Editorial

Plant waterworld

Globally, plants lose at least 100 times more water vapour through their stomata than they gain carbon dioxide. So carbon gain is an expensive process in terms of water requirements and species differ considerably not only in their efficiencies of water use but also in their temporal responses to changing dynamics of water availability. While fixed carbon dioxide becomes the long-lived plant skeleton the rapidly exchanging plant water has a short life in the plant, constantly requiring replenishment. Replenishment by root uptake occurs over a very wide range of soil conditions from dry to waterlogged to saline. Although species differ in their capacities for water uptake over this range, it appears that all plants from primitive bryophytes to flowering plants counteract variations in soil water supply to living cells by gating aquaporin water channels in the plasma membrane. Aquaporins can be closed during drought or during waterlogging (Törnroth-Horsefield *et al.*, 2006), in order to maintain cellular homeostasis. Montalvo-Hernández *et al.* (2008) show nicely that aquaporins play a key role in drought tolerance of the common bean. In a drought-tolerant variety, water translocation is strongly confined to transport within the vascular system, and restricted from parenchymatous tissue. A drought-sensitive variety showed no such preferential pathways during reduced water supply. Arbuscular mycorrhizas can also influence root hydraulic properties, probably through changing the gating of aquaporins in the roots, affecting control of root hydraulic conductance (Aroca *et al.*, 2007). Aquaporins also appear crucial in that most extreme of environments inhabited by mangroves. In phosphorus (P)-limited sites, water uptake is greatly reduced (Lovelock *et al.*, 2006), probably by reduced opening of aquaporins.

Research on aquaporins illustrates the cellular mechanisms by which species differ in their responses to water supply; however, there is still an abundance of questions on water relations requiring observation and quantification before they can be reduced to cellular understanding. Although aquaporins may be implicated in the reduced hydraulic conductivity of P-limited intertidal mangroves, changes in xylem anatomy are also crucial as salinity varies (Sobrado, 2007), and in parallel with the regulation of ion uptake (Carter *et al.*, 2006). Intertidal seaweeds are exposed to environmental extremes ranging from full immersion to full drying exposure over a matter of hours. The resistance of the seaweeds to these extremes is well established and new molecular techniques demonstrate very rapid changes in gene expression, notably of antioxidant genes under full drying conditions (Collen *et al.*, 2007).

For amphibious plants, which can be fully submerged or emergent during a growing season, carbon dioxide supply can be a major limitation when submerged leaves receive high irradiance. Under such conditions photoinhibition may occur, disrupting the antennal system in the chloroplasts. Species differ in their responses. *Nessea crassicaulis* is fully adapted to a submerged habitat, with low concentrations of chlorophyll and no apparent mechanism for protection against photoinhibition (Nielsen & Nielsen, 2006). By contrast, *Lobelia cardinalis* is adapted to an emergent habitat, with effective nonphotochemical quenching to protect against photoinhibition. A question here – Fig. 1 shows a water droplet on a leaf: what can be seen and is the leaf from a plant that is adapted to full submergence?

For intertidal seaweeds environmental seasonality has an hourly time frame; for most terrestrial plants seasons occur at a more leisurely pace. In savanna environments the dry season generally leads to leaf fall but, surprisingly, many trees leaf out before the end of the dry season. The massive baobab tree supports this precocious leaf production using water stored in the trunk – however, the stomata remain closed with minimal transpiration until the rains begin (Chapotin *et al.*, 2006). Dry alluvial terraces in the Alps are often dominated by Scots pine (*Pinus sylvestris*). Dominance is assured in this environment not by a fixed response to the season but by flexibility of root growth responses to seasonal inter-annual differences in soil water supply (Polacek *et al.*, 2006).

Overlying these plant responses are those that are genetically hardwired, such as high rates of water supply to leaves of shrubs in the chaparral during dry summers (Bhaskar *et al.*, 2007), the production of root aerenchyma in *Juncus effusus* (Visser & Bögemann, 2006) and the drive for plants of *Ranunculus nodiflorus* to search out puddles of water as preferred habitats (Noel *et al.*, 2006). Spermatophytes are generally thought to survive extreme environments because the gametophytic stage of the life cycle is protected from environmental extremes. By contrast, the free-living fern gametophyte is generally considered intolerant of drought. A recent investigation of tropical ferns indicates that the gametophyte is commonly not just drought resistant but also desiccation tolerant in many species (Watkins *et al.*, 2007).

In semi-arid climates precipitation occurs episodically, with dry intervening periods. Dry periods are usually considered no more than periods for plants to endure; however, in the semi-arid monsoonal climate of Arizona, droughts before precipitation events actually stimulate ecosystem productivity (Potts *et al.*, 2006). Ecosystem productivity is stimulated not through the responses of the dominant grasses but because of a reduction in ecosystem respiration – particularly the microbial component.

Plant responses to reduced water supply are of clear ecological and agricultural importance. Stomatal responses play key roles in rapid drought responses (Flexas *et al.*, 2006; Monclus *et al.*,

Fig. 1 Photograph of a water droplet (5 mm diameter) on a leaf. What can be seen and is the leaf from a plant that is adapted to full submergence?

2006; Galmes *et al*., 2007), although down-regulation of photochemistry is also crucial (Galle *et al*., 2007), as are interactions between drought and irradiance in influencing longer-term growth responses (Sanchez-Gomez *et al*., 2006). Although diminishing water supply leads to conservative responses of plant water status, these responses can be disrupted by pollutants such as ozone, which can reverse these responses (McLaughlin *et al*., 2007a,b).

