Published in Fungal Diversity 49, issue 1, 93-100, 2011,
which should be used for any reference to this work

SSU rRNA reveals major trends in oomycete evolution

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Abstract Oomycetes are a group of heterokonts that have a huge impact on the environment as well as on human welfare, due the parasitic nature of many species. However, their evolutionary patterns are still not well understood, due in part to the lack of molecular markers suited to resolve the deep phylogeny of this group. Here, we propose a phylogeny of the whole clade based on the nuclear ribosomal small subunit gene, that comprises both culture and environmental studies derived sequences. Our analysis shows notably that i) plant pathogenesis occurred only rarely in oomycete evolution in comparison to animal parasitism ii) obligate symbiosis happened only in a few derived groups and iii) transitions from soil/freshwater to marine environment (and viceversa) are common unlike for most eukaryotic groups. This study illustrates the complexity of evolutionary patterns and will help to better understand the emergence of pathogenicity in the different oomycete groups.

Keywords Oomycetes · Pythium · Phytophthora · Albugo · Phylogeny · Plant diseases · Evolution

Introduction

Oomycetes are a large and ubiquitous group of eukaryotic microorganisms. Its most famous representatives are certainly the plant pathogenic species, like *Phytophthora infestans*, which had a dramatic impact on agriculture which resulted historically in famines and massive emigration (Kamoun 2001). In present times, a species like *Phytophthora sojae* is hampering soybean production in several continents (Kamoun 2001). Other plant pathogens, such as *Phytophthora cinnamomi, P. pinifolia* and *P. ramorum* are causing major damages to forest ecosystems and to the wood industry in Australia, Chile and the USA (Duran et al. 2010; Rizzo et al. 2005). In addition, several species are pathogenic to animals, such as *Saprolegnia parasitica*, which has a major impact on salmon farming (Phillips et al. 2008), *Pythium insidiosum*, a human pathogen (Mendoza and Newton 2005) or *Leptolegnia chapmani*, a parasite of mosquito larvae (Zattau and McInnis 1987). Other hosts than plants and animals are red and brown algae (Gachon et al. 2009), and diatoms (Thines and Kamoun 2010). Finally, several species are saprotrophic and can be found in soils without being able to parasitize a host. Thus, they can be considered as one of the eukaryotic groups which have the greatest impact on human health and welfare, and also on the ecosystems.

Oomycetes have been traditionally considered as fungi because of their morphology (mycelial growth) and their ecology. However, molecular phylogeny placed them within the eukaryotic supergroup heterokonta (also called stramenopiles), which comprises also the diatoms and the brown algae (Ben Ali et al. 2001, 2002), a relationship that appears more obvious when the biflagellated life stage and its typical heterokont morphology is inspected. This aspect had already been noticed by early researchers such as Karling (1942) and Bessey (1942) who hypothesised, respectively, that oomycetes had evolved from heterotrophic flagellates and photosynthetic algae, both categories having representatives within in the heterokonts. The shape and size of oogonia, antheridia, and oospores has been retained as one of the criteria to define genera within the oomycetes, together with the shape of the sporangium (Uzuhashi et al. 2010). With the use of molecular methods in taxonomy, several previously acknowledged genera

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appeared to be paraphyletic, such as for instance *Pythium* and *Peronospora*, which implied several taxonomic emendations which are still ongoing (van der Plaats-Niterink 1981). However, the whole oomycete tree remained still poorly resolved.

Genetic markers used in oomycete research are generally highly variable ones, such as ITS, *cox*2 or partial 28S rRNA sequences (Cooke et al. 2000). This is justified by the need to discriminate closely related strains that can be potential pests. However, such markers are too variable to build an overall picture of the oomycete tree that shows their major evolutionary trends, due to the impossibility to build reliable sequence alignments. Here, we propose a reconstruction of the whole oomycete phylogeny which relies on complete sequences of the SSU rRNA, the most commonly used marker for eukaryotic phylogeny, a slow-evolving gene. For this purpose, we added sequences obtained from environmental DNA surveys to the database of sequenced identified strains provided by GenBank. In addition, we sequenced the SSU rRNA gene of *Albugo candida*, an obligate plant pathogen whose taxonomic affiliation was considered as unresolved (Thines and Kamoun 2010). These results offered us the possibility to draw conclusions on evolutionary pathways followed by oomycetes with special emphasis on their parasitic functions, and also on their ecology.

