Short-communication

Title: Old fashion or trendy? Which is the best approach for assessing diversity of aquatic decomposer fungi in streams?

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Abstract

We assessed fungal diversity in Autumn and Spring on leaves decomposing in 5 streams along a gradient of eutrophication in the Northwest of Portugal, through microscopy-based (spores morphology identification) and DNA-based techniques (Denaturing Gradient Gel Electrophoresis and 454 pyrosequencing). Pyrosequencing revealed 5 times more diversity then DGGE. About 21% of all aquatic hyphomycete species were exclusively detected by pyrosequencing and 26% exclusively by spores identification. In some streams, more than half of the recorded species would be unnoticed if we had relied only on spores identification. Nevertheless, in Spring fungal diversity was higher based on spore identification, probably because many species occurring in this season are not connected to any ITS barcode in genetic databases yet. Pyrosequencing was a powerful tool for revealing fungal diversity on decomposing plant litter in streams and we strongly encourage researchers to continue the effort in barcoding fungal species.

Index descriptors: aquatic hyphomycetes; streams; fungal diversity; 454 pyrosequencing; denaturing gradient gel electrophoresis; fungal spores morphology

Introduction

Freshwater fungi, in particular aquatic hyphomycetes, play a pivotal role in organic matter turnover in headwater streams (Gessner et al. 2007, Graça and Canhoto 2006). By producing a vast array of extracellular enzymes that break down complex plant polymers and improve leaf palatability, aquatic hyphomycetes transform plant material into a more suitable and nutritious food source for invertebrate shredders (Graça 2001, Suberkropp 1998). Conventionally, stream-dwelling decomposer fungal communities are characterized by the microscopic identification of spores released from colonized plant litter (Bärlocher 2005, Gessner et al. 2003). However, the absence of spores may be due to absence of the species or presence of non-sporulating mycelium (Duarte et al. 2010, Nikolcheva et al. 2003). Molecular approaches, based on nucleic acids such as Denaturing Gradient Gel Electrophoresis (DGGE), have been used successfully to circumvent these hurdles (for a review see Duarte et al. 2012). Although DGGE community fingerprints give an estimate of species richness, it does not provide information of species identity. On the other hand, high-throughput sequencing techniques allow the identification of species within communities through direct extraction of DNA sequences from environmental samples, and comparison with reference sequences in genetic databases (e.g. Duarte et al. 2015, Kerekes et al. 2013).

In this work, we assessed fungal diversity on *Quercus robur* leaves decomposing in 5 streams along a gradient of eutrophication in the Northwest Portugal (Duarte et al. 2015, Dunck et al. 2015, Lima-Fernandes et al. 2015), in Autumn 2012 and Spring 2013, through microscopy-based techniques (identification of spores), and by molecular techniques (DGGE and 454 pyrosequencing) of the transcribed spacer 2 region (ITS2) of ribosomal RNA gene (rDNA). Because fungal reproduction is highly sensitive to nutrients in stream water (Duarte et al. 2009, Suberkropp and Chauvet 1995), we

hypothesized that fungal diversity would be more accurately assessed using molecular approaches particularly in streams with high levels of eutrophication in which fungal reproduction might be compromised. However the success of species identification by using pyrosequencing will greatly depend on the available reference sequences present in genetic databases.

Methods

Sampling sites and experiment setup

The study was conducted in five streams of the Ave River basin (Northwest Portugal). The stream sites span a range of eutrophication as follows: Agra Stream < Oliveira Stream < Andorinhas Stream < Selho River < Couros Stream (for detailed characterization see Duarte et al. 2015, Dunck et al. 2015, Lima-Fernandes et al. 2015). Oak (*Quercus robur* L.) air-dried leaves, collected immediately before abscission in Autumn 2007, were weighed in portions of 3 g (±0.001 g) and placed in plastic mesh bags (30 × 23 cm; 5-mm mesh size; 4 replicates). The bags were immersed at each site on 5th November 2012 (Autumn) and 29th April 2013 (Spring). After 23 days of stream immersion, leaf bags were collected from the stream, placed individually in zip-lock plastic bags, and transported in a cool box (4 ºC) to the laboratory. Leaf material was cut into 12-mm leaf disks and used to induce fungal sporulation and estimate fungal diversity by DGGE and 454 pyrosequencing.

Assessment of fungal diversity

Fungal sporulation was induced by aeration of 8 leaf disks in 75 mL of sterilized stream water (0.22-µm pore size, Sarstedt, Nümbrecht, Deutschland) for 48 ± 4 h, at 16 ºC. Conidial suspensions were mixed with 0.5% triton X-100, filtered (0.45-µm pore

size, Millipore, Merck KGaA, Darmstadt, Germany), and stained with 0.05% cotton blue in lactic acid. At least 300 spores per filter were counted and identified under a light microscope to determine the contribution of each species to the total conidia produced by each fungal assemblage.

