The Contribution of DNA Metabarcoding to Fungal Conservation: Diversity Assessment, Habitat Partitioning and Mapping Red-Listed Fungi in Protected Coastal *Salix repens* Communities in the Netherlands

József Geml1,2, Barbara Gravendeel1,2,3, Kristiaan J. van der Gaag4, Manon Neilen1, Youri Lammers1, Niels Raes1, Tatiana A. Semenova1,2, Peter de Knijff4, Machiel E. Noordeloos1

1 Naturalis Biodiversity Center, Leiden, The Netherlands, 2 Faculty of Science, Leiden University, Leiden, The Netherlands, 3 University of Applied Sciences Leiden, Leiden, The Netherlands, 4 Forensic Laboratory for DNA Research, Human Genetics, Leiden University Medical Centre, Leiden, The Netherlands

### Abstract

Western European coastal sand dunes are highly important for nature conservation. Communities of the creeping willow (*Salix repens*) represent one of the most characteristic and diverse vegetation types in the dunes. We report here the results of the first kingdom-wide fungal diversity assessment in *S. repens* coastal dune vegetation. We carried out massively parallel pyrosequencing of ITS rDNA from soil samples taken at ten sites in an extended area of joined nature reserves located along the North Sea coast of the Netherlands, representing habitats with varying soil pH and moisture levels. Fungal communities in *Salix repens* beds are highly diverse and we detected 1211 non-singleton fungal 97% sequence similarity OTUs after analyzing 688,434 ITS2 rDNA sequences. Our comparison along a north-south transect indicated strong correlation between soil pH and fungal community composition. The total fungal richness and the number OTUs of most fungal taxonomic groups negatively correlated with higher soil pH, with some exceptions. With regard to ecological groups, dark-septate endophytic fungi were more diverse in acidic soils, ectomycorrhizal fungi were represented by more OTUs in calcareous sites, while detected arbuscular mycorrhizal genera fungi showed opposing trends regarding pH. Furthermore, we detected numerous red listed species in our samples often from previously unknown locations, indicating that some of the fungal species currently considered rare may be more abundant in Dutch *S. repens* communities than previously thought.

### Citation

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* Email: Jozsef.Geml@naturalis.nl

### Introduction

Fungi represent one of the largest groups of living organisms. However, fungi are still poorly understood and appreciated compared to plants and animals and our knowledge of fungal diversity lags far behind [1]. Approximately 100,000 species of Eumycota (the true Fungi) have been described. Already well before the routine use of DNA sequencing in fungal biodiversity assessments, their true diversity was estimated to be around 1.5 million species [2], while more recent estimates suggest there may be 0.7 to 5 million species [3],[4]. Despite the differences among various estimates, it is clear that we currently know only a fraction of the total fungal biodiversity.

The task of discovering a significant portion of yet unknown species has only recently become possible with the advent of high-throughput DNA sequencing of environmental samples that has enormous potential to further boost data acquisition in biodiversity research [5],[6],[7],[8],[9],[10],[11],[12],[13],[14],[15],[16]. This methodology involves identification of multiple species from environmental samples using targeted loci specifically selected with the purpose of identification. These loci are typically the same ones that are used in specimen-based large-scale DNA barcoding efforts (e.g., [17],[18]), hence providing both appropriate power of resolution for the group of interest and maximum amount of reference sequences available from voucheded specimens.

The fungal diversity assessment presented here focuses on *Salix repens* L. sand dune communities along the North Sea coast, because these areas are highly important for nature conservation, water resource management, and recreational purposes. Coastal sand dunes include an outstanding ecological diversity in terms of environmental heterogeneity and species composition [19]. In the Netherlands, they are home to more than half of all higher plant species, in spite of covering only 1% of the land area [20],[21]. In Europe, coastal dune areas have been reduced by ca. 70% in the last century [22], and biodiversity in the remaining dune ecosystems is highly endangered [23].
however, coastal dunes have been relatively well preserved, with less than 10% area loss, largely due to their roles in drinking water management and as barriers against the sea [21]. These dunes represent one of the last nutrient-poor habitats in the Netherlands and most plant species featured on the Dutch Red List now have their main distribution in these coastal areas. Moreover, the reserves featured in this manuscript are part of Natura 2000, an ecological network of protected areas in the territory of the European Union (http://www.natura2000.nl/pages/kaartpagina.aspx).

The creeping willow (Salix repens) is a widespread shrub in western Europe and inhabits a wide range of vegetation types. It forms both ectomycorrhiza and arbuscular mycorrhiza [24],[25], often supporting a diverse community of symbiotic fungi in its extensive root system [26]. It is a particularly characteristic component of coastal sand dune ecosystems where it is often the only ectomycorrhizal (ECM) host plant [27],[26]. Therefore, it has been repeatedly noted that the presence of *S. repens* adds wealth to the overall fungal diversity in sand dune ecosystems [25],[26]. Somewhat surprisingly, species composition of fungi in *S. repens* communities have not been previously investigated using molecular methods to our knowledge. To date, characterizations of fungi in *S. repens* assemblages have been based on morphology-based identification of fruitbodies and ECM root structures. For example, in one of the early studies comparing above- and below-ground diversity of ECM fungi, Van der Heijden et al. [25] found fruitbodies of 78 ECM taxa belonging to 12 genera and identified fifteen ECM root morphotypes in *S. repens* communities in the Netherlands. Watling [26] reported almost 80 species of macrofungi from *S. repens* beds of the northern Scottish islands based on fruiting records. Despite these outstanding pioneer works, important gaps remain in our knowledge about total fungal diversity in this important ecosystem.

In this study, we collected soil samples at multiple locations in strictly protected coastal areas and conducted a large-scale biodiversity assessment of soil fungi inhabiting the rhizosphere of *S. repens* using pyrosequencing techniques. The aims of this work were 1) to characterize fungal communities found in *S. repens* vegetation; 2) compare the effects of environmental factors (e.g., soil pH, geological district) on communities through multiple sites; and 3) to supplement sporocarp-based mapping data points of rare fungi with soil-based data points. Our results provide the first kingdom-wide fungal diversity assessment in coastal sand dune communities and demonstrate the potential use of high-throughput DNA sequencing of environmental samples to fungal conservation.