Materials and methods

DNA extraction, amplification and sequencing

Spores were extracted from *Albugo candida* which grew on turnip leaves (*Brassica rapa*). DNA was extracted using a guanidine thiocyanate protocol (Chomczynski and Sacchi 1987). Almost complete SSU rRNA gene was amplified through PCR using the eukaryotic domain-specific primers EK 82F and EK1498R (Medlin et al. 1988), with the following cycling profile: initial denaturation of 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 58°C for 15 s and 72°C for 90 s, with a final elongation of 72°C for 7 min. PCR products were then purified and sequenced directly using a BigDye197 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analysed with an ABI-3130xl 48-capillary DNA sequencer (Applied Biosystems).

Database construction and phylogenetic analysis

An alignment with 238 SSU rRNA gene sequences comprising also *Albugo candida* (both environmental and culture-derived) was constructed using using the BIOEDIT 7.0.9 sequence alignment editor (Hall 1999) and manually refined. Publicly available oomycete SSU rRNA environmental sequences were downloaded from GenBank through the taxonomy web site at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.-gov). The sequences were found by BLAST searches using query sequences from all main oomycete genera from which SSU rRNA sequences were available, including the most divergent ones (i.e., *Peronospora*, *Pythium*, Saprolegniales, *Haptoglossa, Eurychasma, Olpidiopsis, Haliphthoros, Halocrusticida).* In turn, all sequences derived from environmental surveys were checked against the database to ensure that all available sequences, even the most derived ones, were included in our analyses. These searches were finalized on May 11th 2010.

All ambiguously aligned positions were not taken into account for the phylogenetic analysis; we used a total of 1,549 positions. *Developayella*, *Pirsonia* and the hyphochytrids plus some environmental clones were proposed as outgroups, following the heterokont phylogeny proposed by Cavalier Smith and Chao (2006) Trees were constructed by maximum likelihood using the program TREEFINDER (Jobb et al. 2004), applying a GTR+G+I model of nucleotide substitution (Rodriguez et al. 1990). All necessary parameters were estimated from the data sets. Bootstrap values were calculated from 1,000 replicates. Alternatively, we built a tree using the program RAxML (Stamatakis et al. 2008) and compared the topologies obtained for the two trees.

Results

The topologies of the trees obtained independently with RAxML and TREEFINDER were identical; therefore, we will refer in the following of the manuscript only to the tree obtained with TREEFINDER. Our phylogeny confirms the existence of two main groups corresponding to the informally called peronosporalean and saprolegnialean “galaxies” (Sparrow 1976), supported 100% boostrap values. These two groups formed together a robust clade (98%), together with some sequences with an unidentified abalone parasite (AB178865) and *Sapromyces elongatus* (AB548399) whose affiliation to any of the galaxies remained uncertain.

Some sequences derived from environmental surveys were included within each of these “galaxies”. The environmental clones DGSM_38 and 18BR19 (respectively, AB275038 and EF219018), of marine origin, branched both within the peronosporalean “galaxy” as well many clone sequences derived from freshwater and soil environment. Many clones derived from freshwater and peat-bog environment (but no sequence from soil or marine environment) branched within the saprolegnialean

"galaxy". In addition, several taxa which were previously unassigned branched with relatively good support with the saprolegnialean "galaxy": *Chlamydomyzium* sp. (EU271965), *Apodachlya brachynema* (AJ238663) and *Atkinsiella dubia* (AB284575).

At a basal position with respect to these two groups, appear three diversely supported clades, which comprise some sequences derived from identified species: these are (1) the group of *Haliphthoros*, *Halocrusticida* and *Halodaphnea* (99% bootstrap) (2) *Olpidiopsis* (73% bootstrap) and (3) *Haptoglossa* and *Eurychasma* (98% bootstrap). Each of these clades includes at least one environmental clone sequence. Besides these groups, several environmental sequences with no close affinities to any of the mentioned clades also occupy basal positions with respect to the aforementioned "galaxies". These environmental sequences are originating mostly from marine environments, with exception of PR3_4E_61 (GQ330583), which derived from a molecular diversity survey of a peat bog (Lara et al. 2010).

