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Host plant development, water level and water parameters shape *Phragmites australis*-associated oomycete communities and determine reed pathogen dynamics in a large lake

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Abstract

In a 3 year study, we analysed the population dynamics of the reed pathogen *Pythium phragmitis* and other reed associated oomycetes colonizing fresh and dried reed leaves in the littoral zone of a large lake. Oomycete communities derived from internal transcribed spacer clone libraries were clearly differentiated accord ing to substrate and seasonal influences. In fresh leaves, diverse communities consisting of *P. phragmitis* and other reed associated pathogens were generally dominant. *Pythium phragmitis* populations peaked in spring with the emergence of young reed shoots, and in autumn after extreme flooding events. In summer it decreased with falling water levels, changing water chemistry and rising tempera tures. Another *Pythium* species was also highly abundant in fresh leaves through out the year and might represent a new, as yet uncultured reed pathogen. In dried leaves, reed pathogens were rarely detected, whereas saprophytic species occurred abundantly during all seasons. Saprophyte communities were less diverse, less temperature sensitive and independent of reed development. In general, our results provide evidence for the occurrence of highly specialized sets of reed associated oomycetes in a natural reed ecosystem. Quantitative analyses (clone abundances and quantitative real time PCR) revealed that the reed pathogen *P. phragmitis* is particularly affected by changing water levels, water chemistry and the stage of reed development.

Introduction

Oomycetes (*Peronosporomycetes*), in particular species of the genera *Pythium* and *Phytophthora* are well known as devas tating pathogens of a large array of agricultural crop plants. More recently, the investigation of largely unexplored non agricultural ecosystems and niches showed several pre viously undescribed oomycete species, some of which were shown to be involved in severe plant diseases like 'sudden oak death' caused by *Phytophthora ramorum* (Rizzo *et al*., 2002), the death of alders caused by *Phytophthora alni* (Brasier *et al*., 2004), or European oak and beech decline caused by several *Phytophthora* species (Jung *et al*., 1999; Jung *et al*., 2005). Aside from the major plant pathogen genus *Phytophthora*, *Pythium* species are also considered ecologically significant in natural situations, as they might be capable of determining plant community composition via seedling infection in the field (Packer & Clay, 2000).

Environmental studies focusing on *Phytophthora* com munities occurring in natural or semi natural ecosystems revealed that major plant pathogens often appear to be embedded in diverse communities of more or less closely related species. Several of these studies have suggested possible environmental factors that influence the structure of such communities and might explain the emergence of a particular pathogen (Balcì & Halmschlager, 2003; Jung *et al*., 2005; Jung, 2009; Moralejo *et al*., 2008). In the genus *Pythium* and other oomycetes, culture based studies have delivered detailed data on possible correlations between community fluctuations in water and soils and environ mental factors to explain the changes observed (van der Plaats Niterink, 1975; Misra, 1982; Ali Shtayeh *et al*., 1986; Hardman & Dick, 1987; Abdelzaher *et al*., 1995; Marano *et al*., 2008). However, so far, no particular attention has been given to the competition between saprophytic and plant pathogenic species and the potential impact of

environmental influences on *Pythium* mediated plant diseases in natural ecosystems.

Pythium phragmitis is a newly described water borne pathogen of common reed (*Phragmites australis*). It was consistently isolated from flooded soils and water in reed stands at Lake Constance, Germany, a large prealpine fresh water lake. It proved to be highly aggressive towards seedlings as well as mature leaves of reed. Because of its potential as an aggressive leaf pathogen, it has been postulated that *P. phragmitis* might play a significant role in the aetiology of reed decline, and thus pose a major threat to Lake Constance and other European reed stands (Nechwatal *et al.*, 2005, 2008b). A recent qualitative account of oomycete communities in reed stands of Lake Constance provided evidence that *P. phragmitis*, too, is associated with a diverse community of several other reed associated *Pythium* species, competing for similar niches (Nechwatal *et al.*, 2008a). Specific adaptations to particular environmental conditions would most likely account for seasonal changes in the composition of the reed associated oomycete community at a given site, and would explain the potential competitive advantage of a pathogen emerging from a diverse community.

Here, we quantitatively analyse the occurrence and dynamics of oomycete communities, including the reed pathogen *P. phragmitis* in an undisturbed reed stand in the littoral zone of Lake Constance, and point out factors that modify these communities during the growing season. We applied molecular community profiling methods with a focus on potential oomycete pathogens in a natural, nonagricultural ecosystem. We used oomycete clone libraries and quantitative real time PCR (qPCR) to test whether these two methods would provide similar estimates of oomycete occurrence and activity in the field. The survey was conducted during three seasons and over a total of three consecutive years, and addressed the following questions: (1) How diverse are oomycete communities competing with *P. phragmitis* in a littoral reed belt? (2) Can we detect seasonal and structural dynamics of reed associated oomycete communities? (3) What are the environmental factors influencing these dynamics?

Materials and methods

Reed bait experiment and sampling site

For the analysis of oomycete communities associated with live and decaying reed under flooded field conditions, we used leaf discs (18 mm diameter) punched from mature field site reed using a cork borer on three occasions in May/June (week 21 25), August (31 35) and October (39 43) during three consecutive years (2004 2006). Half of the discs were brought to the laboratory and dried at 60 °C for 24 h to serve as a model for dead leaves. The other half was used immediately, as a substitute for live leaves. Ten of these either 'dried' or 'fresh' discs each were placed in sealed nylon mesh bags (5 × 5 cm, pore size 125 μm) and floated in the littoral zone of Lake Constance (47°41.830′N, 009° 11.496′E), *c.* 10 m off shore, close to a declining reed stand, by tying them to a pole standing in (depending on season) 0.5 1.2 m deep water. At each occasion, eight bags with fresh and eight bags with dried reed discs were used to allow sampling of two dried and two fresh bags each after 1 4 weeks, respectively. Water temperature, chemistry and water level data at each sampling date were obtained from local environmental agencies (Fig. 1; Supporting Information, Table S1).