Materials and Methods

The Study Region

The following environmental agencies granted permission for the fieldwork (with sampled field sites): Amsterdamse Waterleidingduinen (Haarlem-Bentveld), Dunca (Wassenaar-Meijendel), Natuurmonumenten (Goeree, Callantssoog-Zwanenwater), and Staatsbosbeheer (Terschelling). GPS coordinates of the sampling sites are listed in Table 1. The field sampling only included soil and did not involve endangered or protected species. The selected sites represented sand dune *S. repens* beds (European Environment Agency habitat code: 2170) in an extended area of joined nature reserves located along the North Sea coast of the Netherlands, representing a south-north transect (Figure 1, Table 1). This coastal dune system is characterized by an oceanic climate with mean annual temperature of 9–10°C (July, 16–17°C; January, 2–3°C) and precipitation of 675–800 mm [21]. Sand-lime content varies between 0.1 and 10% [21], with generally calcareous soils in the southern Renodunal district (represented by the sampling sites Goeree, Meijendel, and Haarlem-Bentveld) and more acidic soils in the northern Wadden district (Zwanenwater, Terschelling). In most areas, the height of the dunes reaches 15–25 m [29]. Current management policies are directed toward multiple purposes, such as drinking water supply, defense against storm floods, recreation, and nature conservation [29]. Soil samples were taken at sites monitored by Natuurmonumenten (Goeree and Zwanenwater), Dunca (Meijendel), Staatsbosbeheer (Terschelling), and the Amsterdamse Waterleidingduinen (Haarlem-Bentveld). In March of 2010, 20 soil cores, 2 cm in diameter and ca. 20 cm deep, were taken at each site in a way that cores were at least 2 m from each other to minimize the probability of sampling the same genet repeatedly. For every plot, the 20 cores were pooled, resulting in a composite soil sample for each plot. Soils were deposited in 50 mL Falcon tubes and stored at −80°C until lyophilization.

DNA Extraction, PCR, and Pyrosequencing

We follow the recently proposed guidelines for standardizing the description of next-generation sequencing datasets in publications as proposed by Nilsson et al. [30]. Genomic DNA was extracted from 8 g of lyophilized soil from each composite sample using the Mo Bio PowerSoil kit following the manufacturer’s instructions. We used high-throughput tag-encoded 454 GS-FLX amplicon sequencing to assess fungal diversity in the selected plant communities. Amplicon libraries were performed using a combination of tagged primers designed for the variable ITS region, as recommended for the tag-encoded pyrosequencing method [31]. Genomic DNA samples were amplified using the fungal-specific primer pair ITS1F (5’-CTCTGTCATATAGAGGAAATTC-3’) and ITS4 (5’-TCCTCGCTTATTGATATGC-3’) and tagged with sample identification. Barcodes differed from all other barcodes by at least two nucleotides. Four PCR reactions were performed per sample using the following temperature program: 95°C/2 min, 25 cycles of 95°C/30 s, 54°C/1 min, 72°C/2 min, and 72°C/10 min. One µl of DNA template was used for the 40 µl PCR reaction containing 25.6 µl of MQ water, 4 µl of 10x buffer, 1.5 µl dNTP’s (2.5 mM), 1.5 µl of reverse and forward primers (10 mM), 4 µl MgCl₂ (50 mM), 0.5 µl BSA (10 mg/ml) and 0.4 µl BIOTAQ polymerase (5 U/µl). PCR products were pooled for each sample, purified and sequencing-adapters/tags were integrated via MegaPrimers (PCR). The amplicon length and concentration were estimated, and an equinolar mix of all amplicon libraries was used for pyrosequencing (with the ITS4 primer). Tagged samples were pooled and sequenced using the Genome Sequencer GS FLX System (454 Life Sciences/Roche Applied Biosystems, Nutley, NJ, USA) at GATC Biotech AG (Konstanz, Germany) and the Leiden Genome Technology Center (Leiden, the Netherlands).

Bioinformatic Work

Pyrosequences resulted in 688 434 reads following quality checks and trimming, with median read length of 284 bp. The raw data have been submitted to the European Nucleotide Archive (ERP001713). We used a parallel version of MOTHUR v. 1.32.1 [32] installed at the University of Alaska Life Sciences Informatics Portal for sequence analyses. We filtered sequences by quality score with a sliding window approach as in Brown et al. [33]. The FASTQ files were converted to FASTA and QUAL files, and the sequences were subjected to quality filtering whereby each
sequence was screened for thresholds for average Phred score of $Q \geq 25$ in a sliding window of 50 bp ($q_{\text{windowaverage}} = 25$, $q_{\text{windowsize}} = 50$), no ambiguous bases ($\text{maxambig} = 0$) and homopolymers no longer than 8 bp ($\text{maxhomop} = 8$). Sequences shorter than 150 bp or longer than 400 bp were omitted from further analyses ($\text{mintlength} = 150$, $\text{maxlength} = 400$). A total of 369,181 sequences remained after quality filtering and trimming. Because next-generation sequencing libraries generally vary in size, we normalized the number of sequences for all samples prior to OTU clustering, as recommended by Gihring et al. [34], to ensure that estimators across all samples are comparable. For this purpose, we randomly subsampled the number of trimmed and quality-filtered reads to the size of the smallest library (30,497). The resulting 304,970 sequences served as input for operational taxonomic unit (OTU) clustering using OTUPIE [35] based on 97% sequence similarity. Such 97% sequence similarity cut-off value is routinely used in fungal community studies as a proxy for species, accounting for intraspecific variation and errors generated by PCR (e.g., [36],[37],[38],[7],[39],[40],[9],[12],[15],[41]). We simultaneously removed 13,279 putatively chimeric sequences, using a curated dataset of fungal ITS sequences as reference dataset [42]. Pyrosequencing datasets comprising orders of magnitude more sequences per taxa than traditional methods tend to contain a higher proportion of artifactual OTUs, because sequencing errors tend to be unique, while the accumulation of real taxa starts to level off [43],[12]. Because most pyrosequencing

Figure 1. Sampling sites along the coast of the Netherlands. Sites corresponding to the Renodunal and Wadden geological districts are marked with black and white squares, respectively. doi:10.1371/journal.pone.0099852.g001
Comparing Fungal Communities among Sampling Sites

We used the 97% ITS sequence similarity OTUs inferred earlier as input data for the various ordination methods. To visualize the variation in the occurrence of OTUs across sites, we carried out ordination using non-metric multidimensional scaling (NMDS) with the quantitative version of Sorensen similarity (Bray-Curtis index) with 500 iterations in PC-Ord [47]. Read abundance in 454 sequencing has been shown to be approximately quantitative only within species, while between-species comparisons can be biased by innate sequence structure [39]. Because of such uncertainties regarding the reliability of read abundance, we carried out the ordination analyses with square-root transformed abundance to moderate the influence of OTUs with high sequence counts, while maintaining some approximation of abundance that often reflects ecological significance. The solution with the lowest stress was derived from 250 runs using real data and was then subjected to 250 randomized runs using a Monte Carlo test to evaluate the probability of final the NMDS patterns being greater than chance occurrences. Orthogonal rotation of the resulting NMDS solution was used to maximize correlation between the strongest environmental variables (as indicated by Pearson $r$ values) and major axes. The solution with the lowest number of dimensions was selected when the decrease in the final stress was <5 by adding another dimension [47]. The Pearson correlation coefficient ($r$) values between environmental and fungal community variables and axes 1 and 2 were calculated. Soil pH and the number of OTUs representing various taxonomic groups per site were included in a site environmental factor matrix as quantitative variables. For this latter, we used only taxonomic orders and genera represented by at least 3 OTUs were included for a site as quantitative variables (see Table 2 for full list). Geological district (Renodunal = 1, Wadden = 0) was included as categorical variable. Following NMDS ordination of sites, we examined the Pearson correlation values between the community ordination axes and these variables. We also tested whether fungal communities were statistically different across the sites using a multiresponse permutation procedure (MRPP), also in PC-Ord. For the MRPP analyses, three categorical variables were tested: geological district, as described above, habitat type (wet dune slack vs. dry dune side and top), and soil pH (acidic = 0, non-acidic = 1, based on a pH = 6.5 threshold following the USDA soil classification for acidic and non-acidic soils). Finally, we determined any preferences of individual OTUs for pH categories using indicator species analyses.