The peronosporalean "galaxy" appears in our tree composed of the following robustly supported clades: (1) *Phytophthora* and related genera. The Peronosporales *sensu* Sparrow (Sparrow 1976; called Peronosporales in short for clarity reasons), retrieved with maximum bootstrap support branched together with most *Halophytophthora* species, *Pythium monospermum*, *P. cylindrosporum* and many environmental soil and freshwater clones in a robust clade (bootstrap support =98)(2) *Pythium boreale* and related genera (3) *Pythium insidiosum* and *P. aphanidermatum* clade and (4) *Lagenidum callinectes* and *L. thermophilum*. *Albugo candida* branches robustly within this last clade. Next to these clades, a variety of environmental and cultured derived sequences show no clear affinity to each other. The monophyly of the Lagenidiales (i.e. *Lagenidium* + *Myzocytiopsis*) was not retrieved; genus *Lagenidium* appears paraphyletic. Neither was the monophyly of genus *Halophytophthora*, with some sequences branching inside the Peronosporales and some others with (2). Some species, like *Halophytophthora elongata, H. polymorphica* and *H. batemanensis* do not appear monophyletic.

In contrast, the genera composing the saprolegnialean "galaxy" (*Leptolegnia*, *Sapromyces*, *Aphanomyces* and others) are robustly supported, with the exception of *Achlya* and of one *Leptolegnia* isolate (AJ238662) which falls outside the *Leptolegnia* cluster, but whose affinities appear uncertain.

Discussion

General oomycete phylogeny

The picture of the oomycete tree obtained here is, broadly, congruent with previous phylogenies obtained with other markers such as *cox*2 and LSU (Hudspeth et al. 2000; Riethmüller et al. 2002), which also show the "galaxy split" described by Sparrow (1976) which separates the *Peronospora*-related clades from the *Saprolegnia*-related, and also some basal-branching groups.

As shown in other studies (Beakes and Sekimoto 2009; Uzuhashi et al. 2010), genus *Pythium* appears paraphyletic. One of the main characteristics that define this genus is the formation of zoospores (i.e. the flagellated stage) within a vesicle, a feature which is used to discriminate this genus from the morphologically similar *Phytophthora* and *Halophytophthora* (Van der Plaäts-Niterink 1981). However, our tree suggests that this character might be an ancestral feature in the peronosporalean "galaxy", because it is also shared by genus *Lagenidium*, whose sequences are also basal in the peronosporalean "galaxy". More derived genera might have lost it in the course of evolution. Other characters, such as hyaline and coenocytic hyphae without cross septa are also shared by most peronosporales and also other oomycetes (Beakes and Sekimoto 2009). Thus, there is a lack of valid synapomorphies that can be used to define genus *Pythium sensu lato*, it can be considered invalid as it is.

Uzuhashi et al. (2010) have proposed to split genus *Pythium* into five different taxonomic units based on the shape of the sporangium and on LSU rRNA gene and *cox*2-based phylogenies. One of these units is genus *Ovatisporangium*, and corresponds actually to the K clade suggested by Lévesque and De Cock on the base of ITS sequences (2004). Members of this genus are characterised by an oval or at least oboval sporangium. This clade was highly supported in the LSU and *cox*2 trees, and appears also robust in our SSU rRNA gene-based analysis (bootstrap value 96%). Our analysis differs nevertheless from the one of these authors concerning the clade for which they chose to keep the name *Pythium*. If our analyses show a robust clade (bootstrap value 99%) that unites *Pythium insidiosum*, *P. aphanidermatum*, "*Lagenidium" myophilum* and several parasites isolated from *Daphnia* (Wolinska et al. 2009), they disagree on the inclusion of *Pythium monospermum* within that clade (left within genus *Pythium* by Uzuhashi et al. 2010). Likewise, our analysis does not support the reunion of *P. splendens* and *P. cylindrosporum* in a single clade (called *Globisporangium,* Uzuhashi et al. 2010). These last two clades are only weakly supported in the original study of Uzuhashi et al. (2010), both with LSU rRNA and *cox*2 genes. In our study, *Pythium monospermum* and *P. cylindrosporum* branch together with the Peronosporales and several environmental clones in a robust clade (bootstrap value 98%).