This brief view of some of the research published in *New Phytologist* on the plant waterworld indicates a rich variety of mechanisms by which plants adjust to variations in water supply. Given levels of light and growing season temperatures I think it is quite easy to predict the rate of photosynthesis of a leaf – even in shade. However, it is much more difficult to predict the rate of water loss, and when the predicted photosynthetic rate is incorrect this is commonly attributable to changes in water supply and flow through the plant. Such variation will be a continual source of enquiry.

F. I. Woodward

Editor-in-chief

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Key words: amphibious plants, aquaporins, ecosystem respiration, root hydraulics, stomata, transpiration, xylem anatomy, water availability.

Commentary

Testing the Holy Grail framework: using functional traits to predict ecosystem change

Use of plant functional traits, rather than species identities, to generalize complex community dynamics and predict effects of environmental changes has been referred to as a 'Holy Grail' in ecology. Particularly in species-rich communities or in large-scale vegetation models, this approach could prove very powerful in the quest for general rules associating biota and environmental conditions. One framework designed to predict vegetation responses to environmental change factors and changes in important ecosystem functions simultaneously is an effect–response framework. In this framework, plants can be classified in terms of their response to environmental factors (via response traits) and in terms of their effects on ecosystem properties (via effect traits) (Chapin *et al*., 2000; Lavorel & Garnier, 2002) (Fig. 1). As such, it describes an approach to scale up from individuals to communities and ecosystems in the context of environmental change predictions. While this framework is highly relevant and has the potential to advance how we approach questions of vegetation change, empirical tests are rare. In this issue of *New Phytologist*, Gross *et al*. (pp. 652–662) take an important step forward by testing this framework for water availability.

'This striking disparity between conceptual appeal and empirical application probably indicates uncertainty in how to translate the framework into empirically robust tests.'

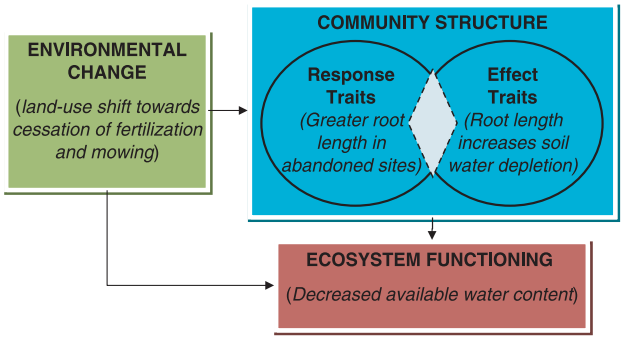

Fig. 1 The response-and-effect framework for scaling through the community level distinguishes between the species (and their functional traits) that respond to environmental change (i.e. response traits) and the species and traits that impact ecosystem function (i.e. effect traits). The center diamond indicates the varying degree of overlap between response and effect traits: two trait groupings may completely overlap (as in Gross *et al*.), reinforcing the effects of environmental change through community dynamics; there may be functional redundancy in response groupings; or no relationship between effect and response traits. We show the specific trait linkages found in Gross *et al*. in parentheses.

The revitalization of the functional traits concept

Classification of plants according to functional traits has a long tradition in plant ecology (Raunkiaer, 1937; Grime, 1979), regardless of whether the traits are called life forms, strategies, syndromes, or functional types. Common to all trait classifications has been the search for a functional description of the vegetation, based on attributes that show a common response to the environment, independent of phylogeny. In parallel to the development regarding functional response, classification of vegetation based on the effects on ecosystem processes has an equally long history (Jenny, 1941). Both investigations are based on the reasonable premise that physiological and demographic constraints, together with trade-offs in life-history, should result in predictable changes in trait representation across environmental gradients.

These investigations took largely separate trajectories until the last decade, when several conceptual advances concerning the links between the effects of environmental changes on vegetation and vegetation effects on ecosystem functioning occurred. Chapin *et al*. (2000) proposed a conceptual framework where modifications of species composition resulting from environmental change translate into modifications of ecosystem functioning via changes in the representation of species traits. Lavorel & Garnier (2002) expanded upon the framework to articulate the environmental response and ecosystem effects through varying degrees of overlap between relevant traits. As one metric of their importance, these two papers have received almost 450 and 170 citations, respectively, at the time of writing this commentary.

While plant functional-type approaches, and in particular the concept of effect and response traits, have been garnering broad interest over the past decade, empirical tests of the concepts have been slower to appear. For instance, of the papers citing Lavorel & Garnier (2002), only 10% measured both effect and response traits. A literature search for 'effect traits' brought up only five relevant experimental studies. This striking disparity between conceptual appeal and empirical application probably indicates uncertainty in how to translate the framework into empirically robust tests.

Testing the effect-and-response framework

Are understanding and incorporating functional traits a key to successful prediction of the effects of environmental change, or are they an appealing conceptual way of looking at change with less potential for empirical application? The work presented by Gross *et al*. is an important demonstration of how the framework can be tested. The authors were able to determine how land-use change in subalpine grasslands affected the community distribution of plant traits related to water use and, in a separate experiment, to determine how the vegetation associated with each land use affected water availability. The authors identified a suite of traits that mediated plant community response and soil water availability across this environmental gradient, validating their model with relationships at other subalpine grassland sites. Here we use their paper as an example that empirical application of the functional effect-and-response framework can be accomplished, aiming to point to ways that other research could follow this lead.