Escherichia coli oomycete libraries

After retrieval, leaf discs were washed and fresh weights were recorded. Ten dried or fresh leaves each were pooled, ground in liquid nitrogen and DNA was extracted from the equivalent of the mean disc fresh weight using the DNeasy Plant Mini Kit (Qiagen). In order to analyse mature, reed associated oomycete communities, we used the baits after 4 weeks of incubation in the lake to generate 18 *E. coli* oomycete libraries (two bait types × three seasons × 3 years). Oomycete specific PCR was performed with primer internal transcribed spacer (ITS) 4Oo and a modified primer ITS5 (Table S2, 0.2 μM each), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.04 U μL^{-1} Taq DNA polymerase (Fermentas) and 1 μL DNA. The cycling conditions were 94 °C 150 s, 34 cycles at 94 °C 30 s, 54 °C 30 s, 72 °C 60 s and final extension (72 °C) of 10 min. To avoid possible individual primer bias towards unbalanced DNA amplification (Jumpponen, 2007), additional testing for consistent amplification of various oomycete species and genera was carried out. The primer pair was shown to equally amplify DNAs of all major groups of oomycetes in these tests (Table S3). After purification of reed bait derived PCR fragments (ENZA Cycle Pure Kit, Peqlab) the PCR Cloning Kit (Qiagen) was used for ligation and transformation according to the manufacturer's instructions. Restriction fragment length polymorphism (RFLP) analysis of inserts was performed with colony PCR products from positive clones (using the above primers and conditions) to reveal RFLP types [operational taxonomic units (OTUs)]. PCR products (10 μL) were digested with 1 U of enzyme according to the manufacturer's instructions. Enzymes used were AluI, HinfI, TaqI, Bsp119I (Fermentas) and Hpy188I (New England Biolabs). After separation of fragments on 3% agarose gels, computer aided evaluation of fragment lengths was carried out using QUANTITYONE 4.5.2 (BioRad). All tentative OTUs were sequenced at least two times and from different clone libraries. Sequencing was conducted using plasmid specific M13 primers (Table S2). To prevent diversity overestimates through PCR generated

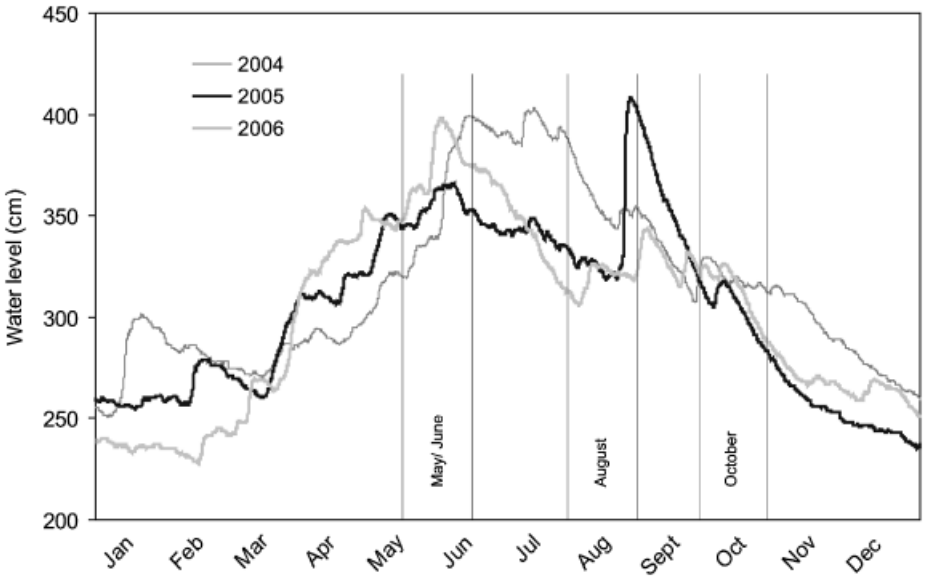

Fig. 1. Interannual variation of the 2004 2006 water level progression (gauge Konstanz); sampling dates (May/June, August, October) are highlighted. Data from LUBW, http://www.hvz.baden wuerttemberg.de

Table 1. Genus and clade affiliation, closest BLAST hit and GenBank accession numbers (ITS sequence) of oomycete OTUs from reed sites detected in this study, as revealed by *Escherichia coli* libraries

Taxon/OTU	Genus/*Pythium* clade*	Best BLAST hit	Identity (%)	ITS GenBank accession no.
Type A	B1e	*P. arrhenomanes*	94	DQ887064
Type B	B1e	*P. phragmitis*	100	DQ887065
Type C	B1d	*P. graminicola*	96	DQ889212
Type D	B1a	*P. catenulatum*	100	DQ889214
Type E	B2	*P. dissotocum*	97	DQ889215
Type F	B2	*P. aquatile*	97	DQ889216
Type G	Unknown	*Pythium*		DQ889217
Type H	Unknown	*Pythium*		DQ889218
Type I	*Aphanomyces*	*A. cladogamus*	94	DQ889219
Type J	*Aphanomyces*	*A. astaci*	84	DQ889220
Type K	A	*P. porphyrae*	99	EU136622
Type L	*Aphanomyces*	*A. astaci*	84	EU136623
Type M	B1d	*P. kashmirense*	97	EU136624
Type N	B1d	*P. inflatum*	96	DQ889213