Genus *Halophytophthora* appears also paraphyletic in our analysis. Although most of the sequences branch in a

robust (bootstrap value 100%) clade at the base of the Peronosporales, several other sequences branch within well-defined clades. One example is *H. kandeliae*, a marine species which branches in our tree within the *Ovatisporangium*/clade K clade. This species forms zoospores within a vesicle, and this feature should be sufficient to exclude it from *Halophytophthora*. However, its inclusion within genus *Ovatisporangium* would be justified by the characteristic oval shape of its sporangium (Ho et al. 1991), and also its SSU rRNA gene sequence. In addition to the misplacement of certain strains within *Halophytophthora*, certain species appear paraphyletic in our analyses (like *Halophytophthora elongata, H. polymorphica* and *H. batemanensis*). This might be due to misidentification of strains deposited in culture collections.

Our analyses show also that several genera described as of uncertain affinities branch robustly together with the saprolegnialean "galaxy": *Atkinsiella dubia*, *Apodachlya brachynema* plus several environmental clones. These genera share together with *Sapromyces elongatus* (whose phylogenetic position still remains uncertain) and the saprolegnialean "galaxy" the capacity to synthesize sterols *de novo*, a biochemical pathway which is absent in the peronosporalean "galaxy" (Ludwig-Köhn et al. 1982; Petersen and Rosendahl 2000). *Chlamydomyzium*, a nematode parasite, also branches together with these genera, albeit with moderate support. A study of its sterol metabolism would confirm its position within an enlarged saprolegnialean "galaxy".

Position of *Albugo candida*

Our analysis showed that *Albugo candida* branched within the peronosporalean "galaxy", together with the marine parasitic *Lagenidium thermophilum* and *L. callinectes* with a strong support (90%). This result is in agreement with a phylogeny obtained with LSU rRNA sequences, although the relationship was not supported. In contrast, *cox*2 placed *Albugo* with some *Pythium* isolates, without statistical support (Uzuhashi et al. 2010). This situation can be explained by the long branches formed by *Albugo* spp. with those genes, whereas SSU rRNA gene gives shorter branches and seems to be, therefore, a better suited genetic marker to solve this particular problem.

In our tree, the order Myzocytiopsidales (which comprises, amongst others, genera *Lagenidium* and *Myzocytiopsis*; Glockling and Beakes 2006) does not appear monophyletic. Our analyses, however, do not support the opposite assumption, i.e. that genus *Lagenidium* is paraphyletic. Therefore, based on the morphological assumptions by Glockling and Beakes (2006), we consider it as a natural grouping (to the exclusion, however, of *Lagenidium myophilum* AB284577, whose sequence might be the product of a misidentification). The placement of *Albugo candida* within Myzocytiopsidales contradicts traditional taxonomy, which tended to classify it within its own group, separately from the other oomycete groups (Gaumann and Bünzli 1986). Some morphological characters, like the irregular structure on the mature oospore wall of *Myzocytiopsis* are found solely in *Albugo candida,* and not on any *Pythium*-related species (Beakes 1981). In addition, several species of *Myzocytiopsis* and *Lagenidium* are obligate (animal) parasites, a feature which would prefigure the adaptation of *Albugo* to obligate plant parasitism.

Uncultured diversity

The addition of environmental clones to phylogenies that are based solely on sequences derived from cultured strains has already proved to be useful by reducing the phylogenetic artefacts caused by taxonomic undersampling (Moreira et al. 2004). In addition, this practice can reveal the existence of uncultured clades whose existence was previously ignored (Holzmann et al. 2003; Berney and Pawlowski 2004; Massana et al. 2004; Lara et al. 2010, 2011), or an unsuspected diversity (Bass and Cavalier-Smith 2004; Lara et al. 2011; Brate et al. 2010a).

Here, the existence of six environmental clones at base of the "galaxies" that are not related to any known isolate strongly suggests the existence of major oomycete clades for which there are no sequences available. It is probable that these clades, for which most of the clones derive from the marine environment (largely undersampled for oomycetes), are still completely unknown. It is also very likely that an unsuspected diversity will be retrieved if oomycete-specific SSU rRNA primers are used for marine environments. This approach has already proved to be fruitful for other clades (see references above). The occurrence of marine clades at the base of the oomycete tree is already known, and has lead Beakes and Sekimoto (2009) to propose a marine origin for oomycetes, in spite of the fact that the also basal-branching genus *Haptoglossa* has mostly terrestrial representatives. Here, clone PR3_4E_61 (GQ330583) from an acidic, mineral-poor peat-bog has also a basal position with respect to the "galaxies". We suggest that a larger sampling effort should be undertaken before assigning a freshwater, terrestrial or marine origin to the oomycetes.