Results

OTU Diversity and Taxonomic Affiliations

Out of the 688,434 original reads, 402,500 sequences passed the various filtering steps. After normalizing the library size across all samples, 304,970 sequences were assembled into 7,156 OTUs of which 1788 were singletons. After excluding all singletons and OTUs with <80% similarity or <100 bp pairwise alignment length to a fungal sequence as performed by USEARCH, 3611 OTUs were retained for subsequent analyses. The number of OTUs at a given site ranged from 660 (Amsterdamse Waterleidingduinen, R-H2) to 971 (Goeree: Middelduinen, R-G1) (Table 1).

Ascomycota was the dominant phylum and accounted for 33.18% of the OTUs, followed by Basidiomycota (22.73%), Glomeromycota (5.29%), Mucoromycota, incertae sedis (1.94%), and Chytridiomycota (0.53%), while unidentified fungal OTUs with most similar sequences to other environmental sequences without assignment to phylum accounted for 36.33% (Figure 2a). In Ascomycota, the ranking of taxonomic orders based on the number of representative OTUs was as follows: Helotiales (171), Pleosporales (136), Hypocreales (114), Pezizales (92), Archaeorhizomycetales (71), Chaetothyriales (64), Capnidiaceae (51), Sordariales (45), Eurotiales (34), Xylariales (19), Diaportheales (17), Verrucariales (13), Lecanorales (13), Thelidiales (12), Botryosphaeriales (10), followed by numerous other orders with <10 OTUs. In addition, there were 49 ascomycete OTUs with incertae

### Table 1. Sampling sites of coastal Salix repens communities used in this study.

| Site Code | Geological district, habitat | Latitude, longitude | pH | OTUs |
|-----------|-----------------------------|---------------------|-----|------|
| R-G1      | Renodunal, wet              | 51.827238; 3.943405 | 6.01 | 971  |
| R-G2      | Renodunal, wet              | 51.810850; 3.861609 | 6.73 | 934  |
| R-M1      | Renodunal, dry              | 52.137439; 4.341960 | 7.3  | 691  |
| R-M2      | Renodunal, dry              | 52.142707; 4.333034 | 7.42 | 790  |
| R-H1      | Renodunal, wet              | 52.338001; 4.531978 | 7.11 | 727  |
| R-H2      | Renodunal, dry              | 52.337312; 4.533910 | 6.56 | 660  |
| W-Z1      | Wadden, dry                 | 52.822073; 4.709229 | 5.71 | 867  |
| W-Z2      | Wadden, wet                 | 52.821516; 4.708264 | 5.32 | 762  |
| W-T1      | Wadden, wet                 | 53.364741; 5.201769 | 6.39 | 718  |
| W-T2      | Wadden, dry                 | 53.398326; 5.269489 | 5.78 | 804  |

Code, geological district, habitat type (wet dune slack vs. dry dune side and top), geographic coordinates, soil pH, number of quality-filtered sequences and the number of 97% ITS2 sequence similarity OTUs are displayed for each site.

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|                  | Axis 1 |     | Axis 2 |     | Axis 1 |     | Axis 2 |     |
|------------------|--------|-----|--------|-----|--------|-----|--------|-----|
|                  |  \(r\) | \(r^2\) |  \(r\) | \(r^2\) |  \(r\) | \(r^2\) |  \(r\) | \(r^2\) |  \(r\) | \(r^2\) |
| **pH**           | 0.718  | 0.516 | 0.094  | 0.009 | -0.229 | 0.052 | 0.011  | 0.012 |
| **OTU richness** | -0.263 | 0.069 | 0.294  | 0.084 | -0.594 | 0.353 | -0.144 | 0.021 |
| **Ascomycota**   | 0.228  | 0.001 | 0.306  | 0.093 | 0.594  | 0.353 | -0.144 | 0.021 |
| **Basidiomycota**| 0.321  | 0.103 | 0.503  | 0.253 | -0.074 | 0.049 | 0.479  | 0.229 |
| **Glomeromycota**| 0.33   | 0.109 | 0.197  | 0.039 | 0.655  | 0.429 | -0.142 | 0.02  |
| **Mucoromycota** | -0.318 | 0.101 | 0.529  | 0.28  | 0.854  | 0.729 | -0.527 | 0.278 |
| **Chytridiomycota** | -0.585 | 0.342 | 0.159  | 0.025 | 0.98   | 0.777 | -0.406 | 0.165 |
| **Ascomycete orders** |  |  |  |  |  |  |  |  |
| **Fusarium**     | 0.318  | 0.101 | 0.529  | 0.28  | 0.854  | 0.729 | -0.527 | 0.278 |
| **Archaeorhizomycetales** | 0.704 | 0.495 | 0.479  | 0.229 | 0.655  | 0.429 | -0.142 | 0.02  |
| **Capnodiales**  | -0.371 | 0.138 | 0.223  | 0.05  | 0.73   | 0.532 | -0.092 | 0.008 |
| **Geomyces**     | 0.242  | 0.059 | -0.282 | 0.079 | 0.344  | 0.119 | -0.213 | 0.046 |
| **Devriesia**    | 0.202  | 0.041 | 0.479  | 0.221 | 0.854  | 0.729 | -0.527 | 0.278 |
| **Exophiala**    | -0.599 | 0.359 | 0.017  | 0.005 | 0.154  | 0.024 | 0.199  | 0.04  |
| **Gliocladium**  | 0.012  | 0  | 0.258  | 0.067 | 0.02   | 0  | 0.107  | 0.011 |
| **Melanocarpus** | 0.418  | 0.175 | 0.254  | 0.065 | 0.392  | 0.154 | 0.195  | 0.038 |
| **Geotrichum**   | -0.624 | 0.389 | -0.058 | 0.003 | 0.437  | 0.191 | -0.092 | 0.008 |
| **Penicillium**  | -0.274 | 0.075 | 0.177  | 0.031 | 0.287  | 0.083 | 0.155  | 0.024 |
| **Phialophora**  | 0.829  | 0.687 | -0.441 | 0.194 | 0.63   | 0.397 | 0.113  | 0  |
| **Microsphaera** | 0.195  | 0.038 | -0.257 | 0.066 | 0.17   | 0.029 | -0.26  | 0.068 |
| **Onygenales**   | 0.643  | 0.414 | -0.749 | 0.561 | 0.899  | 0.808 | -0.309 | 0.096 |
| **Oribatiales**  | 0.596  | 0.359 | 0.017  | 0.029 | 0.359  | 0.129 | -0.332 | 0.11  |
| **Pezizales**    | 0.921  | 0.848 | -0.154 | 0.023 | 0.873  | 0.761 | -0.566 | 0.32  |
| **Phyllumenales**| 0.419  | 0.176 | 0.014  | 0.029 | 0.04   | 0.002 | 0.167  | 0.028 |
| **Saccharomycetales** | 0.759 | 0.577 | -0.184 | 0.034 | 0.588  | 0.346 | -0.414 | 0.171 |
| **Sordariales**  | -0.563 | 0.317 | 0.227  | 0.074 | 0.817  | 0.667 | -0.211 | 0.044 |
| **Verrucariales**| -0.535 | 0.286 | 0.045  | 0.045 | 0.551  | 0.303 | -0.435 | 0.189 |
| **Xylariales**   | -0.037 | 0.001 | 0.57   | 0.325 | 0.63   | 0.397 | 0.052  | 0.003 |