Measuring functional response

A survey of papers that cite Lavorel & Garnier (2002) indicate that investigating functional response is about four times as common as investigating functional effect. The primary approach in identifying response traits is to evaluate how environmental change may alter trait representation via community composition. Environmental change factors can either be simulated in experimental manipulations or evaluated using a correlative approach across an environmental gradient. Gross *et al*. used 12 grassland sites of different land-use histories, which formed a gradient in water availability and several other covarying environmental factors. Experimental manipulations (e.g. Engelhardt, 2006; Bret-Harte *et al*., 2008) are less confounded by other site variables, but the results may indicate transient or timelag effects and may not as clearly follow natural gradients. Probably a duel approach is most robust.

To scale up from species-level traits to a community-level response, Gross *et al*. aggregated aboveground traits using a mass-ratio approach, in which the trait of each species was weighed by the relative abundance of the species at each site. This approach has been utilized by other studies which found that community-aggregated functional traits were correlated with ecosystem process (Garnier *et al*., 2004; Vile *et al*., 2006). For belowground traits, Gross *et al*. measured aggregate traits directly by measuring root traits at each site and bypassing

species identification entirely. This approach blurs the line between traditional community and ecosystem measurements, having the advantage that is it much less time intensive than species-by-species measures. Additionally, these measurements take into account the nonadditive effects of species interactions, while the mass-ratio approach assumes additivity (Suding *et al.*, 2008).

Measuring functional effect

To investigate community effect on soil moisture, Gross *et al.* compared soil moisture in plots at each site where they had removed all vegetation to plots where vegetation was intact. This removal manipulation allowed the separation of plant effects from land-use effects on soil moisture. Removal manipulations provide a strong test of community effects through comparison between manipulated and unmanipulated plots. Similarly, the effects of individual trait groups on ecosystem functioning can be evaluated by selective removals (Cross & Harte, 2007; Bret-Harte *et al.*, 2008) or in single-species monocultures (Eviner, 2004; Engelhardt, 2006; Pontes *et al.*, 2007). While these are all reasonable methods to estimate ecosystem effect, the total community-removal approach by Gross *et al.* has the advantage that it is relatively simple to conduct and interpret at the community level. Several other studies have simultaneously identified changes in trait groups and ecosystem function along a gradient (Garnier *et al.*, 2004; Quetier *et al.*, 2007). These studies do not directly separate site from species effects on ecosystem functions, providing weaker tests of the framework.

Relating response to effect

A major distinguishing factor of the response-and-effect framework is that it simultaneously incorporates both changes in community structure as a result of the response and effects of these changes on ecosystem function. Gross *et al.* demonstrate that community-aggregated response traits translate the effects of these changed plant communities on soil water availability. In this case, without information on both response and effect at the community level, impacts of land-use change on soil moisture would be underestimated.

Species that respond to environmental change may share effect traits, leading to shifts in ecosystem processes, or alternatively may vary in traits so that the effect is less than expected, or even in the opposite direction (Suding *et al.*, 2008). While Lavorel & Garnier (2002) emphasize several scenarios where effect and response traits may differ, Gross *et al.* focused on traits that are related to both response and effect (which they refer to as response–effect traits). Gross *et al.* assume that the same traits important to the response to an environmental change factor are those that are critical in determining the effect of that factor on ecosystem function. While assuming that response and effects traits are one and the same would simplify relationships, we do not know how often this correlation should occur, or what situations or habitats may be particularly prone to a particular scenario of trait linkages. This correlation is supported for succession by the findings of Garnier *et al.* (2004), for grazing by Blanco *et al.* (2007) and for water drawn down in macrophytes by Engelhardt (2006). In contrast, other work has found that response and effect traits did not strongly overlap (Cross & Harte, 2007; Bret-Harte *et al.*, 2008). Further understanding of response-trait linkages is critical for successful application of the framework.

Future prospects

Using functional response and effect traits as 'common currency' has great potential to scale up from individuals to communities and ecosystems in the context of environmental change predictions. Given the relative popularity of the conceptual framework but the paucity of the empirical tests, it is essential that methods are developed for application of these frameworks. While the research need is immense, studies such as those by Gross *et al.* substantiate the importance of community-centered scaling via functional traits to provide more relevant information on the large, but often neglected, role of community dynamics in environmental change.

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Key words: community dynamics, ecosystem change, effect traits, effect–response framework, functional traits, response traits, vegetation models, water availability.

Tree root architecture – form and function

Over 25 years ago, Fitter (1982) recognized that the form (architecture) of the branching root system was almost certainly related to the acquisition of essential soil resources (function). Since then, there have been many studies that have advanced our understanding of how plasticity in the birth and death of lateral root branches might confer a competitive advantage on individual plants and may structure plant communities, and the topic continues to generate significant scientific interest and debate to this day (e.g. Kembel & Cahill, 2005; de Kroon & Mommer, 2006; Hodge, 2006; Grime, 2007; Kembel *et al.*, 2008). The primary focus to this point in the ecological literature has been on understanding rates of root length proliferation and we know that root length can sometimes respond dramatically to increased availability of 'patchy' soil resources. However, missing from many studies of root proliferation and acquisition of essential soil resources is a detailed understanding of how plants have altered their form (morphology and especially anatomy). Guo *et al.* (this issue; pp. 673–683) make an important contribution by comparing the lateral root branch anatomy of 23 species of temperate trees. They address the following questions. How are multitasking temperate tree root systems designed? Is the branching root system anatomically similar across species? Are lateral branches constructed so that only the most distal roots are responsible for absorption of nutrients?