**Pythium* clades after Lévesque & de Cock (2004).

cloning and sequencing errors (Cline *et al.*, 1996; Acinas *et al.*, 2005), we defined an OTU threshold level of 99% sequence similarity, resulting in single ambiguous position in some types. Furthermore, to prevent artefact sequences from being included in the analysis, only OTUs with $>$ 96% sequence similarities to GenBank entries, or occurring more than once and in more than one clone library, were counted as positive oomycete clones. Detection of chimeric se quences was carried out by independent BLAST searches for ITS1 and ITS2 at GenBank and using the CHIMERA CHECK software of the Ribosomal Database Project (RDP, version 2.7, Cole *et al.*, 2003). Sequences of each of the defined OTUs were submitted to GenBank (Table 1). Completeness of sampling within each *E. coli* library was estimated by saturation curves and further tested by Chao half and first order Jacknife using Species Diversity and Richness (SDR) v. 3.03 (Pisces Conservation Ltd).

Community comparison and data analysis

To compare species richness and diversity in clone derived oomycete communities from different seasons (six libraries for each season) and substrates (nine libraries for each substrate), the Shannon Wiener and the Simpson index were calculated and compared using SDR. Average sequence divergence (θ_π) for clone derived communities was calcu lated using ARLEQUIN 2.00 (Schneider *et al.*, 2000). Aligned complete ITS1, 5.8S and partial ITS2 sequences for each

taxon obtained during this study and their relative frequencies were used for analysis. The degree of differentiation between communities from fresh and dried leaf disc derived clones or different seasons was estimated by computing F_{st} statistics using ARLEQUIN. Methods for molecular diversity calculation have been described by, for example, Mortiz & Faith (1998), Martin (2002) and Jones & Martin (2006). Correlations between environmental factors (water temperature) and species diversity were calculated with GRAPHPAD PRISM 3.00 (GraphPad Software). Canonical correspondence analysis (CCA) was used to analyse the influence of substrate and seasonal variations in water temperature, water chemistry (NO_3, NH_4, Ca, Mg and O_2) and flooding depth on the oomycete libraries. According to mean water level course, periods of rising water levels in May were labelled '1', while August and October were labelled '0' in this analysis. A Monte Carlo permutation test based on 999 random permutations was used to test the null hypothesis that oomycete communities were unrelated to environmental influences. Analyses were performed using PC ORD 5.10.

qPCR of *P. phragmitis* in reed baits

The successive colonization of reed baits during 1–4 weeks of exposition, in different substrates, at different seasons and over three consecutive years was evaluated by qPCR. For the detection of the highly abundant oomycete *P. phragmitis* (OTU type B) we used specific ITS primers Phrag fw and Grp3 rev (Table S2) that were screened for specificity using DNA extracts of several oomycete and fungal species. The primers were shown to be highly specific, and did not amplify DNAs of several closely related species (Table S3). We used the QuantiTect SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions with 0.5 μM primer concentration and 1 μL of template DNA. qPCR was conducted on a Smart Cycler (Cepheid) using the following program: 95 °C for 10 min, 45 cycles 95 °C for 20 s, 61 °C for 20 s and 72 °C for 20 s. A standard curve was generated to quantify the 232 bp *P. phragmitis* rDNA product in comparison with the total DNA of an average leaf disc of the respective sampling. DNA was extracted from *P. phragmitis* mycelia growing on V8 agar using the DNeasy Plant Mini Kit (Qiagen). A standard curve ($y = -3.266x + 31.345$; $r^2 = 0.96$) was inferred from three independent dilution series. Amplification occurred down to 0.3 pg of template DNA. Reed leaf discs before exposition in the littoral water were used as negative controls at each sampling date. To determine whether the presence of plant DNA and secondary metabolites affect detection, experiments were performed using reed DNA amended with *P. phragmitis* DNA. No effect of plant material on DNA amplification could be detected. We used qPCR to verify the clone library results by comparing the relative abundance of *P. phragmitis* per library (mean of nine fresh and dried libraries for substrate comparison, and the three fresh substrate libraries for season comparison, respectively) to the percentage of *P. phragmitis* rDNA per leaf disc of the 4th week baits. For comparison of *P. phragmitis* abundance among different substrates, Mann Whitney's test, and among different seasons Kruskal Wallis test was performed using GRAPHPAD PRISM v. 3.00.

Results

Molecular diversity of oomycetes in reed baits

We constructed 18 oomycete clone libraries after PCR with total DNA from dried and fresh reed leaf discs. In total, we screened 1105 positive oomycete clones, resulting in 14 different OTUs (Table 1, Fig. 2). In each library, one to three taxa were predominant, while others appeared rather sporadically (see also Table S4). The completeness of sampling within each library was proven by species richness estimators, revealing a complete or nearly complete saturation of taxa within the sampled libraries (Table 2). Sequencing of the cloned rDNA ITS fragments revealed that a majority of 11 OTUs were *Pythium* spp., mostly from clade A and B as defined by Lévesque & de Cock (2004), comprising species with filamentous sporangia, and three were *Aphanomyces* spp. (Table 1). BLAST searches confirmed that only three of the OTUs could unambiguously (≥99% sequence identity) be assigned to already known species. Three OTUs had sequence similarities to GenBank entries of 97%, the remaining had < 97% identity to any published sequence. Four OTUs could be assigned to a genus level only: two *Aphanomyces* spp. with 84% similarity to GenBank entries and two *Pythium* spp. matching only in the 5.8S rRNA gene (Table 1).