**Basidiomycete orders**

|                  | Axis 1 |     | Axis 2 |     | Axis 1 |     | Axis 2 |     |
|------------------|--------|-----|--------|-----|--------|-----|--------|-----|
| **Agaricales**   | 0.479  | 0.229 | -0.137 | 0.019 | 0.255  | 0.065 | -0.331 | 0.11  |
| **Auriculariales** | -0.796 | 0.634 | 0.122  | 0.015 | 0.445  | 0.198 | -0.118 | 0.014 |
| **Boletales**    | 0.015  | 0  | 0.213  | 0.045 | 0.63   | 0.397 | 0.052  | 0.003 |

**Ascomycete genera**

- **Acremonium**
- **Alternaria**
- **Archeorhizomyces**
- **Cadophora**
- **Cladonia**
- **Devriesia**
- **Exophiala**
- **Geoglossum**
- **Geomyces**
- **Geotrichum**
- **Geotrichum**
- **Gliocladium**
- **Geotrichum**
- **Phialophora**
- **Phialophora**
- **Phyllumenales**
- **Phyllumenales**
- **Saccharomycetales**
- **Sordariales**
- **Verrucariales**
- **Xylariales**
- **Basidiomycete genera**

**Basidiomycete genera**

- **Ceratoasidium**
- **Claviceps**
- **Clavulina**
Table 2. Cont.

| Order               | Axis 1  | Axis 2  | Axis 1  | Axis 2  |
|---------------------|---------|---------|---------|---------|
|                     | $r$     | $r'$    | $r$     | $r'$    |
| Cantharellales      | -0.179  | 0.032   | -0.292  | 0.085   |
| Corticales          | -0.186  | 0.035   | -0.19   | 0.036   |
| Filobasidiales      | -0.398  | 0.158   | -0.023  | 0.001   |
| Hymenochaetales     | -0.465  | 0.216   | -0.208  | 0.043   |
| Polyporales         | -0.212  | 0.045   | -0.376  | 0.142   |
| Russulales          | -0.447  | 0.2     | 0.026   | 0.001   |
| Sebacinales         | 0.39    | 0.152   | -0.13   | 0.017   |
| Sporidiobolales     | -0.372  | 0.138   | 0.122   | 0.015   |
| Thelephorales       | 0.824   | 0.679   | -0.456  | 0.208   |
| Tremellales         | 0.069   | 0.005   | 0.106   | 0.011   |
| Trichosporonales    | -0.853  | 0.727   | 0.243   | 0.059   |
| Rickenella          | -0.465  | 0.216   | -0.208  | 0.043   |
| Russula             | -0.477  | 0.227   | 0.471   | 0.221   |
| Sebacina            | -0.115  | 0.013   | 0.288   | 0.083   |
| Sporobolomyces      | -0.004  | 0       | 0.265   | 0.07    |
| Stephania           | 0.356   | 0.127   | 0.026   | 0.001   |
| Thanatephorus       | 0.515   | 0.265   | -0.237  | 0.056   |
| Thelphora           | -0.633  | 0.4     | -0.223  | 0.05    |
| Tomentella          | 0.939   | 0.882   | -0.403  | 0.163   |
| Trichosporon        | -0.853  | 0.727   | 0.243   | 0.059   |

Ordinations were performed with square-root transformed abundance in the OTU vs. site matrix. Final stress value for the 2-dimensional NMDS solution was 0.03062. Variables with $|r| \geq 0.5$ values are shown in bold and are displayed in the NMDS ordinations in Figure 3.
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Diversity and Ecology of North Sea Dune Fungi

(a) Distribution of OTUs among fungal phyla

- Ascomycota, 1198
- Basidiomycota, 821
- Glomeromycota, 191
- Mucoromycotina, 70
- Chytridiomycota, 19
- unidentified, 1312

(b) Ascomycete orders

(c) Basidiomycete orders

- Agaricales
- Atheliales
- Auriculariales
- Boletales
- Cantharellales
- Corticales
- Cystofiliobasidiales
- Entylomatales
- Exobasidiales
- Filobasidiales
- Geastrales
- Hymenochaetales
- Incertae sedis
- Leucosporidiales
- Microbotryales
- Phallales
- Polyporales
- Pucciniales
- Russulales
- Sebacinales
- Sporidiobolales
- Thelephorales
- Tremellales
- Urocystidales
- Ustilaginales

OTUs
for characterizing changes in fungal community structure along a gradient. MRPP tests confirmed the importance of soil pH in shaping fungal community structure, with the Wadden communities being more similar to each other than to other Renodunal sites. However, there was also a strong structuring of the occurrence of fungal OTUs according to soil pH, co-correlated with the geological district. Sites belonging to the Wadden district were clustered on the left side of the ordination plot, with strongly negative axis 1 values. Even though the Renodunal district sites were more scattered, the Wadden and Renodunal sites formed non-overlapping clusters (Figure 3). However, one Renodunal site (Goeree: Middelduinen, R-G1), that had an acidic soil pH value, did not cluster with the Wadden communities but was more similar to the Renodunal communities than to other Renodunal sites.

MRPP tests confirmed the importance of soil pH in shaping fungal community composition (effect size $A = 0.04259$, probability $p = 0.00463$), while the effect of geological district was somewhat weaker ($A = 0.02786$, $p = 0.03157$). Habitat type (wet dune slack vs. dry dune side and top) did not seem to have a significant influence on community composition ($A = 0.01113$, $p = 0.17961$).