'The questions of how plant root systems are constructed and how their form is related to the capture of essential soil resources have intrigued ecologists and plant biologists for decades ...'

Multitasking root systems in patchy soil

Trees, like all plants growing in the wild, must solve a host of problems using their root systems. Root systems anchor the plant, sometimes from gale-force winds. Perennial plants have a growth rhythm designed to help the plant survive periods of cold, drought and defoliation, and roots (as well as shoots) store nonstructural carbohydrates to provide the plant with the energy it needs to survive changes in climate and periodic disturbances that alter whole-plant source–sink relationships. Roots are a part of the plant vascular system that takes up soil solution and transports water and other compounds (Pratt *et al.*, 2008). Finally, roots and their associated symbiotic bacteria and fungi are responsible for fixing atmospheric nitrogen and acquiring the essential nutrients required for growth.

The questions of how plant root systems are constructed, and how their form is related to the capture of essential soil resources, have intrigued ecologists and plant biologists for decades because we have learned that there can be as much variation in the availability of essential nutrients across 20 cm of soil as there is across an entire field or across a significant ecological gradient (Gross *et al.*, 1995; Farley & Fitter, 1999). An interesting example of how microsite mineralization of soil nitrogen can be influenced by plant roots and their associated mycorrhizas is presented by Schimel & Bennett (2004). Plants are basically sessile organisms, so the only way that they can 'forage' for 'patchy' essential soil resources is to dynamically change their form or their physiology, or both.

Lateral roots – dynamic plant modules

Lateral roots are the plant modules that grow and die on small spatial scales in the soil and they, along with their associated mycorrhizas, are primarily responsible for nutrient acquisition. Variations in the size, shape, surface area and demography of lateral branches and associated mycorrhizas, along with concomitant changes in root physiology, are the way in which a plant can 'forage' for water and nutrients in the soil. Lateral root branches arise in the pericycle of the parent root, grow through the cortex and form lateral branches, which are sometimes complex in their architecture (Pregitzer, 2002; Pregitzer *et al.*, 2002). The plant root system can sense a

change in resource availability in the soil and initiate new lateral roots in 'hot spots', places in the soil where essential resources are more available (Walch-Liu *et al*., 2006; Nibau *et al*., 2008).

Some years ago, Grime (1965) argued that comparative patterns in trait variation could tell us something about functional specialization. This is essentially the approach that Guo *et al*. have taken. They systematically dissected the lateral branches of 23 species of temperate trees growing in China following the protocol of Pregitzer *et al*. (2002). They also quantified and used the anatomy of the distal root branches as a surrogate for distinguishing root branches involved in active metabolic uptake of nutrients vs transport and storage. In addition to quantifying the anatomy of branching root segments, they quantified which of the lateral branch orders were colonized by mycorrhizal fungi.

The results of Guo *et al*. suggest that most of the active absorption of nutrients occurs in first-order roots, the tiny lateral branches at the very distal end of the root system (Pregitzer *et al*., 2002). Mycorrhizas were associated with the first three orders of roots, although the degree of activity of mycorrhizas in the second-order and third-order roots is not clear from their results. Based on the results of Guo *et al.* and what we know about the relationship between root nitrogen concentration and rates of root respiration (Reich *et al*., 2008), I suspect that the first-order roots are the primary carbon depot for mycorrhizal hyphae, which ramify away from the root tip to forage widely in the spatially and temporally heterogeneous soil. Guo *et al.* also found that the morphology and anatomy of the root system seems to be conserved within a species, an observation increasingly reported in the literature (Pregitzer *et al*., 2002; Kembel & Cahill, 2005; Grime, 2007). The implication is that different species have evolved specialized mechanisms to sense changes in the availability of essential soil resources and to alter their lateral branch architecture and demography to compete effectively for limiting water or nutrients (Walch-Liu *et al*., 2006; Nibau *et al*., 2008). However, the evolutionary costs and benefits of lateral root plasticity have not yet received the attention they deserve (de Kroon & Mommer, 2006).

Unanswered questions

The results of Guo *et al*. raise several unanswered questions. To start with, the systematic dissection of lateral root branches into orders is an arbitrary approach. Granted, this systematic approach has led us to understand that most of the absorptive length and metabolic activity in tree roots is correlated with the distal ends of the branching root system (Pregitzer *et al*., 1998; Pregitzer *et al*., 2002; Reich *et al*., 2008). However, we still do not really understand the variation in morphology and anatomy of lateral branches that arise in the pericycle. We know that the plant can sense external changes in essential soil resources, alter endogenous factors that regulate lateral root development and produce a plastic response – but how plastic? Do trees primarily produce new first-order roots and alter mycorrhizal fungi associations in response to changing soil conditions or are new lateral branches more complex in architecture? Is lateral root branch architecture highly conserved within a species, or highly plastic? How heritable is lateral root branch architecture?