Impact of substrate on oomycete community structure

Oomycete communities were significantly correlated with substrate type, as demonstrated by CCA. Substrate type (corresponding to axis 1) explained 27% of the observed variation, and fresh and dried communities grouped opposite in the plot (Fig. 3). The OTU type B, equivalent to the reed pathogen *P. phragmitis*, in particular, showed a significant association with fresh plant material that could be confirmed by qPCR. In addition, another OTU (type C, closely related to *Pythium graminicola*) was highly abundant only in fresh leaf discs (Fig. 2). Both are members of the grass pathogen cluster B1e/d as defined by Lévesque & de Cock (2004), which was dominant in fresh leaf discs (types A, B, C, M and N = 70.5% mean abundance in all fresh substrate libraries). Furthermore, OTU type I (closely related to *Aphanomyces cladogamus*) and OTU type G

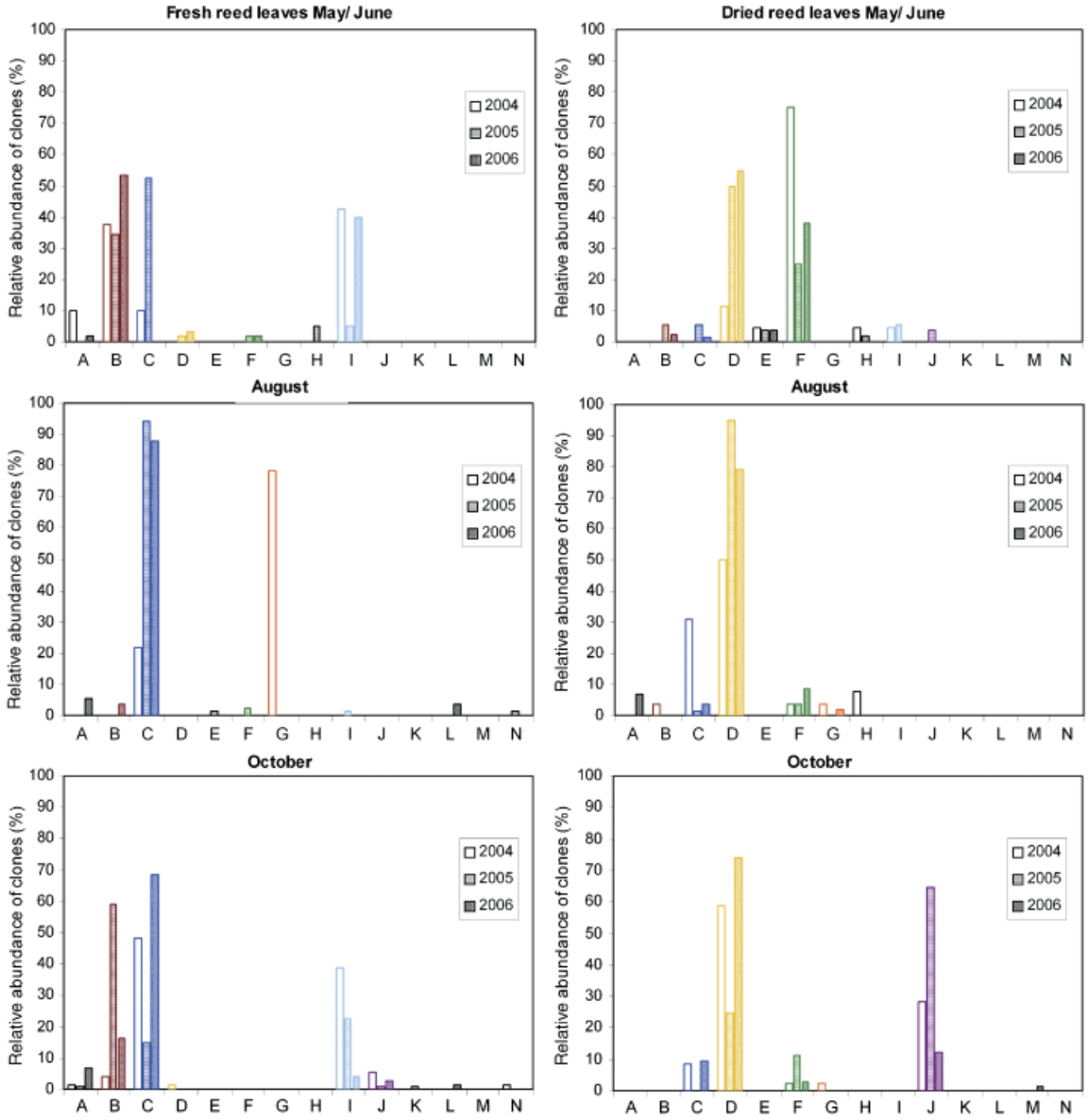

Fig. 2. Relative abundance (%) of oomycete taxa in 18 *Escherichia coli* libraries (two substrates × three seasons × 3 years). For individual data see Table S4.

(*Pythium* sp.) were associated with fresh leaf material (Fig. 2). In dried leaf discs, mainly *Pythium* OTUs belonging to clade B1a and B2 (Lévesque & de Cock, 2004) were detected. Here, the predominating taxon was OTU type D (=*Pythium catenulatum*). Two more OTUs, close to *Pythium aquatile* (F) or *Aphanomyces* sp. (J), respectively, were predominantly found in dried leaf discs (Fig. 2).

Species diversity was significantly higher in fresh reed baits as compared with dried material (Table 2). Sequence divergence (θ_π) of clone communities too was significantly higher in fresh compared with dried leaf discs, as indicated by F_{st} statistics (Table 2). Considering the colonization of both substrate types in relation to continuously available water temperature data, a significant negative correlation could be observed between water temperature and commu nity diversity data (as expressed by the Simpson index) in fresh reed baits (Fig. 4a), while no such correlation could be found for dried baits (Fig. 4b).