The joint plots illustrate the strength and direction of correlation of variables to each ordination axis (Figure 3). The correlation of soil pH with axis 1 was strong ($r = 0.718$). At the phylum level, only Chytridiomycota showed correlation with axis 1 ($r = -0.385$) and was more diverse in the acidic sites, while diversity in other phyla did not show significant trends. At the taxonomic order level, the ascomycete Archaeorhizomycetales, Helotiales, Euroti-ales, Sordariales, and Verrucarioideae and the basidiomycete Trichosporonales, Auriculariales, and the basidiomycete Thelephorales were found to be more diverse in the acidic sites (Figure 3, Table 2). Orders showing greater richness in the alkaline soil samples included the ascomycete Pezizales, Lecanorales, Pleosporales, Oomycetales and the basidiomycete Thelephorales. At the genus level, the diversity in ascomycete genera Archorhizomycetes, Lachnum, Penicillium, and Verticillium and basidiomycete genera Trichosporon, Thelephora, Clavulinaceae, Carinarius, Clavulina, and Mycena were negatively correlated with pH, while the ascomycete Phoma, Exophiala, Podospora, Cladonia, Teratospora, Geopora, Caldodora, Alternaria, Scolobasidium and the basidiomycete Tomentella, Inocybe, Panellus, and Tanatephora were more diverse in the alkaline samples (Figure 3, Table 2).

There were 96 OTUs identified as significant ($p<0.05$) indicators for a certain pH category. Of these, 82 were indicators for acidic and 14 for alkaline sites. Several indicators belonged to root-associated fungi. For example, an unidentified root-endophytic Phialocephala and the ECM Russula pascua (SH117152.05FU), Thelephora albomarginata, Thelephora sp. (SH112629.05FU), and Tulasnellula sp. (SH117022.05FU) were associated with acidic sites, while the ECM Inocybe dianthus (SH110718.05FU) and an unidentified Tomentella were characteristic of the alkaline sites. In addition, there were several saprobic that were found almost exclusively in the acidic soil samples, such as Cryptococcus sp. (SH117277.05FU), Fusarium sp. (SH106152.05FU), various Mortierellales species, Mycena sinian (SH102569.05FU), Penicillium adamentii, Rickenella fihala (SH109302.05FU), Schizolobastosporon starkhymenici, Umbelopsis raveniana (SH100505.05FU), and a Verticillium sp. (SH111596.05FU), some of which can be potentially pathogenic. An unidentified species of the nematode pathogen Pochonia genus (SH106625.05FU) also showed strong preference for acidic sites. There were only two saprobic species identified as indicators for the alkaline soils: Fusarium torulosum and a Podospora species. The full list of indicator OTUs is shown in Table 3.

**Discussion**

**Acidic vs. Non-acidic Sand Dune Fungal Communities**

While environmental factors influencing the distribution of plants and animals have been thoroughly studied, environmental factors controlling the spatial patterns and community composition of soil microorganisms are still poorly understood [49]. Recent molecular studies on various microbial groups (mostly bacteria) have started to explore the distributional patterns of soil communities, however, many of them applied DNA-fingerprinting techniques or phospholipid analyses that do not permit the taxonomic surveys and comparisons of different communities [50],[51]. Soil pH is generally implicitly considered important for shaping fungal communities with some supporting evidence from experimental studies mostly with AM fungi [52],[53] and, in recent years, from joint pyrosequencing analyses of bacterial and fungal communities in agricultural soils and land use types [54],[49]. Unfortunately, both of these latter sequencing studies relied on nuclear small-subunit (SSU) ribosomal genes, meaning that their power of taxonomic resolution was at family-level groups at best. Therefore, information on the effect of soil pH on the
distribution of specific fungal taxa and on the composition of their communities in natural habitats remains surprisingly scarce.

Our large-scale comparison of various acidic and non-acidic coastal dune Salix repens beds clearly indicates that soil pH strongly correlates with the composition of fungal communities. In NMDS ordinations, soil pH had high Pearson correlation values with the dominant ordination axis and overrode the effect of the geological district (Waddenz vs. Renodunal) on community composition in both the NMDS and the MRPP analyses (Figures 3 and 4). This is in agreement with the findings of Van der Heijden et al. [25] that soil pH significantly contributed to the variation explained in ECM morphotype composition of communities associated with S. repens. In our NMDS ordinations and MRPP analyses, Inocybe and Tomentella showed strong preference for alkaline soils, while Clavulinia, Cortinarius, Thelephora, and to some extent Lactarius, were more diverse in acidic sites (Figure 3, Table 2). It is worth noting, that because our study focuses on natural systems, it is impossible to determine based on our current data whether the communities are structured directly by pH or indirectly via the interaction of pH with soil communities and various environmental factors (e.g., nutrient availability, organic C characteristics, vegetation differences etc.). Because results from previous studies using a pH gradient with controlled variables suggest no or only weak effect of pH on community structure [54],[49] and because many fungal species have a relatively wide pH optimum (e.g. [55],[56]), it is likely that the observed correlation of pH with community composition is mainly indirect, e.g., via nutrient availability and altered competitive interactions between soil fungi and bacteria [57], and other soil biota. While our data suggest that the effect of soil pH on fungal community composition may be stronger in relatively undisturbed sites than in agricultural settings, disentangling causal relationships is beyond the scope of our work.

Root-associated Fungi

Although taxa detected by our sequencing efforts featured groups from a wide variety of ecological guilds, including many saprobie and/or plant pathogenic fungi (e.g., Fusarium cereale, Gibberella zeae, Phoma multiaristata) as well as animal or fungal pathogens (e.g., Hypomyces chrysospermus, Metaehtazium ansiophae, M. flavosordire and other Hypocreales), we focus our discussion on groups that are most relevant to current conservation priorities, such as fungi with macroscopic fruitbodies and/or with symbiotic associations with S. repens or with red listed plants (e.g. orchids) in these habitats.

Previous studies based on sporocarps and ECM root structures have shown that Salix repens communities generally harbour numerous ectomycorrhizal basidiomycetes [25],[26]. This was confirmed by our large-scale soil sequence data, as we found 418 ECM OTUs. The majority of these showed similarity to basidiomycete genera known to form associations with willows (e.g. [58],[59],[15]), such as Inocybe (95 OTUs), Thelephora/Tomentella (83), Cortinarius (36), Helvelona (10), Russula (7), Laccaria (7), Clavulina (6), Lactarius (5), Paxillus (2). Also, there were 4 OTUs (Amanita pantherina, Hydnellum rufescens, Suillus luteus and Tulasnella sp.) that represent species that are known to form ECM associations with other host trees found near the sampling sites, such as oak, pine or poplar. In addition, we found 52 OTUs belonging to the Sebacinaceae, a taxonomically difficult group with diverse ecological roles, including various forms of mycorrhizae (ECM, orchid, ericoid, arbutoid) and other associations with plant roots [60]. We also identified several ectomycorrhizal ascomycetes: Ceratocyclosium geophilum, one of the most widespread and abundant ECM fungus with a wide host range [59], Groppa arnicae and G. cervina, a genus known to occur in sand dunes [61], Helvella compressa and H. maculata, and an unidentified Trichophaea species. In addition, we found four truffles that are likely symbionts of Qericea that is widespread in the dunes: Tuber annueae, and three unidentified Tuber species.

