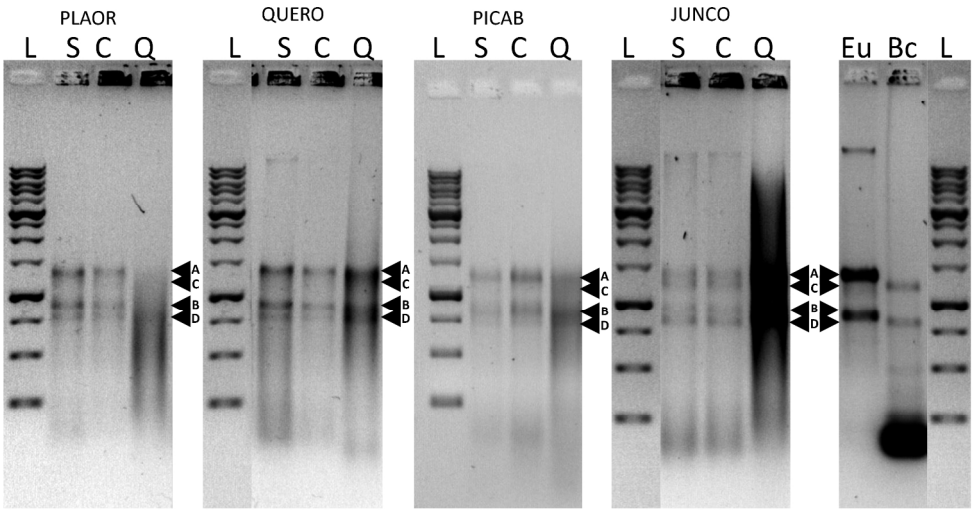

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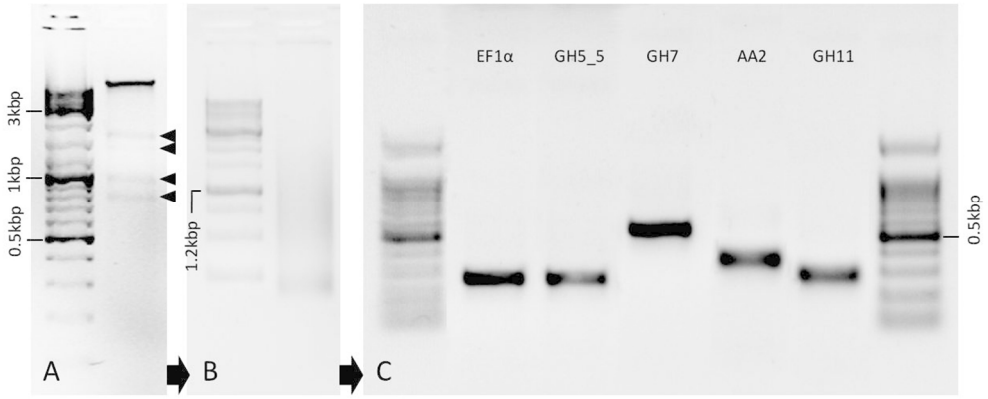