Impact of season and year on oomycete community structure

The oomycete community structure of littoral reed stands correlated with the seasonally fluctuating factors tempera ture, water chemistry and flooding, represented by correla tion with axis 2 in the CCA ordination (explaining 16% of

Table 2. Comparison of diversity indices and estimated species richness in clone derived oomycete communities from different seasons and substrates

	Species diversity						Molecular diversity	
			Estimated species richness					
Community	Species richness	Shared species	Chao 1/ 2	Jacknife	Simpson's D*	Shannon Wiener*	Usable loci	θ (SD) (intracommunity genetic diversity)**
May/June	9		9	9	5.389a	1.798a	315	39.14 (18.85)a
August	11	6	12	14	3.136b	1.373b	346	33.45 (16.16)b
October	12		14	15.33	5.049c	1.756a	316	45.46 (21.83)c
Fresh discs	13	10	13.03	13	3.492a	1.517a	319	43.83 (21.05)a
Dried discs	11		14.01	16	2.782b	1.400b	332	36.08 (17.39)b

*Significant differences between seasons and substrates are indicated by different letters.

**Significant differences between seasons and substrates are indicated by different letters, as revealed by F_{st} statistics ($P < 0.001$).

F_{st} values for intercommunity genetic diversity: May/June vs. August = 0.126; August vs. October = 0.136; May/June vs. October = 0.051; fresh vs. dried reed discs = 0.125.

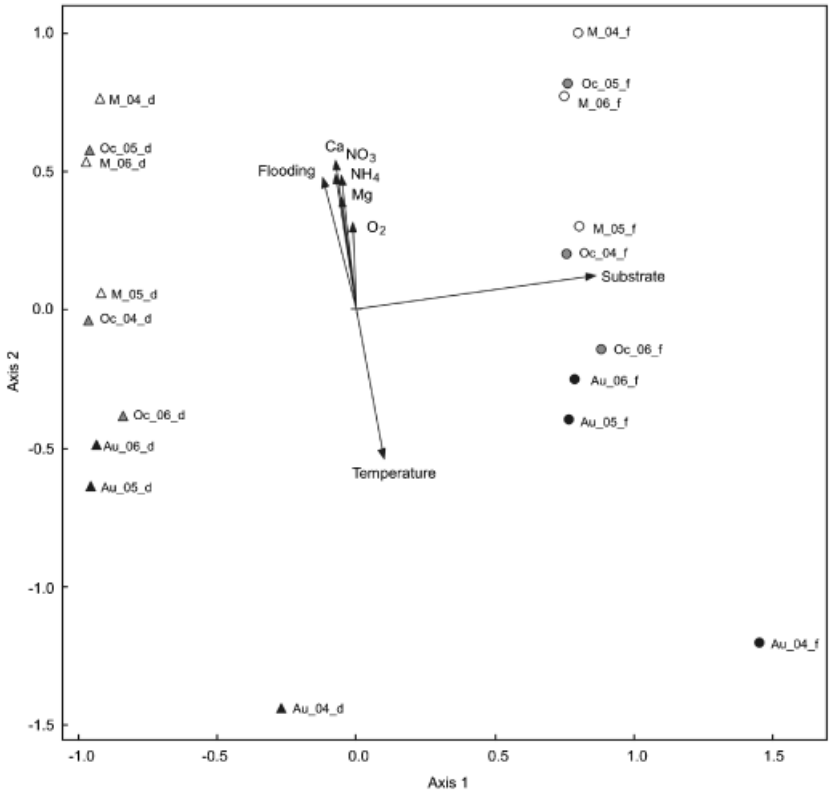

Fig. 3. CCA biplot ordination on the effects of substrate, water chemistry, temperature and level (represented as arrows) on 18 oomycete clone libraries. Axes 1 and 2 explain 26.9% and 15.6% of the variation, respectively. Library labels: f, fresh substrate (circles); d, dried substrate (triangles); M, May/June (white); Au, August (black); Oc, October (grey); 04, 05, 06 = 2004, 2005, 2006, respectively; Monte Carlo permutation test on axis 1: $P = 0.001$.

the variation). Oomycete communities in October and May grouped distinct from August communities, with October 2005 samples being similar to the May samples (Fig. 3). In May and October, fresh reed discs were dominated by types B (=*P. phragmitis*), C (close to *P. graminicola*) and I (*Aphanomyces* sp.), and dried leaf discs by type D (=*P. catenulatum*) and J (*Aphanomyces* sp.). In August, *P. phragmitis* was hardly ever detected, and predominating oomycetes were type C in fresh and type D in dried baits. In August 2004, an additional *Pythium* sp. (G) was abundantly found in fresh substrates. This taxon did only occur sporadically in 2005 and 2006 (Fig. 2). A significant diversity reduction in August was shown by several diversity estimators; clone libraries from summer samples revealed significantly lower species and molecular diversities, as compared with the spring and autumn samplings (Table 2).

qPCR of *P. phragmitis* in reed baits

We used qPCR to analyse the progression of *P. phragmitis* in reed baits, and to confirm the results obtained from the clone libraries. We observed a linear increase of mean

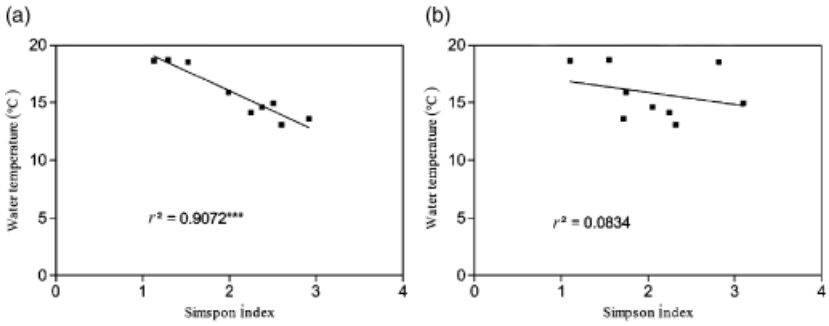

Fig. 4. Correlation of lake water temperature and clone library diversity as expressed by the Simpson index, in fresh (a) or dried (b) reed baits. ***, $P \leq 0.0001$.