One of the dangers in trying to make global generalizations about root form and function is the comparison of roots that differ significantly in how they are constructed and how active they are metabolically. Guo *et al*. demonstrate that root order predicts root anatomy fairly consistently across 23 species of temperate trees. However, if we were to compare trees with perennial forbs and annual grasses we would find a different outcome. Studies of root relative growth rate in 'patchy' soil mostly ignore the fact that lateral roots can differ dramatically in both form and function (Pregitzer, 2002). Cumulative evidence now strongly suggests that the lateral roots of trees vary in their construction and maintenance costs depending on their position on the branching root system. As time progresses and our understanding of tree root systems improves, it increasingly seems as if 'fine roots' – roots actively involved in the uptake of nutrients and roots that have short life expectancies – consist primarily of first-order to third-order lateral roots that arise dynamically from the pericycle in response to changes in soil resource availability. In reality, this generalization is a gross over-simplification. The distal branches of the perennial roots system are the hub of mycorrhizal activity and the host to root hairs (Fig. 1). A more detailed understanding of the dynamic and integrated 'root–fungal module' awaits the attention of innovative new studies. Clearly, the

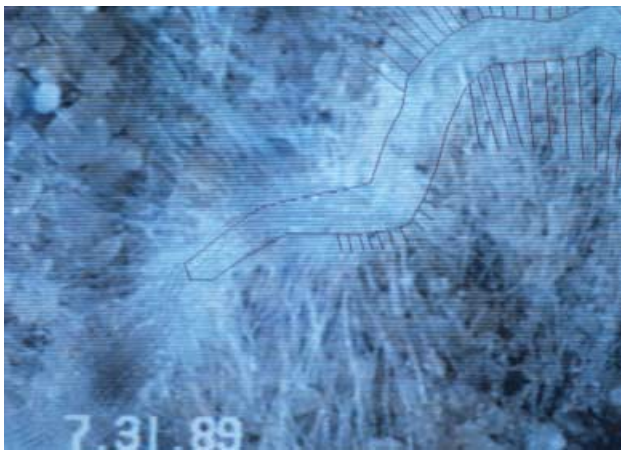

Fig. 1 *Prunus pennslvanica* L. (pin cherry) lateral root tip and associated roots hairs growing ('foraging') in a patch of nitrogen-enriched sandy soil at the University of Michigan Biological Station in 1989. Individual grains of sand and scores of root hairs are visible in the image. The outline shows the position of the root and root hairs 12 h previously. Lateral root architecture, root hairs and mycorrhizal hyphae are all responsive to changes in soil resource availability, but relationships between root form and function remain relatively poorly understood. (See Pregitzer *et al*., 1993 for details of this particular study.)

metabolic action in woody plants with complex lateral branching root systems is at the tips of the lateral root branches. It seems that the functional architecture of plant roots is as diverse and interesting as the functional architecture of shoots. Perhaps we should not be surprised by this because biotic diversity is high, competition is keen and resource capture is spatially and temporally complex in the patchy soil.

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Key words: lateral roots, root anatomy, root demography, root turnover, root update.

Letters

Multilocus genotyping of arbuscular mycorrhizal fungi and marker suitability for population genetics

Arbuscular mycorrhizal fungi (AMF) are an ecologically important group of plant symbionts and their species richness has been shown to influence plant diversity and productivity (Van der Heijden *et al.*, 1998). Genetic diversity within AMF species is important as genetically different isolates have been shown to differentially affect plant growth and nutrition (Munkvold *et al.*, 2004; Koch *et al.*, 2006). The study of AMF diversity in ecosystems, particularly identifying which AMF species associate with different host plants, requires reliable identification of different AMF. It has long been recognized that identifying AMF across broad geographical ranges requires molecular tools for fast and reliable genotyping directly from soil material.

Previously, genotyping methods for distinguishing AMF species have mostly been restricted to ribosomal DNA (rDNA) sequences. The advantage of these loci is the potential for cross-species amplification using universal primers, and the relative ease of amplification from different material (e.g. colonized root pieces, single spores, etc.). A large body of studies have identified the species composition of AMF communities in many different ecosystems (Öpik *et al.*, 2006; Rosendahl, 2008). However, studies of genetic variability within AMF species are important for understanding the basic biology, genetics and ecology of AMF fungi, which cannot be addressed at the community level. For example, a hierarchical study of genetic variability from the local scale within populations right up to an inter-continental scale is lacking. Such hierarchically designed studies could lay the foundation that will allow us to answer fundamental questions about the biology of AMF, their genetics, whether they form recombinant populations, the amount of genetic exchange among populations, the importance of drift and selection in AMF species, and the distribution of genetic and functional diversity in AMF over different geographic scales, and allow us to examine the co-evolutionary relationships between AMF genotypes and their host plant genotypes.

For most of these applications ribosomal markers are unsuitable because of a lack of sufficient within-species variability and are potentially problematic because of confounding intra-sporal variability (Sanders *et al.*, 1995) and copy number polymorphism (Corradi *et al.*, 2007). A population genetics approach to the study of AMF requires multilocus genotyping of nonribosomal loci. Stukenbrock & Rosendahl (2005a,b) first developed and applied this approach by amplifying three different loci in a large set of spores of three *Glomus* species harvested from the field. However, ideally, multilocus genotyping should comprise a much larger number of loci. Two simultaneously published studies (Croll *et al.*, 2008; Mathimaran *et al.*, 2008), describing genetic markers for AMF, should now make this possible. Both studies identified multiple loci that were variable among isolates of a commonly studied AMF, *Glomus intraradices*. Length differences among the alleles were used to identify genetic differences. Part, but not all, of the variation was found in repeat regions, and both studies referred to the markers as either microsatellites or simple sequence repeat markers. The simultaneous publication of the two studies might lead to some confusion for researchers who may now want to use these markers. Here, our aim is to clarify how many new and different loci have actually been identified and which loci are likely to be suitable for population genetics studies, to highlight potential problems with the genotyping techniques used, and to discuss future approaches to their use in AMF population biology.