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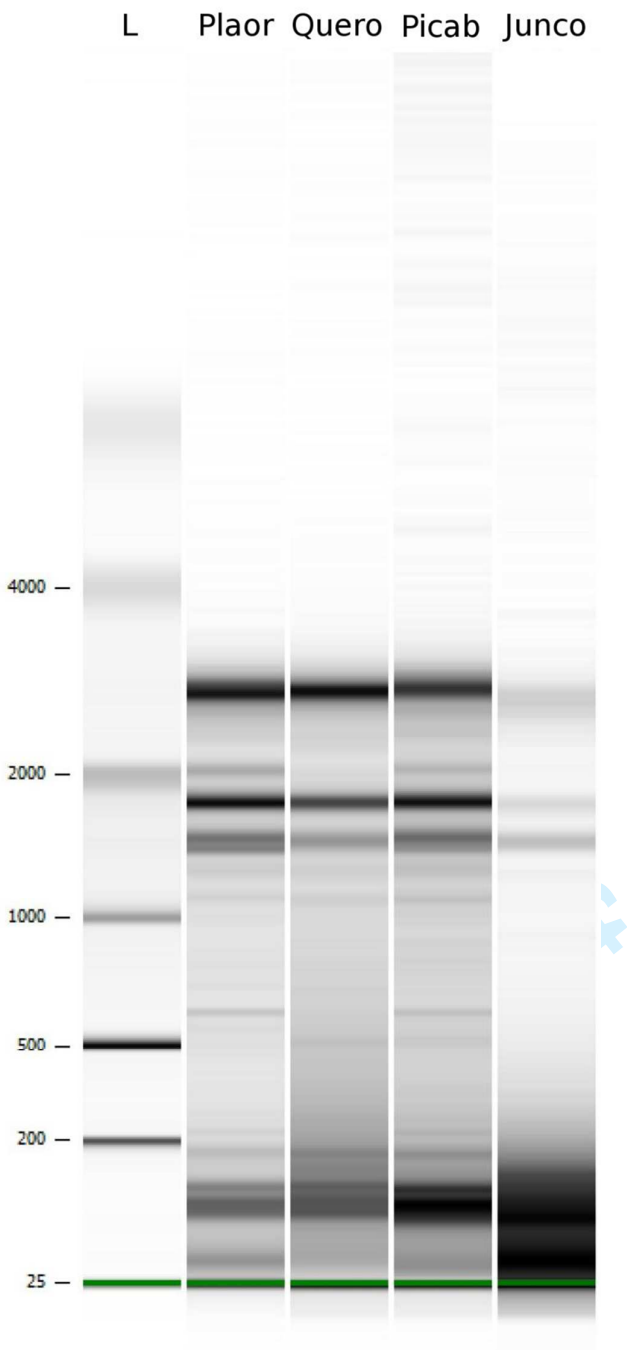
Plant species	Sampling date	Location	Coordinates	Wood moisture [%]	RNA yield [µg/100 mg]	260:280	260:230	260:270
Acer sp.	10/25/2015	Coubon (FRA)	44° 99'N, 3° 90'E	68.96 ± 9.43	1.03 ± 0.33*	1.38 ± 0.10	0.65 ± 0.08	1.03 ± 0.11
Alnus glutinosa	10/25/2015	Coubon (FRA)	44° 99'N, 3° 90'E	62.23 ± 0.57	1.41 ± 0.55*	1.62 ± 0.27	0.72 ± 0.08	1.13 ± 0.1
Betula pubescens	11/3/2015	Venaria Reale (ITA)	45° 18'N, 7° 55'E	44.1 ± 0.83	2.36 ± 1.5	1.61 ± 0.33	0.70 ± 0.11	1.12 ± 0.1
Carpinus betulus	11/3/2015	Venaria Reale (ITA)	45° 18'N, 7° 55'E	47.22 ± 1.98	2.11 ± 1.45	1.60 ± 0.27	0.81 ± 0.35	1.15 ± 0.12
Castanea sativa	11/8/2015	Entracque (ITA)	44° 23'N, 7° 42'E	37.06 ± 13.39	0.59 ± 0.36*	1.50 ± 0.23	0.65 ± 0.36	1.12 ± 0.14
Corylus avellana	11/3/2015	Venaria Reale (ITA)	45° 18'N, 7° 55'E	54.34 ± 10.17	1.09 ± 0.91*	1.48 ± 0.18	0.80 ± 0.24	1.11 ± 0.13
Crataegus monogyna	11/3/2015	Venaria Reale (ITA)	45° 18'N, 7° 55'E	26.13 ± 0.56	0.8 ± 0.2*	1.38 ± 0.15	0.72 ± 0.22	1.09 ± 0.1
Fagus sylvatica	11/3/2015	Venaria Reale (ITA)	45° 18'N, 7° 55'E	46.33 ± 0.68	1.42 ± 2.46	1.53 ± 0.21	1.13 ± 0.30	0.7 ± 0.1
Fraxinus excelsior	10/24/2015	Entracque (ITA)	44° 23'N, 7° 42'E	30.68 ± 5.8	1.21 ± 0.59*	1.64 ± 0.26	1.01 ± 0.29	1.12 ± 0.12
Juniperus communis	10/24/2015	Entracque (ITA)	44° 23'N, 7° 42'E	40.5 ± 0.68	7.633 ± 0.77*	1.26 ± 0.02	0.77 ± 0.02	0.94 ± 0.11
Larix decidua	6/18/2015	Vinadio (ITA)	44° 20'N, 7° 14'E	38.72 ± 4.38	0.97 ± 0.24*	0.96 ± 0.01	0.30 ± 0.00	1.26 ± 0.12
Laurus nobilis	12/8/2015	S. Margherita Ligure (ITA)	44° 33'N, 9° 22'E	47.66 ± 0.41	2.13 ± 0.54	1.37 ± 0.08	0.90 ± 0.30	1.07 ± 0.1
Malus sylvestris	10/24/2015	Entracque (ITA)	44° 23'N, 7° 42'E	50.99 ± 0.91	1.64 ± 1.46	1.46 ± 0.12	1.03 ± 0.19	1.13 ± 0.15
Picea abies	10/24/2015	Entracque (ITA)	44° 23'N, 7° 42'E	67.74 ± 0.79	2.24 ± 2.32*	1.50 ± 0.23	0.68 ± 0.08	0.98 ± 0.14
Pinus sylvestris	10/25/2015	Solignac sur Loire (FRA)	44° 58'N, 3° 53'E	51.33 ± 1.07	2.54 ± 2.73	1.4 ± 0.07	0.63 ± 0.03	1.11 ± 0.11
Platanus orientalis	11/5/2015	Torino (ITA)	45° 05'N, 7° 68'E	71.74 ± 0.56	0.252 ± 0.24*	1.54 ± 0.05	1.02 ± 0.06	1.03 ± 0.17
Populus tremula	10/25/2015	Solignac sur Loire (FRA)	44° 58'N, 3° 53'E	43.3 ± 1.38	2.59 ± 0.66	1.34 ± 0.02	0.75 ± 0.06	1.12 ± 0.12
Prunus avium	10/24/2015	Entracque (ITA)	44° 23'N, 7° 42'E	62.59 ± 1.0	0.95 ± 0.39*	1.44 ± 0.04	0.87 ± 0.13	1.08 ± 0.1
Quercus robur	11/3/2015	Venaria Reale (ITA)	45° 18'N, 7° 55'E	29.44 ± 3.87	0.796 ± 0.83	1.47 ± 0.02	0.76 ± 0.13	1.52 ± 0.11