P. phragmitis rDNA in fresh leaf samples during the sampl ing period (1 4 weeks of exposition in the littoral zone) (Fig. 5a). In contrast, in dried plant material, a decrease of pathogen rDNA per leaf disc could be observed during the 4 weeks of incubation (Fig. 5b). The rDNA quantities per leaf disc of the 4th week (Fig. 6a) corresponded to the abun dance of OTU type B (*P. phragmitis*) in the clone libraries (Fig. 6b), regarding both substrate and seasonal trends. Accordingly, *P. phragmitis* rDNA was mainly detected in fresh substrate, significantly smaller amounts were detected in dried leaf discs, and *P. phragmitis* rDNA quantities per leaf disc showed a strong decline in August.

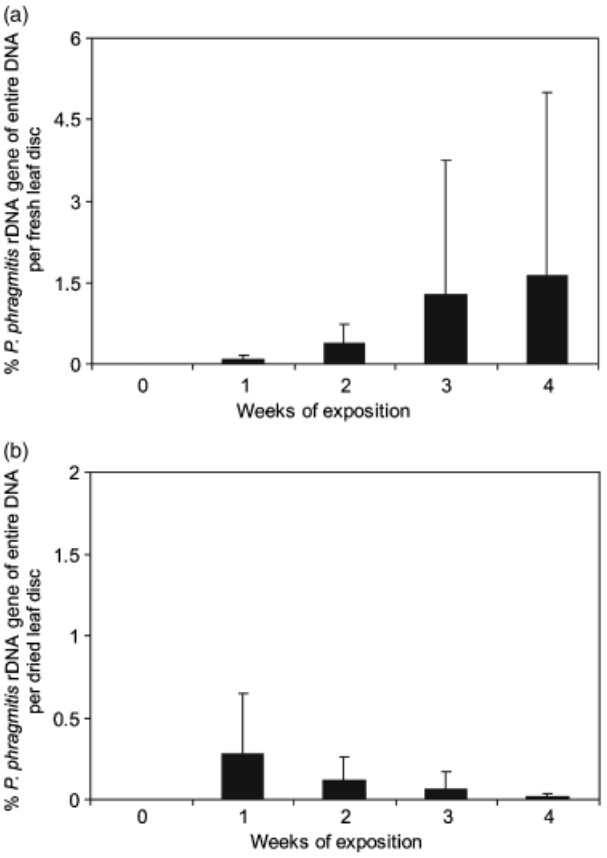

Fig. 5. Successive colonization of reed discs by *Pythium phragmitis* during 1 4 weeks of exposition in the littoral on either fresh (a) or dried plant material (b), as revealed by real time PCR. Mean values and SD of all nine samplings (irrespective of season) are given for each week, plus nine negative controls. Note different scaling of *y* axes in (a) and (b).

Discussion

This study for the first time investigates the abundance of oomycete species in a natural ecosystem using cultivation independent molecular methods. We used reed leaf baits to create clone libraries of oomycetes specifically associated with reed. The results obtained were confirmed by a qPCR assay setup for the well characterized reed pathogen *P. phragmitis*. Both methods revealed largely similar results for both substrate specificity and seasonality, indicating that the two techniques used are reliable, producing highly reproducible datasets. Furthermore, our results were con firmed by repeated sampling for three consecutive years. We could show substrate and season dependent differences in oomycete communities, with similar trends every year, and the predominating taxa being detected regularly. This strongly indicates that the among year bias introduced by the methods used was small, and our results could be used to deduce season and substrate dependent preferences within oomycete communities.

Overall species richness in the present study is consistent with previous traditional isolation studies on oomycete diversity of aquatic environments, where similar species numbers could be found (e.g. van der Plaats Niterink, 1975; Hallett & Dick, 1981; Abdelzaher *et al.*, 1995). A qualitative isolation study on similar sites at Lake Constance using reed baits revealed eight oomycete species (Nechwatal *et al.*, 2008a), five of which could also be detected within our study (≥99% ITS sequence identity). The difference in species number and composition between the two ap proaches might be assigned to the proposed advantage of molecular over traditional methods, i.e. the detection of species difficult to culture or even unculturable. For exam ple, the absence of any significant GenBank match for OTU types G, H, J and L indicates that these might comprise