The study by Mathimaran *et al.* (2008) identified 18 loci and Croll *et al.* (2008) showed polymorphism in 13 loci, of which two had previously been identified by Raab *et al.* (2005). The two studies used similar, but not identical, strategies to identify repetitive DNA stretches by searching publicly available databases (Table 1). Candidate sequences were then amplified in a set of isolates and potential length polymorphism was scored. In both studies, loci were amplified in a number of isolates from different geographic locations. It should be noted that one locus described by Mathimaran *et al.* (2008) is the same as one polymorphic locus identified by Croll *et al.* (2008) but has been given two different designations. The variation in two more loci reported by Mathimaran *et al.* (2008) is documented in previously published work. We hope that Table 1 will help researchers who intend to use these markers to identify the different loci for which primers have been developed and prevent unintentional studies of the same locus under two different names.

Locus *Glint08* identified by Mathimaran *et al.* (2008) is identical to locus Bg348 from Croll *et al.* (2008), even though the primers are located at different distances from the repetitive sequence region. Loci *Glint09* and *Glint18* identified by Mathimaran *et al.* (2008) were previously published by Corradi & Sanders (2006) and described as genes encoding P-type IID ATPases. Corradi & Sanders (2006) reported polymorphism in a population of *G. intraradices* based on a comparison of different alleles at the same locus. Furthermore, the gene was found to exist in two variants in each of several isolates and in three variants within one isolate (Corradi & Sanders, 2006). Locus *Glint09* is based on the sequence of the third variant; however, the primers designed by Mathimaran *et al.* (2008) are not specific for this particular variant. As a consequence, the primers based on locus *Glint09* potentially amplify up to three different locations in the genome within a single isolate. Locus *Glint18* was identified in an assembled sequence (contig) that matches the P-type IID ATPase variants. However, the resulting consensus sequence does not exactly match any of the original P-type IID ATPase variants, probably as a consequence of the contig being assembled from several different variants (i.e. a chimaeric contig). Consequently, primers for locus *Glint18* do not specifically amplify one of the several variants. Loci *Glint09* and *Glint18* are separated by approx. 500 bp. In our opinion, these two loci are unsuitable for most population genetic studies because of the multi-copy nature of the gene they are located in, unless primer sequences are chosen that restrict the amplification to one variant.

The studies of Mathimaran *et al.* (2008) and Croll *et al.* (2008) both describe polymorphic loci exhibiting size differences of 1 or 2 bp among some alleles. Scoring such a polymorphism is potentially problematic even if PCR products are separated on a capillary sequencer, Spreadex polymer or polyacrylamide gels. These methods offer a high resolution of allele length differences, but the amplification of repeat motifs often leads to the presence of stutter peaks (or shadow bands) as a result of DNA polymerase error. Where small length differences are observed among alleles, it is advisable to