Oomycetes from the peronosporalean "galaxy" are noticeably present in the marine environment; several sequences from cultured marine organisms are interspersed within that group (such as *Lagenidium callinectes*, *L. thermophilum*, *Halophytophthora* spp.). In addition, three clones are derived from surveys of marine molecular diversity (T41.118, DGSM_38 and 18BR19). Most interestingly, marine clone Biosope T41.118 (FJ537328)

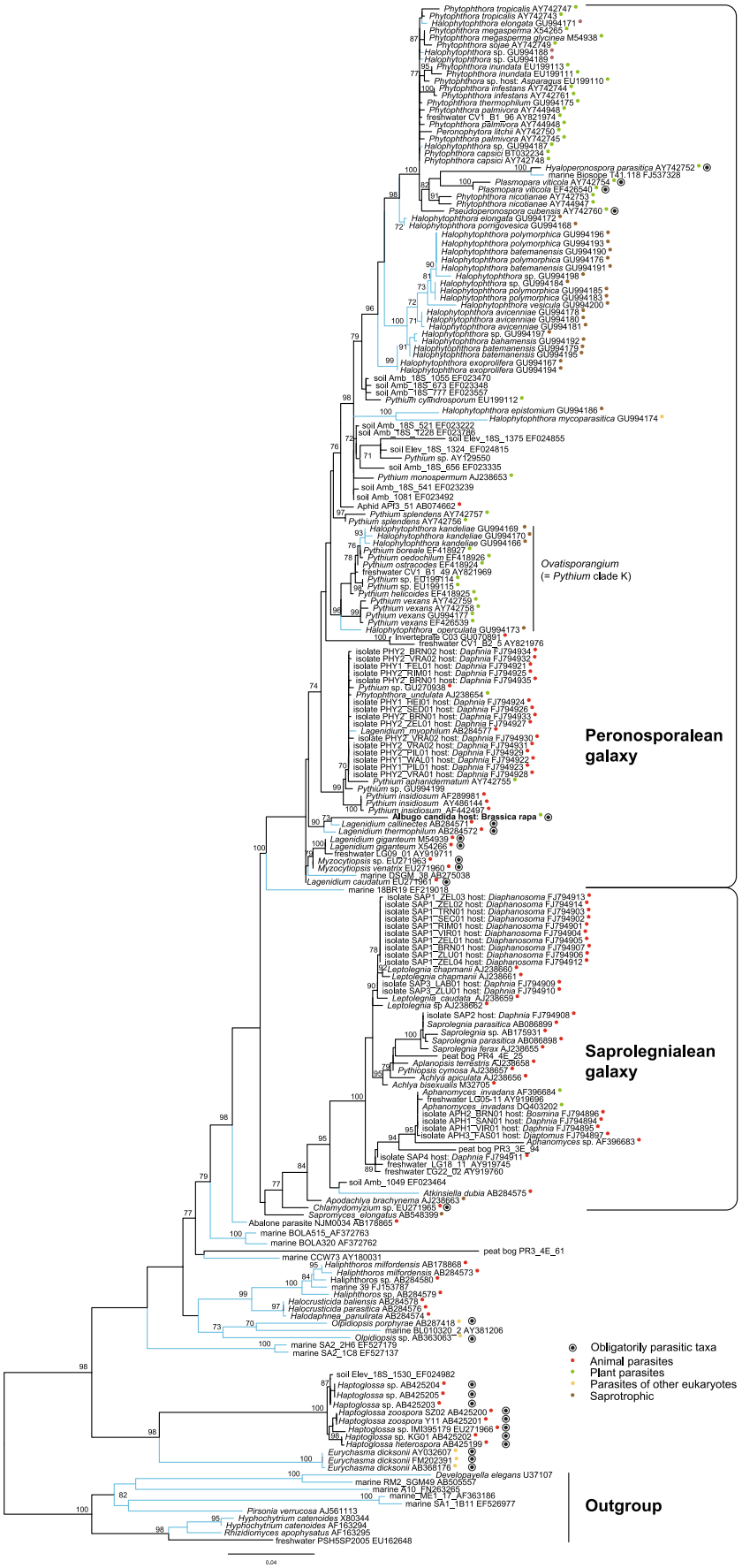

Fig. 1 Phylogenetic tree of the oomycetes, based on culture-derived and environmental sequences. Branches leading to marine taxa are illustrated in light blue. Identical environmental clone sequences are not illustrated. Rounded symbols indicate obligatory parasitic taxa. Red, green, yellow and brown dots refer to animal parasites, plant parasites, other eukaryotes parasites and saprotrophic taxa. The numbers at the nodes indicate bootstrap values as obtained with the TREE-FINDER Maximum Likelihood algorithm

clusters strongly with *Hyaloperonospora parasitica*, an obligate parasite of members of the Brassicaceae family; this suggests that new *Hyaloperonospora* species infecting unsuspected hosts are likely to be discovered in the future. The saprolegnialean "galaxy" in a strict sense does not include sequences from marine environment. However, *Atkinsiella dubia*, a marine crustacean pathogen, is basal to that clade with a strong support (95%) in our analysis, and could thus be associated to that clade. Altogether, it seems therefore that transitions from marine to freshwater environment happened relatively often in the evolutionary history of the oomycetes, in sharp contrast to what has been observed in most eukaryotes, such as the thalassiosiroid diatoms (Alverson et al. 2007), dinoflagellates (Logares et al. 2007), cryptomonads (Shalchian-Tabrizi et al. 2008) and euglyphid testate amoebae (Heger et al. 2010). The parasitic nature of many oomycetes does not explain alone their tendency to cross easily the salinity barriers; the equally parasitic Perkinsea show very few transitions (Brate et al. 2010b) and the Syndiniales have no known representative or environmental sequences in freshwater environments (Guillou et al. 2009).

However, the largest part of the diversity is still encountered in soil and freshwater environments, were a large amount of environmental clones have been retrieved, showing a diversity of potentially new species or even higher clades which remain still unexplored.

Evolution of parasitism in oomycetes

Parasitism is thought to have evolved early in the oomycetes (Beakes and Sekimoto 2009). In this, they can