Quercus ruber	11/3/2015	Venaria Reale (ITA)	45° 18'N, 7° 55'E	69.14 ± 0.25	0.41 ± 0.17*	1.37 ± 0.20	0.55 ± 0.16	1 ± 0.1
Robinia pseudoacacia	11/3/2015	Venaria Reale (ITA)	45° 18'N, 7° 55'E	65.94 ± 1.49	0.99 ± 0.95*	1.36 ± 0.17	0.76 ± 0.26	0.72 ± 0.18
Salix sp.	10/24/2015	Entracque (ITA)	44° 23'N, 7° 42'E	37.31 ± 3.35	1.77 ± 1.21	1.39 ± 0.06	0.78 ± 0.07	1.10 ± 0.1
Fagus sylvatica; Corylus avellana; Quercus robur; Carpinus betulus	2/28/2015	Venaria Reale (ITA)	45° 18'N, 7° 55'E	54.81 ± 3.87	1.30 ± 0.29	1.35 ± 0.01	0.76 ± 0.06	1.4 ± 0.09
Quercus pubescens, Prunus avium; Cotynus coggygria	4/1/2015	Bussoleno (ITA)	45° 14'N, 7° 10'E	57.98 ± 1.03	4.42 ± 1.07	1.18 ± 0.02	0.66 ± 0.02	1.25 ± 0.14
Acer campestre; Fraxinus excelsior	6/10/2015	Laives (FRA)	46° 67'N, 4° 82'E	59.36 ± 5.88	0.49 ± 0.044*	1.77 ± 0.14	1.08 ± 0.07	1.04 ± 0.14
Abies alba; Fagus sylvatica; Laburnum alpinum	6/15/2015	Chiusa di Pesio (ITA)	44° 20'N, 7° 68'E	50.16 ± 11.365	4.57 ± 2.10	1.44 ± 0.05	0.79 ± 0.05	1.14 ± 0.12
Larix decidua	6/18/2015	Vinadio (ITA)	44° 20'N, 7° 14'E	38.72 ± 4.38	0.97 ± 0.24*	0.96 ± 0.01	0.30 ± 0.00	1.15 ± 0.12