DNA was extracted from 4 freeze-dried leaf disks using the MoBio UltracleanTM Soil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions, except for the lysis step that was conducted in the FastPrep FP120 instrument (velocity 5.5, duration 30 sec., twice) (Qbiogene, Heidelberg, Germany). Half of the DNA extracts were used for the PCR amplification followed by DGGE analyses, and the other half was sent to Genoinseq (Biocant, Cantanhede, Portugal) for 454 pyrosequencing analyses.

PCR reactions of amplicons for DGGE analyses were done according to Seena et al. (2012), using the primer pairs ITS3GC and ITS4 (White et al. 1990), which amplify the ITS2 region of fungal rRNA gene. DGGE analyses were performed using a DCode^{TM} universal mutation detection system (Bio-Rad Laboratories, Hercules, CA, USA) according to Duarte et al. (2010) with few modifications: gels had a denaturing gradient from 30 to 60% and were stained with 1x Midori Green (NIPPON Genetics EUROPE GmbH, Düren, Germany) for 10 min. A DNA mixture of 7 aquatic hyphomycete taxa was used to align the gels in further analyses. DGGE gels were aligned and normalized using BioNumerics 7.1 (Applied Maths, Sint-Martens-Latem, Belgium).

For 454 pyrosequencing, DNA extracts from 3 replicate bags were combined, giving a total of 10 DNA extracts (5 streams x 2 seasons). The ITS2 region was amplified with ITS3 and ITS4 primers (White et al. 1990) as described by Duarte et al. (2015). Raw data were analyzed in March 2014, as described by Duarte et al. (2015).

Results and Discussion

Fungal diversity assessed from DGGE operational taxonomic units (OTUs) was higher in Autumn than in Spring for most streams, except for Selho (Fig.1A). The number of DGGE OTUs varied between 16 for Andorinhas in Spring and 35 for Couros in Autumn. Overall, the number of DGGE OTUs in Spring did not change much between streams; but, in Autumn, Couros presented the highest number of DGGE OTUs. On the other hand, the number of pyrosequencing OTUs was on average 5 times higher than that assessed by DGGE (Fig.1B), varying between 61 OTUs for Andorinhas and 167 OTUs for Oliveira, both in Autumn. The discrepancy between the 2 molecular techniques may be due to DGGE limitations: 1) the resolution limit is about 1% of the community population (Muyzer et al. 1993); minor populations may be below the detection limit; 2) different DNA sequences may have similar motilities due to identical GC contents (Muyzer et al. 2004), and so beyond one DGGE band/OTU, DNA from more than one species may be present (Duarte et al. 2010); and 3) the resolution of DGGE gels may be spatially limited by the gel length. But still, substantial information about species composition in complex fungal communities can be obtained from DGGE, which can be particularly valuable for an initial screening of fungal diversity. More detailed analyses of communities' composition may be then obtained by pyrosequencing of selected samples.

Aquatic fungal diversity (as aquatic hyphomycete species) increased in streams with moderate eutrophication (Oliveira and Andorinhas) and decreased in the most eutrophic streams (Selho and Couros) (Duarte et al. 2015, Dunck et al. 2015, Lima-Fernandes et al. 2015) (Fig.S1). Also, a higher number of aquatic fungal species was observed in Spring for most streams, contrarily to overall diversity assessed from

DGGE and pyrosequencing, which was higher in Autumn. Probably, both molecular techniques detected other species besides aquatic fungi (Duarte et al. 2015). In fact, since DGGE gives an estimation of the total fungal richness within assemblages this will overestimate aquatic fungal diversity when relying only on DGGE (Duarte et al. 2010). This issue may be circumvented by developing new primers with higher specificity for this particular group of fungi.