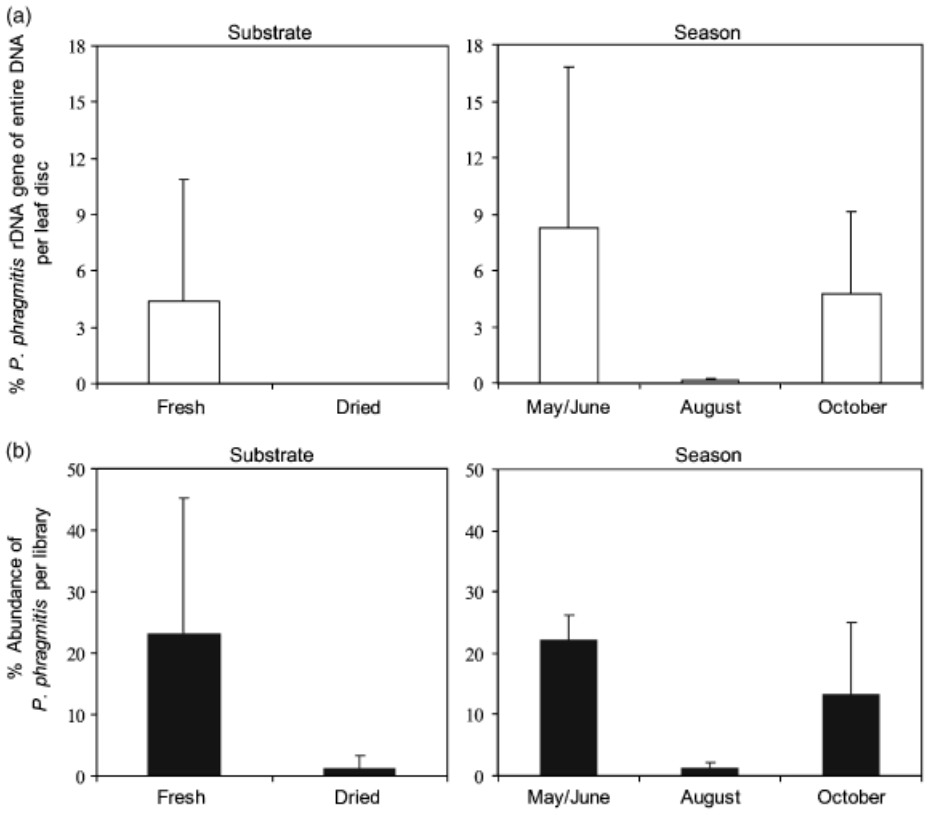

Fig. 6. Comparison of oomycete clone library and qPCR data. (a) Relative amount (%) and SD of *Pythium phragmitis* rDNA in relation to total DNA of an average leaf disc as revealed by real time PCR. (b) Relative abundance (%) of *P. phragmitis* clones in *Escherichia coli* libraries. Left, substrate comparison (3 years × three seasons); right, season comparison (3 years, fresh substrate).

previously unknown taxa that are unculturable under stan dard conditions. It might, however, also be due to the longer sampling and baiting periods of this study, and the different habitats investigated.

Total diversity in our study was markedly higher as compared with other recent studies on *Pythium* spp. asso ciated with agricultural soils or greenhouse crops, using both traditional (Moorman *et al*., 2002) or molecular methods (Arcate *et al*., 2006; Schroeder *et al*., 2006). It was equally high as in a recent study using highly sensitive DNA macroarrays (Tambong *et al*., 2006). Natural habitats might generally be highly diverse in *Pythium* spp., casting doubt on the traditional view that uncultivated sites are relatively poor in members of this genus (van der Plaats Niterink, 1981).

As in other studies focusing on oomycete diversity in unexplored natural ecosystems, a high number of 'new', previously undescribed taxa was detected. Still, the defini tion of species boundaries and the assignment of OTUs to taxa is not unambiguous. In oomycetes, degrees of similarity in ITS sequences between well defined (morpho) species within a single genus range from 80% to 99% (e.g. Jung *et al*., 2003; Nechwatal *et al*., 2005; Nechwatal & Mendgen, 2006) and the level of intraspecific variation is unknown in most taxa. Thus, the definition of fixed threshold levels for species delineation does not seem appropriate or even possible (Arnold *et al*., 2007). Based on other studies with only minor ITS sequence differences between *Pythium* isolates accompanied by a clear morphological discrimina tion (Jung *et al*., 2003; Nechwatal *et al*., 2005, 2008a), we tentatively assigned 'species' rank to all OTUs detected within this study.

Using two different bait types, we observed a distinct substrate differentiation, with oomycete species being asso ciated with either fresh or dried reed leaf discs that were used to mimic two different substrate types (Fig. 3). The taxa most abundantly detected in fresh (i.e. live) leaf baits were *P. phragmitis* and a species close to *P. graminicola*, which, despite extensive isolation efforts (investigating > 80 iso lates), could not yet be isolated (data not shown). As the majority of *Pythium* species from fresh leaf discs, both belong to the grass pathogen cluster B1e/d (Lévesque & de Cock, 2004), and can thus be characterized as presumably pathogenic on reed (Nechwatal *et al*., 2008a). *Aphanomyces* sp. (type I) was also among the predominating species in fresh baits, and although as yet uncultured, might be another undescribed pathogen of reed. qPCR assays target ing *P. phragmitis* confirmed its strong predominance and accumulation in fresh leaf baits during exposition. In contrast, in dried reed baits, growth of *P. phragmitis* was

strongly inhibited, because other, saprophytic species are more competitive in the colonization of dead reed material. Accordingly, the taxa predominantly and abundantly de tected in dried leaf discs, i.e. *P. catenulatum* (type D), *P. aquatile* (F) and *Aphanomyces* sp. (J), do not belong to major grass pathogen groups and obviously have a more saprotrophic lifestyle (van der Plaats Niterink, 1981; Lévesque & de Cock, 2004). Because unspecific cross colonization of nonpreferred substrates, particularly after advanced decay of the fresh discs, and their detection by highly sensitive molecular tools, some of the presumably saprophytic species were sporadically present on fresh baits, and *vice versa*, leading to a relatively high species overlap between the two substrates (Table 2).