Salix repens is one of the very few plants known to form both ECM and AM [24],[62]. In dry coastal foredunes, where phosphorus is the main limiting nutrient, AM fungi and their hosts generally predominate, while nitrogen is the main limiting element in dune slacks, resulting in the dominance of ECM taxa [62]. With the ability to form both ECM and AM associations, Salix repens is particularly well-suited to survive in the highly dynamic dune ecosystems and is able to colonize habitats that range from dry to wet and from acidic to calcareous [62]. We detected 191 glomeromycete OTUs in our samples, representing Glomus (53), Rhizophagus (10), Archeaspora (6), Actinolpora (4), Claroideoglomus (4), Scutellospora (4), Cetraspora (3), Paraglomus (3), Entrophospora (2), Diversispora (1), Racocetra (1), and 100 unidentified glomeromycete OTUs. Because the majority of land plants are known to form AM, it is likely that the majority of these taxa are also associated with a wide range of plants beside Salix repens.

We also found numerous OTUs with very high sequence similarity to known dark sepatate root endophytes (DSE) (e.g., Cadophora, Cladophialophora, Exophiala, Leptodontidiium, Melinomycyes, Phialoschopha, Phialophora spp.). Although DSE colonization and diversity have previously been shown to be higher in acidic conditions in general [63],[64], our data suggest that different genera may prefer different pH conditions. For example, Cadophora and Exophiala were represented by more OTUs in the alkaline sites, while Melinomyces was somewhat more diverse in samples with lower pH (Table 2).

The sampling areas included in our study harbor some of the richest orchid populations in the Netherlands. The diversity of orchid mycorrhizae may be particularly relevant for nature conservation as they provide symbioses to this unique group of plants with high public appeal and conservation value. The majority of orchid mycorrhizal symbionts belong to the Ceratobasidiaceae, Sebacinaeae and Tulasnellaceae [65],[66], although ECM fungi have also been reported to be associated with orchids, albeit mainly with non-photosynthetic, mycoheterotrophic species that often use the ECM fungus as a channel to obtain carbohydrates from the ECM host tree [65],[67]. Among the first group of orchid symbionts that are typically associated with photosynthetic orchids, we found 21 Ceratobasidiaceae and 12 Tulasnellaceae OTUs in addition to the 52 Sebacinaeae OTUs mentioned above. Among other ECM lineages, the basidiomycote Inocybe, Tomentella, and the ascomycete Tuber species have been found to form mycorrhizal associations with the mycoheterotrophic orchid Epipactis helleborine [67],[68]. This orchid is represented by two subspecies in the Netherlands, among these, E. h. ssp. helleborine is widespread in a variety of habitats, while E. h.
| OTU  | pH     | Accession no. | % bp | SH | Name                  | Phylum          | Order           |
|------|--------|---------------|------|----|-----------------------|-----------------|-----------------|
| 1573 | acidic | JQ273136      | 99.1 | 230 | SH14087605FJ          | Agaricomycotina | -               |
| 1200 | acidic | JQ273136      | 80.4 | 337 | SH11310405FJ          | Arthropoidea     | Acarina         |
| 339  | acidic | JQ273136      | 98.6 | 219 | SH11310405FJ          | Arthropoidea     | Acarina         |
| 341  | acidic | JQ273136      | 99.1 | 244 | SH11310405FJ          | Arthropoidea     | Acarina         |
| 372  | acidic | JQ273136      | 99.1 | 314 | SH11310405FJ          | Arthropoidea     | Acarina         |
| 1685 | acidic | JQ273136      | 97.1 | 219 | SH11310405FJ          | Arthropoidea     | Acarina         |
| 735  | acidic | JQ273136      | 97.3 | 257 | SH11310405FJ          | Arthropoidea     | Acarina         |
| 1200 | acidic | JQ273136      | 96.5 | 201 | SH11310405FJ          | Arthropoidea     | Acarina         |
| 2601 | acidic | JQ273136      | 99.5 | 291 | SH11310405FJ          | Arthropoidea     | Acarina         |
| 374  | acidic | JQ273136      | 100  | 221 | SH11310405FJ          | Arthropoidea     | Acarina         |
| 397  | acidic | JQ273136      | 97.3 | 181 | SH11310405FJ          | Arthropoidea     | Acarina         |
| 361  | alkaline | JQ273136    | 100  | 303 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 4597 | alkaline | JQ273136   | 99.2 | 228 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 605  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 540  | alkaline | JQ273136    | 99.1 | 201 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 686  | alkaline | JQ273136    | 99.3 | 228 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 746  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 395  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 480  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 610  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 686  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 4961 | alkaline | JQ273136   | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 480  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 610  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 686  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 347  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 4961 | alkaline | JQ273136   | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 610  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 686  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 347  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 4961 | alkaline | JQ273136   | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 610  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 686  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 347  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 4961 | alkaline | JQ273136   | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 610  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| 686  | alkaline | JQ273136    | 99.7 | 181 | SH11310405FJ         | Arthropoidea     | Acarina         |
| OTU | pH     | p       | Accession no. | % bp | SH     | Name                      | Phylum          | Order          |
|-----|--------|---------|---------------|------|--------|---------------------------|-----------------|----------------|
| 158 | alkaline | 0.0084  | KC180725      | 100  | 154    | Podospora sp.             | Ascomycota      | Sordariales    |
| 859 | acidic  | 0.0047  | UDB015270     | 99.3 | 272    | Rickenella fibula         | Basidiomycota   | Hymenochaetales|
| 1456| acidic  | 0.0468  | FN687605      | 95.7 | 161    | Russula pascua            | Basidiomycota   | Russulales     |
| 627 | acidic  | 0.0468  | UDB017659     | 98.5 | 197    | Russula pascua            | Basidiomycota   | Russulales     |
| 429 | acidic  | 0.0294  | HF558658      | 99.6 | 262    | Schizoblastosporion starkeyiHenricii | Ascomycota | Saccharomycetales |
| 1618| alkaline | 0.0294  | JN802314      | 98.1 | 210    | Sordariales sp.           | Ascomycota      | Sordariales    |
| 346 | acidic  | 0.0084  | JQ761571      | 99   | 196    | Sordariomycetes sp.       | Ascomycota      | -              |
| 555 | acidic  | 0.023   | HQ211703      | 91.6 | 323    | Sordariomycetes sp.       | Ascomycota      | -              |
| 560 | acidic  | 0.047   | UDB017379     | 100  | 216    | Thelephora albo marginata  | Basidiomycota   | Thelephorales  |
| 514 | alkaline| 0.0468  | JN858076      | 100  | 192    | Thelephora sp.            | Basidiomycota   | Thelephorales  |
| 1281| alkaline| 0.0458  | KC840637      | 99.1 | 222    | Tomentello sp.            | Basidiomycota   | Thelephorales  |
| 568 | acidic  | 0.0494  | GQ268679      | 88.4 | 258    | Trechisporales            | Basidiomycota   | Trechisporales |
| 1126| acidic  | 0.0376  | JF691365      | 96.2 | 213    | Trechisporales sp.        | Basidiomycota   | Trechisporales |
| 420 | acidic  | 0.0426  | JF519135      | 100  | 195    | Trechisporales sp.        | Basidiomycota   | Trechisporales |
| 5679| acidic  | 0.044   | JF519135      | 95.5 | 156    | Trechisporales sp.        | Basidiomycota   | Trechisporales |
| 4735| acidic  | 0.0234  | JQ272445      | 96.5 | 173    | Tremellomyctes sp.        | Basidiomycota   | -              |
| 1929| acidic  | 0.047   | JN655663      | 99.2 | 242    | Tulostrella sp.           | Basidiomycota   | Cantharellales |
| 2522| acidic  | 0.0494  | EU715662      | 97.4 | 193    | Umbelapsis ramanniana     | Mucoromycotina  | Mucorales      |
| 540 | acidic  | 0.0252  | KC489481      | 96.4 | 195    | Umbelapsis sp.            | Mucoromycotina  | Mucorales      |
| 561 | acidic  | 0.0166  | HQ157863      | 98.4 | 253    | Umbelapsis sp.            | Mucoromycotina  | Mucorales      |
| 731 | acidic  | 0.033   | KC489481      | 99   | 204    | Umbelapsis sp.            | Mucoromycotina  | Mucorales      |
| 1205| acidic  | 0.047   | DQ914739      | 97.4 | 229    | Verticillium sp.          | Ascomycota      | Incertae sedis |
| 1037| acidic  | 0.044   | KC588535      | 89.4 | 227    | uncultured fungus         | -               | -              |
| 1076| acidic  | 0.0084  | KC588535      | 86.5 | 296    | uncultured fungus         | -               | -              |
| 1211| alkaline| 0.0049  | AJ875364      | 99.7 | 343    | S100236.05FU              | uncultured fungus | - |
| 1459| alkaline| 0.0486  | JQ312779      | 100  | 209    | uncultured fungus         | -               | -              |
| 1651| acidic  | 0.047   | HM439535      | 96.8 | 220    | S101693.05FU              | uncultured fungus | - |
| 1845| acidic  | 0.0084  | FJ197879      | 93.1 | 232    | uncultured fungus         | -               | -              |
| 1913| acidic  | 0.047   | EF521253      | 80.6 | 345    | uncultured fungus         | -               | -              |
| 1969| acidic  | 0.047   | JQ313102      | 99.6 | 226    | uncultured fungus         | -               | -              |
| 2667| alkaline| 0.049   | EU516756      | 89.6 | 328    | uncultured fungus         | -               | -              |
| 328 | acidic  | 0.0046  | JQ312820      | 98.5 | 259    | uncultured fungus         | -               | -              |
| 358 | alkaline| 0.0458  | JN889968      | 99.4 | 315    | S102623.05FU              | uncultured fungus | - |
| 363 | acidic  | 0.0084  | KC588772      | 100  | 261    | uncultured fungus         | -               | -              |
| 367 | alkaline| 0.0084  | JK316640      | 99   | 306    | uncultured fungus         | -               | -              |
Table 3. Cont.