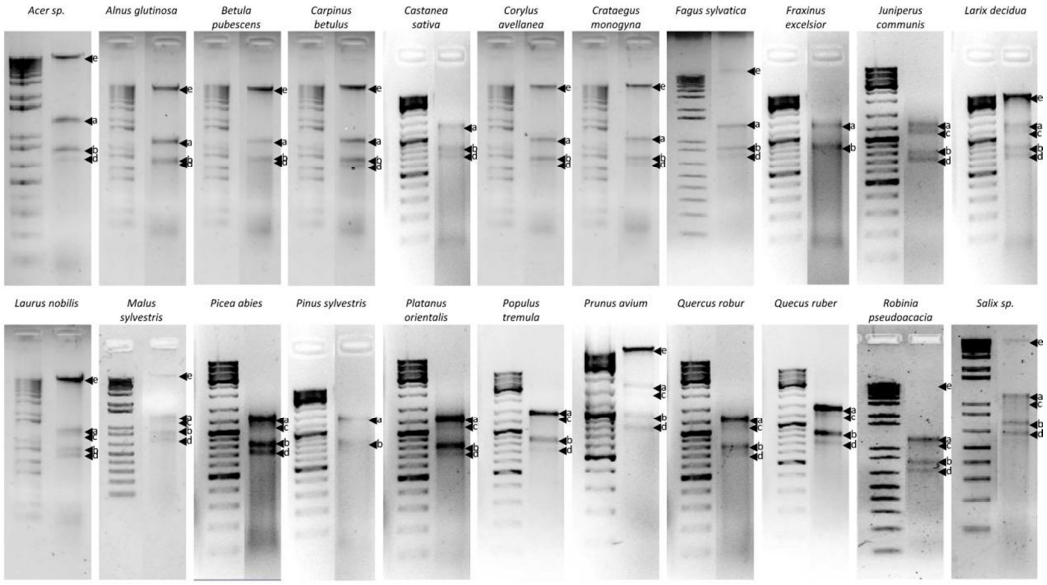
Table 1. Origin and characteristics of the decaying wood samples and of the corresponding RNA extracts with the standard method described in the paper.

(a) "tree-specific" wood samples; (b) "forest-specific" samples; (*) RNA samples for which a final purification step using the “RNA Clean & Concentrator™-5 kit” (Zymo Research) was performed; OD_{230}, $_{260,\ 270}$ and $_{280}$, absorbance of the RNA samples at 230, 260, 270 and 280 nm respectively; FRA, France; ITA, Italy. Manpa, "Mandria Natural Park lowland forest"; Foroa, "Foresto Oasis xerothermic forest"; Valsa, "Val de Saône alluvial forest"; Marpa, "Marguareis Natural Park mountain forest"; Lompa, "Lombarda Pass alpine larch forest".



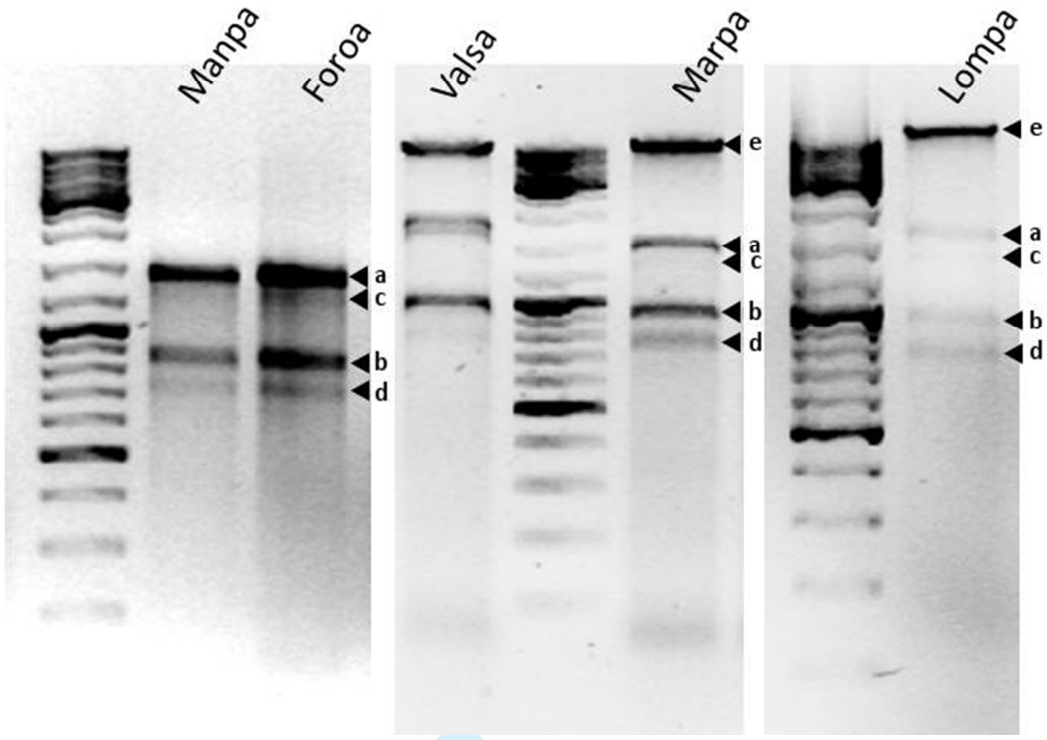
Supplemental Figure 1.