Fifty tree percent of aquatic fungal species was detected by the identification of spore morphology and pyrosequencing, while 26% were detected based on spore morphology and 21% by using pyrosequencing (Fig.2). The percentage of aquatic fungal species identified by pyrosequencing was higher in Autumn than in Spring. In fact, at least 50% of the species would have been unnoticed in Autumn if fungal diversity had been assessed only through spore identification in Agra (the most oligotrophic stream), Selho and Couros (most eutrophic streams) (Duarte et al. 2015, Lima-Fernandes et al. 2015) (Fig.2). Fungal reproduction is known to be sensitive to stream water nutrients (Duarte et al. 2009, Suberkropp and Chauvet 1995); for instance, the absence of spores may not indicate that the species is absent but that its ability to produce spores is compromised at high levels of eutrophication. Contrarily, in Spring fungal diversity was higher based on spore identification. Most species which were only identified through spore morphology (9 species) occurred in Spring (6 species) and are not yet connected to any ITS barcode in genetic databases, and thus its identity cannot be assessed through pyrosequencing (Table S1). In temperate regions, litter fall occurs mainly during Autumn and most barcoded aquatic fungal species may have been isolated in this particular time of the year. However, aquatic fungal diversity is reported to vary with season (Nikolcheva and Bärlocher 2005), and this should encourage

researchers to expand sampling effort, in what concerns fungal isolation and barcoding, throughout the year.

Overall, 454 pyrosequencing proved to be a powerful tool for revealing fungal diversity on decomposing-plant litter in streams and strongly encourages researchers to continue the effort in barcoding fungal species. Meanwhile, traditional microscope-based approaches can complement the trendiest technology to obtain a more complete overview of aquatic fungal diversity in streams.

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References

Bärlocher, F., 2005. Freshwater fungal communities, in: Dighton, J., Oudemans, P., White, J. (Eds.), The fungal community: its organization and role in the ecosystem. Taylor and Francis, CRC Press, Boca Raton, Florida, pp. 39-59.

Duarte, S., Bärlocher, F., Trabulo, J., Cássio, F., Pascoal, C., 2015. Stream-dwelling fungal decomposer communities along a gradient of eutrophication unraveled by 454 pyrosequencing. Fungal Divers. 70, 127-148.

Duarte, S., Cássio, F., Pascoal, C., 2012. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology - insights from freshwaters, in: Magdeldin, S. (Eds.), Gel Electrophoresis - Principles and Basics. InTech, pp. 173-196.

Duarte, S., Pascoal, C., Alves, A., Correia, A., Cássio, F., 2010. Assessing the dynamic of microbial communities during leaf decomposition in a low-order stream by microscopic and molecular techniques. Microbiol. Res. 165, 351-362.

Duarte, S., Pascoal, C., Garabetian, F., Cássio, F., Charcosset, J.-Y., 2009. Microbial decomposer communities are mainly structured by trophic status in circumneutral and alkaline streams. Appl. Environ. Microbiol. 75, 6211-6221.

Dunck, B., Lima-Fernandes, E., Cássio, F., Cunha, A., Rodrigues, L., Pascoal, C., 2015. Responses of primary production, leaf litter decomposition and associated communities to stream eutrophication. Environ. Pollut. 202, 32-40.

Gessner, M.O., Barlocher, F., Chauvet, E., 2003. Qualitative and quantitative analyses of aquatic hyphomycetes in streams. Fungal Diversity Research Series. 10, 127-157.

Gessner, M.O., Gulis, V., Kuehn, K.A., Chauvet, E., Suberkropp, K., 2007. Fungal decomposers of plant litter in aquatic ecosystems, in: Kubicek, C.P., Druzhinina, I.S. (Eds.), The Mycota: environmental and microbial relationships. Springer, Berlin, pp. 301-321.

Graça, M.A.S., 2001. The role of invertebrates on leaf litter decomposition in streams - A review. Int. Rev. Hydrobiol. 86, 383-393.

Graça, M.A.S., Canhoto, C., 2006. Leaf litter processing in low order streams. Limnetica. 25, 1-10.

Kerekes, J., Kaspari, M., Stevenson, B., Nilsson, R.H., Hartmann, M., Amend, A., Bruns, T.D., 2013. Nutrient enrichment increased species richness of leaf litter fungal assemblages in a tropical forest. Mol. Ecol. 22, 2827-2838.

Lima-Fernandes, E., Fernandes, I., Geraldes, P., Pereira, A., Cássio, F., Pascoal, C., 2015. Eutrophication modulates plant-litter diversity effects on litter decomposition in streams. Freshw. Sci. 34, 31-41.

Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C., Schäfer, H., Wawer, C., 2004. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, in: Kowalchuk, G.A., de Bruijn, F.J., Head, I.M., Akkermans, A.D.L., van Elsas, J.D. (Eds.), Molecular microbial ecology manual. Kluwer Academic Publishers, The Netherlands, pp. 743-769.

Muyzer, G., de Waal, E., Uitierlinden, A., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695-700.

Nikolcheva, L.G., Bärlocher, F., 2005. Seasonal and substrate preferences of fungi colonizing leaves in streams: traditional versus molecular evidence. Environ. Microbiol. 7, 270-280.