Table 1 Summary of markers developed for *Glomus intraradices*

Locus	Accession no.	Database	Function	Type	Length polymorphism	Reference
Bg32	CG431930	GSS	Unknown	Probably noncoding	Indels	Croll *et al*. (2008)
Bg42	CG431913	GSS	Unknown	Probably noncoding	(TA) repeat + other indels	Croll *et al*. (2008)
Bg62	CG431880	GSS	RNA polymerase II large subunit	Proximate coding region	(TAAAA) repeat + other indels	Croll *et al*. (2008)
Bg196	CG431972	GSS	Unknown	Probably noncoding	Several repeat motifs + other indels	Croll *et al*. (2008)
Bg235	CG432041	GSS	Unknown	Probably noncoding	Several indels	Croll *et al*. (2008)
Bg273	CG432137	GSS	Unknown	Probably noncoding	(T) + (A) repeats + other indels	Croll *et al*. (2008)
Bg276	CG432062	GSS	Unknown	Probably noncoding	Several indels	Croll *et al*. (2008)
Bg303	CG432175	GSS	Unknown	Probably noncoding	Several indels	Croll *et al*. (2008)
Bg348	CG432294	GSS	Predicted protein of unknown function	Proximate coding region	(TAA) + (TAAA) repeats + other indels	Croll *et al*. (2008)
Bg355	CG432269	GSS	Unknown	Probably noncoding	Several indels	Croll *et al*. (2008)
Nuclear intron	BE603853	EST	Intron in gene of unknown function	Proximate coding region	(T), (A) + (TAA) repeats	Croll *et al*. (2008)
mtLSU int1	AJ973189-193	Standard	Intron in mitochondrial LSU gene	Proximate coding region	Several indels	Raab *et al*. (2005); Croll *et al*. (2008)
mtLSU int2	AJ973189-193	Standard	Intron in mitochondrial LSU gene	Proximate coding region	Indel	Raab *et al*. (2005); Croll *et al*. (2008)
Glint01	CG432086+113*	GSS	Unknown	Coding	(AAAT) repeat + other indels	Mathimaran *et al*. (2008)
Glint02	DT883628	EST	Unknown	Coding	(GAA) repeat only?	Mathimaran *et al*. (2008)
Glint03	BI452162	EST	Unknown	Coding	(TTAT) repeat? + other indels	Mathimaran *et al*. (2008)
Glint04	BM959176*	EST	Unknown	Coding	(TTA) repeat? + other indels	Mathimaran *et al*. (2008)
Glint05	BE603957*	EST	Putative cell wall protein	Coding	(TAT) repeat? + other indels	Mathimaran *et al*. (2008)
Glint06	BM959329	EST	Unknown	Coding	(CAT) repeat? + other indels	Mathimaran *et al*. (2008)
Glint07	BE603778*	EST	Unknown	Coding	(TTA) repeat? + other indels	Mathimaran *et al*. (2008)
Glint08 (same asBg348)	CG432294	GSS	Predicted protein of unknown function	Proximate coding region	(AATA) repeat? but see Bg348 above	Mathimaran *et al*. (2008)
Glint09	AM118108	Standard	P-Type IID ATPase	Coding	(AATG) repeat? + other indels	Corradi & Sanders (2006); Mathimaran *et al*. (2008)
Glint10	BM027318	EST	Unknown	Coding	(AATGGT) repeat? + other indels	Mathimaran *et al*. (2008)
Glint11	BI452145	EST	Unknown	Coding	(CAA) repeat only?	Mathimaran *et al*. (2008)
Glint12	BM959214	EST	Unknown	Coding	(CAA) repeat + other indels	Mathimaran *et al*. (2008)
Glint13	BM959443*	EST	Unknown	Coding	(AAT) repeat? + other indels	Mathimaran *et al*. (2008)
Glint14	BM027461*	EST	Unknown	Coding	(T) repeat only?	Mathimaran *et al*. (2008)
Glint15	BM959581*	EST	Unknown	Coding	(T) repeat only?	Mathimaran *et al*. (2008)
Glint16	CG431704+705*	GSS	Unknown	Probably noncoding	(A) repeat only?	Mathimaran *et al*. (2008)
Glint17	CG431789+901*	GSS	Unknown	Probably noncoding	(T) repeat only?	Mathimaran *et al*. (2008)
Glint18 (same as *Glint09*)	AM118108	Standard	P-Type IID ATPase	Coding	(A) repeat only?	Corradi & Sanders (2006); Mathimaran *et al*. (2008)

Loci are named according to the original publications (Raab *et al*., 2005; Corradi & Sanders, 2006; Croll *et al*., 2008; Mathimaran *et al*., 2008). The putative functions of loci are noted if known from previously published work or if a BLASTX database search on National Center for Biotechnology Information (NCBI) revealed a highly significant match with a known fungal protein (alignment score > 50). Accession numbers show the original sequence of the repeat motif. * denotes accession numbers of loci where highly similar sequences from the database were assembled to make a contig covering the repeat motif. In these cases, the accession number indicates one of the original sequences covering the complete repeat locus. Databases are either the standard nucleotide collection, the genome survey sequences (GSS) or the expressed sequence tag (EST) databases from NCBI. All loci were classified accordingly to their likelihood of being coding or noncoding, depending on whether they are located in an expressed sequence or not. The length polymorphisms among the alleles at each locus were described according to the available sequence data (Croll *et al*., 2008; Mathimaran *et al*., 2008); a question mark has been added to the proposed repeat motif if no sequence data were available. For loci where sequence data were not available for all alleles, the length differences among the alleles were used to determine whether the predicted repeat motif alone can explain the observed length polymorphism or whether other indels must be present among the alleles.

obtain sequences that verify that the differences are real and not an artifact of the electrophoresis. This was not done for all loci showing 1- or 2-bp differences in the study by Mathimaran *et al.* (2008) and we suggest more rigorous testing of these differences before using these markers in genotyping studies. If large sets of isolates need to be analysed, the risk of artifacts in the allele identification may be dramatically reduced by using only loci with 3-bp or longer repeat motifs.

Assuming that the length differences are accurate, most of the markers identified by Croll *et al.* (2008) and Mathimaran *et al.* (2008) are useful for demonstrating genetic differences among *G. intraradices* isolates. This does not, however, mean that they are suitable for studying all aspects of AMF population biology. Mutation rates vary across the genome and it is generally assumed that noncoding regions evolve at a higher rate than coding regions, as a result of selective constraints on proteins encoded by the genes. Therefore, it is important to identify the location of the loci in the genome to predict their suitability for particular studies. Mathimaran *et al.* (2008) mostly identified length polymorphism in expressed sequence tags (ESTs). Repeat motifs identified in ESTs are likely to be under selective pressure to maintain functional integrity of the protein. However, most of the markers reported by Croll *et al.* (2008) and some of those reported by Mathimaran *et al.* (2008) originate from sequences obtained in a genome survey, where regions throughout the genome were randomly sequenced. Because of their random location in the genome, these sequences are likely to be outside of coding regions. However, *G. intraradices* was shown to have a relatively small genome of approx. 15 Mb (Hijri & Sanders, 2004) and, therefore, gene density could be relatively high. Neutral loci are preferable for population genetic studies, as the polymorphism more likely reflects random genetic processes such as mutation, migration or drift. As expected, a majority of the loci from both studies show length polymorphism in the repeat motif. However, a large number of indels and substitutions were also found outside the repeat motif (Table 1). Therefore, the markers do not represent pure simple sequence repeats (or microsatellites) and length differences among alleles should be considered carefully. However, the presence of a large number of substitutions enables researchers to use these markers for a variety of applications such as single nucleotide polymorphism (SNP) genotyping.