| OTU | pH      | p     | Accession no. | % bp | SH            | Name              | Phylum | Order |
|-----|---------|-------|---------------|------|---------------|--------------------|--------|-------|
| 401 | acidic  | 0.0244| KC588704      | 100  | 320           | uncultured fungus  | -      | -     |
| 4316| alkaline| 0.0188| GU308427      | 99.6 | 255           | uncultured fungus  | -      | -     |
| 4368| acidic  | 0.0224| KC416188      | 97.5 | 158           | uncultured fungus  | -      | -     |
| 4423| acidic  | 0.044 | KC416184      | 95   | 161           | uncultured fungus  | -      | -     |
| 451 | acidic  | 0.0084| AF504839      | 99.7 | 364           | SH109673.05FU      | uncultured fungus | -     | -     |
| 466 | alkaline| 0.034 | FN397289      | 100  | 322           | SH112415.05FU      | uncultured fungus | -     | -     |
| 4887| acidic  | 0.0396| JQ312820      | 91.7 | 220           | uncultured fungus  | -      | -     |
| 502 | acidic  | 0.0084| DQ309106      | 97.6 | 328           | SH114572.05FU      | uncultured fungus | -     | -     |
| 573 | acidic  | 0.047 | JQ312957      | 94.5 | 218           | uncultured fungus  | -      | -     |
| 6263| acidic  | 0.047 | JQ312957      | 96.1 | 207           | uncultured fungus  | -      | -     |
| 630 | acidic  | 0.047 | AM260886      | 97.8 | 274           | SH110947.05FU      | uncultured fungus | -     | -     |
| 643 | acidic  | 0.0084| KC588567      | 99.3 | 283           | uncultured fungus  | -      | -     |
| 6519| alkaline| 0.049 | JX316660      | 95.7 | 207           | uncultured fungus  | -      | -     |
| 6520| acidic  | 0.048 | KC588554      | 95.9 | 217           | uncultured fungus  | -      | -     |
| 665 | acidic  | 0.047 | DQ309136      | 99.6 | 271           | uncultured fungus  | -      | -     |
| 711 | acidic  | 0.047 | HM136622      | 99.2 | 355           | uncultured fungus  | -      | -     |
| 713 | acidic  | 0.047 | JQ312796      | 99.5 | 199           | uncultured fungus  | -      | -     |
| 723 | acidic  | 0.036 | DQ3093782     | 97.6 | 329           | SH099908.05FU      | uncultured fungus | -     | -     |
| 762 | acidic  | 0.0458| JQ312820      | 96.4 | 193           | uncultured fungus  | -      | -     |
| 787 | acidic  | 0.0084| JF300531      | 90.2 | 368           | uncultured fungus  | -      | -     |
| 803 | acidic  | 0.0084| JN890270      | 96.3 | 327           | uncultured fungus  | -      | -     |
| 838 | acidic  | 0.0434| FN298702      | 93.3 | 326           | SH104632.05FU      | uncultured fungus | -     | -     |
| 852 | acidic  | 0.0454| JQ312820      | 97.8 | 230           | uncultured fungus  | -      | -     |
| 857 | acidic  | 0.0162| HM037687      | 99   | 196           | SH10459.05FU       | uncultured fungus | -     | -     |
| 899 | acidic  | 0.047 | HM028720      | 99.4 | 334           | SH099545.05FU      | uncultured fungus | -     | -     |
| 828 | acidic  | 0.0308| HQ021815      | 99.7 | 320           | SH108513.05FU      | uncultured fungus | -     | -     |

Where available, the Species Hypothesis (SH) numbers are given for the corresponding sequence as published by Köljalg et al. (2013). doi:10.1371/journal.pone.0099852.t003
Figure 4. Examples for OTUs found in our soil samples showing high sequence similarity with rare, red-listed fungi. Sporocarp records (before and after 1990) are based on the mapping database of the Dutch Mycological Society (http://www.verspreidingsatlas.nl/paddenstoelen).