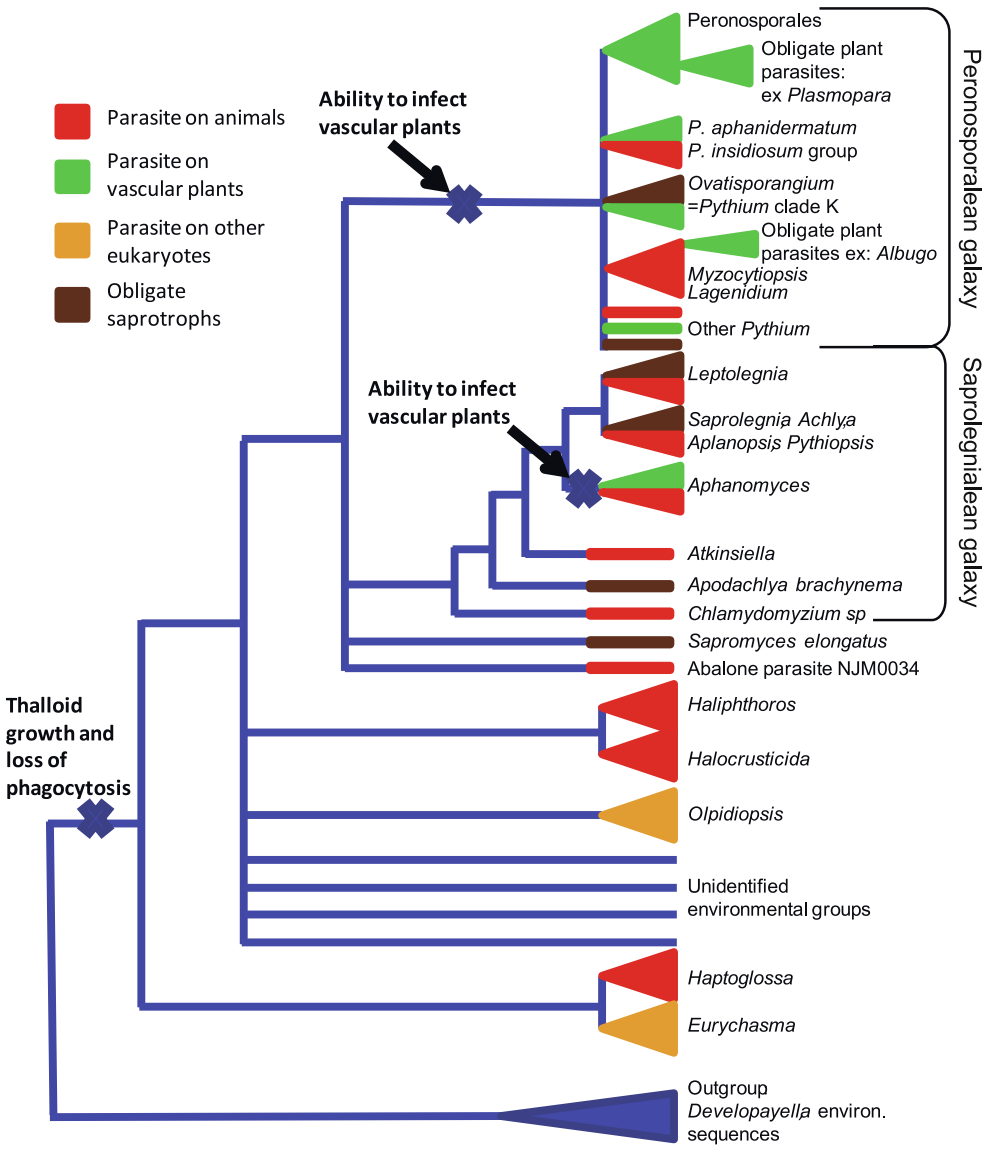

Fig. 2 Illustration of the evolution of types of hosts in oomycetes as inferred from our phylogenetic analysis

be easily compared to fungi, which are also osmotrophic organisms and show the same evolutionary tendency. However, most oomycete parasites behave as opportunistic pathogens; only a small part of the oomycete diversity is obligatorily symbiotic (here represented by genera *Hyaloperonospora*, *Pseudoperonospora, Plasmopara*, *Albugo*, *Lagenidium, Myzocytiopsis, Chlamydomyzium, Olpidiopsis*, *Haptoglossa* and *Eurychasma*). Several species are entirely saprotrophs and have never been reported to be linked to any host, such as for instance *Apodachlya brachynema* or *Sapromyces elongatus*. It is likely that ancestral species started with such a saprotrophic condition, occasionally being able to grow on wounded, stressed or weakened hosts, like modern *Halophytophthora* sp. do (Göker et al. 2007). A step further implies development of specialised structures and biochemical pathways to be able to infect healthy hosts (like in *Phytophthora*), which eventually turns into an obligate symbiosis (like in *Hyaloperonospora*). All these intermediate steps can be found in a single clade, like the one where these examples come from (see Fig. 1).