Nikolcheva, L.G., Cockshutt, A.M., Bärlocher, F., 2003. Determining diversity of freshwater fungi on decaying leaves: Comparison of traditional and molecular approaches. Appl. Environ. Microbiol. 69, 2548-2554.

Seena, S., Duarte, S., Pascoal, C., Cássio, F., 2012. Intraspecific variation of the aquatic fungus Articulospora tetracladia: an ubiquitous perspective. PLoS ONE. 7, e35884.

Suberkropp, K., 1998. Microorganisms and organic matter decomposition, in: Naiman, R.J., Bilby, R.E. (Eds.), River ecology and management: lessons from the Pacific coastal ecoregion. Springer, New York, USA pp. 120-143.

Suberkropp, K., Chauvet, E., 1995. Regulation of leaf breakdown by fungi in streams: influences of water chemistry. Ecology. 76, 1433-1445.

White, T., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: Innis, M., Gelfand, D., Sninsky, J., White, T. (Eds.), PCR Protocols: a guide to methods and applications. Academic Press, New York, pp. 315-322.

Figure legends

Fig.1 Total number of OTUs on decomposing oak leaves in the 5 streams of the Ave River basin in Autumn 2012 and Spring 2013, based on DGGE fingerprinting (A) and 454 pyrosequencing (B) of the ITS2 region of the rRNA gene. Ag, Agra Stream; Ol, Oliveira Stream; An, Andorinhas Stream; Se, Selho River; Co, Couros Stream.

Fig.2 Number of aquatic hyphomycete species identified by spore morphology, 454 pyrosequencing or by using both methods in the 5 streams of the Ave River basin, in Autumn 2012 and Spring 2013.

Figure1
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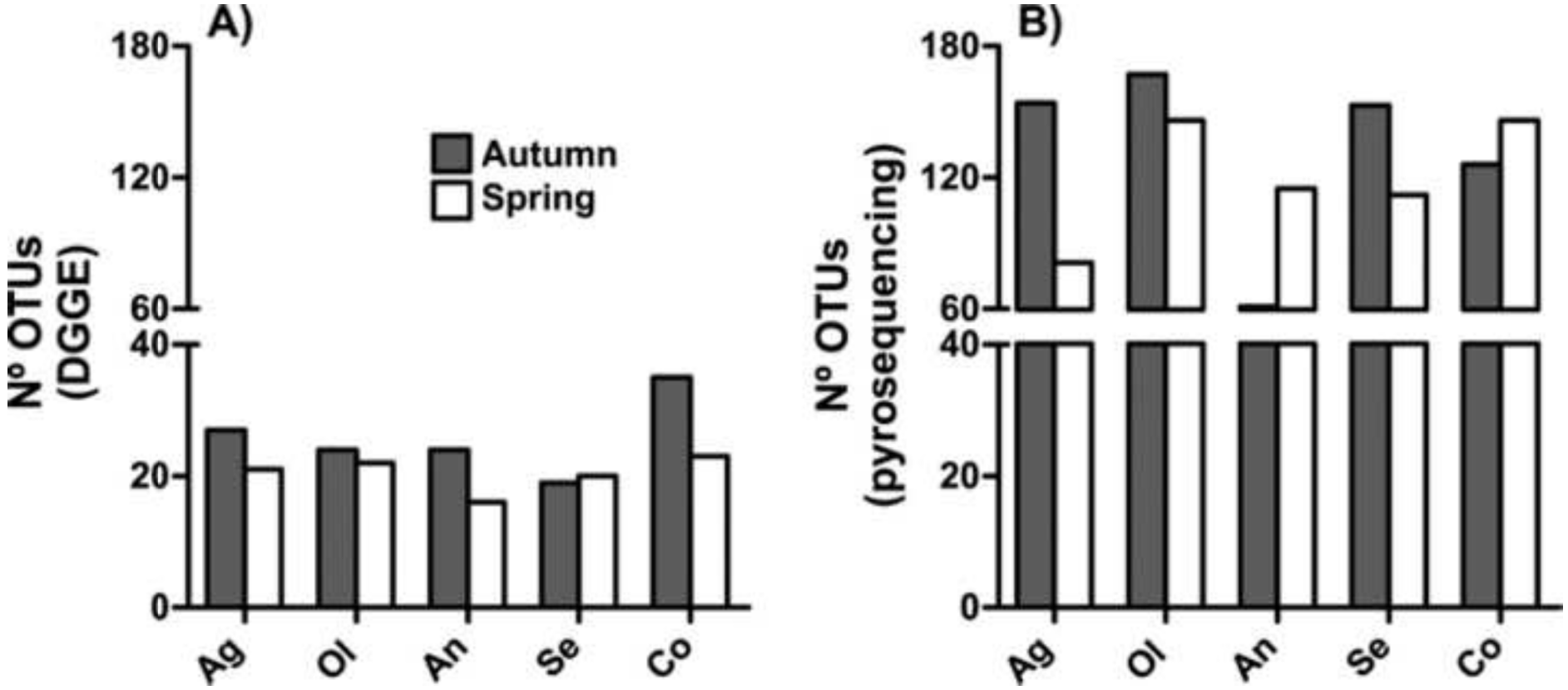