Separation of four selected decaying wood RNA extracts (see abbreviations in Figure 1), obtained using the standard protocol described in the manuscript by capillary electrophoresis (Bionalyzer 2100 and a RNA Pico Chip (Agilent)) Occurrence of sharp bands of ribosomal RNA suggested an absence of RNA degradation during the extraction procedure.



Supplemental Figure 2.

Specific electrophoretic profile of the 22 tree-specific wood RNA extracts. RNA extracts were size fractionated by electrophoresis on non-denaturing agarose gels stained with ethidium bromide. Most extracts are characterized by four prominent ribosomal RNA fragments that co-migrate with eukaryotic 28S (a) and 18S (b) and bacterial 23S (c) and 16S (d) ribosomal RNA. (e), metagenomic DNA that could be removed using a DNase I treatment. 1 Kbp Gene Ruler by Thermo Fisher Scientific was used as ladder for *C. sativa, F. excelsior, J. communis, L. decidua, P. abies, P. sylevstris, P. orientalis*, *P. tremula, P. avium, Q. robur, Q. ruber* samples. In all the others samples 1 kbp Plus Ladder by Thermo Fisher Scientific was used.



Supplemental Figure 3.

Specific electrophoretic profile of the five forest-specific wood RNA extracts. RNA extracts were size fractionated by electrophoresis on non-denaturing agarose gels stained with ethidium bromide. Most extracts are characterized by four prominent ribosomal RNA fragments that co-migrate with eukaryotic 28S (a) and 18S (b) and bacterial 23S (c) and 16S (d) ribosomal RNA. (e), metagenomic DNA that could be removed using a DNase I treatment. 1 Kbp Gene Ruler by Thermo Fisher Scientific was used as ladder.

	Buffer type			
Sample name	Standard	CTAB	p value	
Plaor	0.252 ± 0.238	0.064 ± 0.015	0.835	
Quero	0.796 ± 0.826	0.125 ± 0.015	0.492	
Picab	2.45 ± 2.316	0.093 ± 0.016	0.040	*
Junco	7.633 ± 7.283	0.869 ± 0.775	0.00001	**

Supplemental Table 1.

Addition of CTAB in the RNA extraction buffer reduces the extraction yield (expressed in µg of RNA per 100 mg of wood ± standard deviation). RNA were extracted using either the standard protocol (Standard) without CTAB in the extraction buffer or with a modified version (CTAB) in which 2% CTAB were added to the buffer. Difference were compared using a simple χ^2 test for each of wood samples. *, $p < 0.05$; **, $p < 0.01$.

	260:270 mean value ± st.dev.		
Sample name	1 Chlor. Ext.	3 Chlor. Ext.	p value
Plaor	0.945 ± 0.095	0.998 ± 0.112	1.000
Quero	0.978 ± 0.115	1.008 ± 0.140	1.000
Picab	1.038 ± 0.334	0.940 ± 0.175	0.987
Junco	1.525 ± 0.824	1.153 ± 0.104	0.807

Supplemental Table 2.

Effect of the number of chloroform extractions on RNA purity. $OD_{260:270}$ ratios were calculated for RNA extracts obtained using one single chloroform extraction and for RNA extracts obtained using three consecutive chloroform extractions. The χ^2 test does not highlight any significant difference.