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**Contribution of Soil Data to Mapping Rare Fungi**

The Netherlands boasts one of the most efficient programs in the world for gathering data on macrofungi, where the Netherlands Mushroom Mapping Working Group (Werkgroep Paddebuinenkartering Nederlandse WPN) has been producing distribution maps and fruiting statistics since 1989 with a nationwide network of ca. 600 volunteers that produce approximately 60,000 data points yearly [70],[71],[72]. The long-term mapping program of the WPN has been crucially important in compiling a near complete checklist of macrofungi [73], in highlighting high diversity sites [70], and in drawing attention to the serious decline of macrofungi in the Netherlands [72]. As a result, an official national Red List of fungi was published in 1996, followed by a revision in 2008 [74]. On the other hand, due to inherent limitations of the data collecting method, data on the diversity and distribution of microfungi and taxa with inconspicuous fruitbodies have remained scarce. Building on the methodological advances provided by next-generation sequencing techniques, we can still greatly improve the exhaustiveness of species inventories by providing complementary information, particularly with regard to asexual fungi and species with microscopic and/or inconspicuous fruiting bodies, in addition to providing new spatial data points for macrofungi already featured in the species lists.

The massive amount of sequence data we generated provide the first kingdom-wide fungal diversity assessment for this coastal ecosystem, offering a complementary view on species diversity with a particularly large contribution on asexual fungi and species with microscopic and/or inconspicuous fruiting bodies that fall beyond the scope of the above mapping project. Even for macrofungi, despite the obvious spatial limitations of the methodology we used, we detected numerous red listed species in our samples, often from previously unknown locations. Several taxa with scarce (<5) previous sporocarp records, such as *Inocybe exilis, Pseudobolbitius typhofera, Russula pusca*, *Sectellaria vitroela* and several *Tomentella* and *Sebacina* species to name a few, were found in multiple locations, indicating that they may be more widespread in the Netherlands than previously thought (Figure 4). This may be particularly true for *Tomentella*, a species-rich genus with often inconspicuous sporocarps, that is regularly shown to be much more diverse and abundant in soil and root samples than in sporocarp-based diversity assessments (e.g., [15],[75],[76]). We also provide valuable data for additional taxa that are considered either rare or sporadic, often with “vulnerable” or “endangered” red list categories, e.g., *Clavaria taylorii*, *Clavulinopsis trunculata*, *C. lutea*, *Canocybe subversiphlyctica*, *Cortinarius abietorum*, *C. cusimirii*, *C. cinnamonus*, *C. dusenbergeri*, *C. parcannulatus*, *Entoloma asperrile*, *E. clandestinum*, *E. longistriatum*, *E. turbidum*, *Geastrum stratum*, *Geoglossum felax*, *G. umbreile*, *Geopora arenicola*, *Hbelonema collariatum*, *H. leucocarpus*, *H. nigellum*, *Hemimycena tortuosa*, *Hydnum fusescens*, *Hygrocybe helobia*, *Hygrophorus vitellina*, *Hygrocybe agaridii*, *I. decipiens*, *I. dunensis*, *I. gisseguelata*, *I. jacobi*, *I. nitidissula*, *I. servitina*, *I. vulpinella*, *Lactarius amanitaceus*, *Myccena abellolitica*, *Panuridae papilionaceus*, *Peziza ampliata*, *Pleurotus eryngii*, *Rassula foetens*, *Russula parallata*, *Squamanita odorata*, *Tephrocybe tylidora*, *Thamnophenos cucumeris*, *Tricholoma lacticum*, *Tulostoma brunna*, *Xerocomus riperellus* et al.

Highly similar matching sequences from species not formerly reported from the Netherlands represent a special case and confirming the presence of some species will require additional evidence. Some such examples among ascomycetes are the truffle *Tuber melanosporum*, the earth-tongue *Geoglossum glandulare*, the cup fungus *Geporina cervina*, and the false morel *Verpa digitalisformis*. In Basidiomycota, the list of taxa formerly not reported from the Netherlands and found among the highly similar (>98%) matching sequences of our OTUs includes: *Clavaria californica*, *Cortinarius javieri*, *C. crisipes*, *Heterobasidion parviporum*, *Hygrocybe persistens*, *Inocybe giancima*, *Myccena simia*, *Platyn satur*, *Rassula integra*, *R. nana*, *Sphaerobolus ingoldii*, and *Tubaria minima*. Occurrence data regarding species that usually occur in arctic-alpine regions, such as *Cortinarius javieri* and *Rassula nana*, are particularly interesting, given the geographic position and the lack of mountains in the Netherlands. In the Arctic, these species are symbionts of various dwarf willow species (e.g. *Salix polaris*, *S. reticulata*), [77], [Grem pers. obs.], while in the Netherlands, they appear to grow with *Salix repens*. Both *Cortinarius javieri* and *Rassula nana* have been found associated with *Salix repens* in the oceanic archipelago north of Scotland [26]. It has to be noted that *Rassula lucata*, a species originally described from the Netherlands albeit with no current mapping data, is very closely related to *R. nana* and whether or not they are truly distinct species is currently uncertain. Therefore, detailed taxonomic work is needed to test whether or not the two taxa are conspecific and whether *R. lucata* refer to the same taxon as our OTU (no. 695), in which case the name *R. nana* will be preserved based on priority.

**Conclusions**

Fungal communities are notoriously difficult to fully characterize for ecological and biodiversity studies and for conservation purposes. Even for macrofungi (e.g., such as mushrooms, true and false truffles etc.), that have the longest history of diversity studies among fungi [1],[70],[78], basic questions about the number of species at a given location or differences in species richness among vegetation types have generally remained unanswered due to taxonomic problems and the scarcity of long-term sporocarp-monitoring projects [78].

Our work provides a wealth of information on the kingdom-wide diversity of fungal communities along the protected coastal dune habitats in the Netherlands, extending both the list of species likely occurring in the Netherlands as well as providing new data points for many rare species. Furthermore, our work highlights that fungal communities found in the rhizosphere of *S. repens* can have very different species composition due to edaphic factors and that the protection of areas that harbour such varied *S. repens* beds is vital for the conservation of co-habiting fungi, including many red-listed species. Future fruitbody collecting projects should utilize our data to concentrate sampling and monitoring efforts on selected localities in order to confirm species occurrences that are currently only based on soil sequence data. Although more detailed, taxon-specific studies will follow, this project already provides examples for the potential contribution of high-throughput soil sequencing studies to fungal mapping and conservation.

**Data Accessibility**

The raw data have been submitted to the European Nucleotide Archive (ERP001713).
Supporting Information

Table S1 List of OTUs that showed >97% ITS2 sequence similarity to fully identified fungi in coastal Salix repens communities used in this study with best identified match (GenBank or UNITE accession number, Species Hypothesis, name), similarity (%), pairwise alignment length (bp), sequence counts in the sampled sites, phylum, and order classifications.

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Author Contributions

Conceived and designed the experiments: JG MN. Performed the experiments: JG BKVDG PDK MN. Analyzed the data: JG BKVDG MN YL NR TS PDK MN. Contributed reagents/materials/analysis tools: JG BKVDG PDK MN. Contributed to the writing of the manuscript: JG BKVDG MN YL NR TS PDK MN.

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