Seasonal differences in natural fungal or oomycete com munities have repeatedly been reported to occur in soil samples, tree branches or leaf litter (e.g. Gessner *et al*., 1993; Schadt *et al*., 2003; Fryar *et al*., 2004; Nikolcheva & Bärlocher, 2005). In *Pythium* species, dominance and com munity composition have been observed to be fluctuating both relatively quick as well as on a more long term basis (van der Plaats Niterink, 1975; Ali Shtayeh *et al*., 1986; Hardman & Dick, 1987; Abdelzaher *et al*., 1995). However, the identification of single factors responsible for such changes in natural ecosystems is difficult, because the seasonality recorded is rather the net result of several complex interactions in the ecosystem than being based on distinct correlations with single environmental factors (Hal lett & Dick, 1981). For example, changes in trade offs between fungal, oomycete and bacterial colonizers (Mille Lindblom & Tranvik, 2003; Nikolcheva & Bärlocher, 2004) might also account for seasonal fluctuations in these com munities but were outside the scope of the present study. Nevertheless, some factors likely to be of importance for reed associated oomycete community dynamics could be identified in our study.

First, seasonal variations in species richness are likely to be mediated by water temperature and water chemistry changes (Fig. 3). Temperature and external ion and oxygen concentration changes are known to interact with oomycete growth and sporulation (van der Plaats Niterink, 1981; Walker & van West, 2007), and a decline in oomycete isolation frequencies with rising water temperatures and decreasing oxygen contents during summer has repeatedly been reported (e.g. Hallett & Dick, 1981; Misra, 1982; Ali Shtayeh *et al*., 1986; Abdelzaher *et al*., 1995; Marano *et al*., 2008). In our study, too, significantly higher water tempera tures accompanied by changing ion and oxygen concentra tions and decreasing species diversities during the August samplings were recorded. Correlations between water tem perature and species diversity (Fig. 4) indicated that species sets colonizing fresh baits (i.e. reed pathogens) might be more responsive to rising temperature than saprophytic decomposers feeding on plant litter of diverse origin. The reed pathogen *P. phragmitis*, in contrast, was recently shown to be promoted by high temperatures in *in vitro* tests (Nechwatal *et al*., 2008b). Therefore, the almost complete absence of this species in summer is unlikely to be due to its sensitivity to seasonally higher water temperatures, and might (in addition to water level outlined below) also be linked to seasonally changing water chemistry.

Second, for the reed pathogen *P. phragmitis* in particular, water level fluctuations (Fig. 3) and the stage of reed development might be of significance. Lake Constance is characterized by marked changes in mean water level, due to the absence of water gauge regulation measures. Generally, water level rises during the first sampling period to reach its highest level at the end of June (*c*. 430 cm, gauge Konstanz) as a result of snow melt in the Alps. Subsequently, the level decreases during August and October until winter to *c*. 290 cm (Fig. 1). *Pythium phragmitis* as a leaf rather than a root or rhizome colonizer (Nechwatal *et al*., 2008b) was most abundant in late spring, most probably as a conse quence of the onset of reed shoot growth and rising lake water level. During this season, high amounts of infectable plant material, i.e. submerged shoots and young leaves, are available, while in summer, decreasing water levels bring about a temporal unavailability of such substrates. As *P. phragmitis* was originally isolated from littoral soils (Nechwatal *et al*., 2005), reflooding of banks fallen dry during winter, and subsequent germination of oospores might represent an additional source of inoculum in spring. In October 2005, we observed a marked increase of *P. phragmitis*, and a clear grouping of the two communities with those of May (Fig. 3), as a consequence of an extraordinary high water level in September, which would normally occur in late spring (Fig. 1). This could have led to a considerable inoculum build up in mature wind or flood bent reed during early stages of senescence and decay, and confirms the strong dependency of *P. phragmitis* on the availability of submerged host tissue. These findings are consistent with results of Nechwatal *et al*. (2008b) who correlated *P. phragmitis* infection of reed with leaf submer gence and flooding events. The as yet uncultured *Aphano myces* sp. (I) had similar seasonal patterns and might therefore be subject to similar environmental influences. Type C (close to *P. graminicola*), in contrast, showed weaker seasonal trends, and was dominant throughout the vegeta tion period, peaking in August. This as yet uncultured taxon might represent a new pathogen of reed, well adapted to elevated summer water temperatures and less dependent on reed submergence, for example as a root pathogen.

In summary, the community changes observed during this 3 year study suggest a considerable degree of niche differentiation within the reed associated, littoral oomycete species. Saprotrophic species communities were less diverse,

both at taxa as well as at sequence level, but less sensitive to temperature changes and less dependent on reed development, consistent with their proposed low host specificity and the constant availability of organic matter of diverse origin. The high diversity of grass associated species communities, in contrast, indicates that a highly diverse and adaptive set of species might act as pathogens on reed. Among the mean driving forces for niche differentiation, most likely interlinked with each other, are water temperature and chemistry, water level, the stage of reed development and its inundation depth, that is, host plant availability. This was particularly true for the well characterized reed pathogen *P. phragmitis*. Future efforts should aim at the isolation and characterization of other, as yet uncultured reed associated oomycete pathogens to eventually result in a complete picture of these organisms in a unique, endangered ecosystem.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Water chemistry, water temperature and reed phenology data of Lake Constance at three sampling dates over three consecutive years (2004 2006).
Table S2. Primers used in this study.
Table S3. List of oomycete and nonoomycete species used in specificity tests for oomycete specific primer pair ITS4 Oo and ITS5, and *Pythium phragmitis* specific primer pair Phrag fw and Grp3 rev.
Table S4. Absolute abundance of oomycete taxa (number of clones) in different substrates (fresh/dried), seasons (May/June, August, October) and years (2004 2006).