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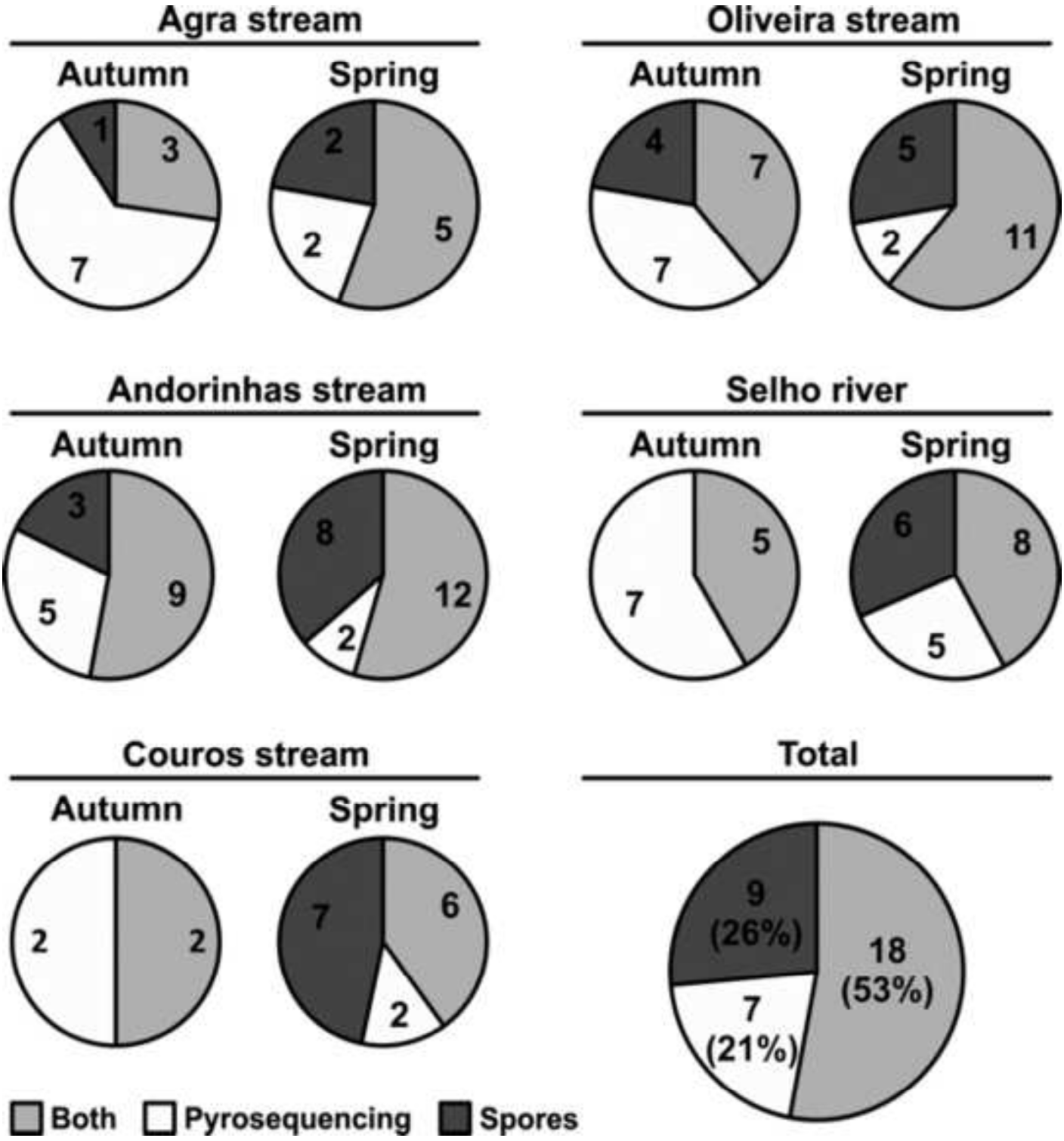

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