However, it seems that not every host is equal against infection. Our tree shows that the ability to infect plant cells has appeared only twice independently in oomycetes, once at the base of the peronosporalean "galaxy" and once at the emergence of genus *Aphanomyces* (see Fig. 2). This is in contrast with the ability to infect animal cells, which is found in almost all clades, except in the obligate plant pathogens; it seems therefore "easier" for an oomycete to infect plant tissue than animal tissue. The reasons do not seem to be related to the presence of RXLR effectors, given that these effectors have been described in both plant pathogen *Phytophthora* spp. (Kamoun et al. 2009) and on the fish pathogen *Saprolegnia* (Van West et al. 2010). Future studies targeting oomycete animal pathogens and non-symbiotic species that received less attention than plant infecting species could provide clues to explain such discrepancy (Phillips et al. 2008).

Acknowledgement This work was supported by the Swiss State Secretariat for Education and Research (grant reference: SER No. C09.0139) and the European Union for the projects ISEFOR "Increasing Sustainability of European Forests: Modelling for Security against Invasive Pests and Pathogens under Climate Change (FP7-KBBE-2009-3 call, Proposal number: 245268) and the COST action FP0801 "Established and Emerging *Phytophthora*: Increasing Threats to Woodland and Forest Ecosystems in Europe". The authors would like to thank Mário Rui Proença Santos (Universidade Tecnica de Lisboa - Portugal) for the *Albugo* infected *Brassica rapa* plant material.

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