Genotyping on a large scale requires amplification of DNA from single spores directly collected from the field, instead of passing through the laborious process of *in vitro* cultivation. However, the small size of *G. intraradices* spores poses a challenge for the amplification of genetic markers because of the very low amount of DNA. Stukenbrock & Rosendahl (2005b) and Mathimaran *et al.* (2008) propose two different approaches to solve this problem. In the first study, a nested PCR was performed and up to five different loci could be amplified. However, it is not known whether this method would perform well with the comparatively small spores of *G. intraradices*. One additional concern is the number of loci that can be amplified simultaneously. Mathimaran *et al.* (2008) chose a promising method called whole-genome amplification (WGA), providing a higher number of template copies of each locus. This method is increasingly used for amplification of DNA from single cells (Spits *et al.*, 2006), unculturable bacteria (Stepanauskas & Sieracki, 2007) or filamentous fungi (Foster & Monahan, 2005), including AMF (Gadkar & Rillig, 2005a,b). While the potential exists to create many template loci from minute samples of cells or spores, several factors may bias the WGA. Notably, WGA is very sensitive to template contamination by other microorganisms as a result of the indiscriminate DNA amplification; a very real concern for spores from pot cultures or the soil (Hijri *et al.*, 2002; Corradi *et al.*, 2004). Furthermore, some parts of the genome tend to be better amplified than others, creating a representation bias in the final product and potentially null alleles (Pinard *et al.*, 2006). In order to apply whole-genome amplification to field-collected spores, the method should be rigorously tested by using well-defined *in vitro* cultivated material as a comparison to whole-genome amplification from single spores of the same culture.

If successfully applied, highly discriminatory markers combined with large-scale hierarchical sampling could elucidate the extent of clonal networks within field sites and resolve patterns of genetic diversity at larger geographic scales. Furthermore, the co-evolution between AMF and their host plants could be studied in detail by identifying spatial distributions of particular genotypes. These areas of investigation have become even more relevant in the context of globally applied inoculum in the absence of data on ecological competitiveness and the potential to persist in the field among native AMF (Schwartz *et al.*, 2006). While the global population genetics of plant pathogenic fungi has received much attention in recent years, studies on plant symbionts will hopefully catch up soon.

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Key words: arbuscular mycorrhizal fungi (AMF), *Glomus intraradices*, microsatellites, multilocus genotyping, population genetics, simple sequence repeats (SSR).

Unexpected vagaries of microsatellite loci in *Glomus intraradices*: length polymorphisms are rarely caused by variation in repeat number only

Microsatellite markers, or simple sequence repeats (SSRs), are widely used as tools to distinguish genotypes or individuals in paternity analyses, forensics and population genetics (Ellegren, 2004). Microsatellites have been studied extensively in many fungal genomes (Lim *et al.*, 2004) but, surprisingly, have not been exploited to study the population genetics of arbuscular mycorrhizal fungi (AMF), a class of important plant symbionts, until two publications independently claimed the utility of these markers for a specific species, *Glomus intraradices* (Croll *et al.*, 2008a; Mathimaran *et al.*, 2008). In a letter contributed to this forum (Croll *et al.*, 2008b), these 'microsatellite markers' were tabulated with the aim of clarifying possible confusions about their suitability in population genetics. The authors of this letter concluded that 'as expected, a majority of the loci from both studies show length polymorphism in the repeat motif' (cited from Croll *et al.*, 2008b). However, only 10% of the length polymorphisms they observed (Croll *et al.*, 2008a) were caused, at least partially, by changes in the repeat motif (Table 1). The vast majority of length polymorphisms (> 90%) were caused by insertions–deletions (indels) in the flanking regions; some of the so-called SSR loci did not contain any repeat longer than two triplets and were not polymorphic in these areas.

Table 1 Nature of polymorphisms observed at simple sequence repeat (SSR) loci in Croll *et al.* (2008a) and this study

Study	Alleles sequenced	Total number of length polymorphisms	Only caused by SSR	Partly caused by SSR	Not at all caused by SSR
Croll *et al*. (2008a)	40	29	2 (7%)	1 (3%)	26 (90%)
Present	36	28[a]	5 (18%)	10 (36%)	13 (46%)

[a]Including length polymorphisms compared with the original sequence obtained from public databases (see Supporting Information Fig. S1).

In our own study, we identified microsatellite loci, using well-defined criteria, in our database screen (at least five identical repeats of two, three or four nucleotides, or a stretch of at least 10 identical single nucleotides). We found clear length polymorphisms in 18 loci selected in this way, examining eight different strains of *G. intraradices*. The target repeat sequence was present in each case, and it would have been logical to assume that the length polymorphisms would have been caused by changes in the numbers of repeat lengths. However, when we sequenced two alleles of different size for each of the 18 loci (see Supporting Information Fig. S1), we found that the length difference was based exclusively on repeat length polymorphism in only 18% of the alleles studied, and at least partially in 36%. For almost half of the alleles studied (46%), the repeat was not affected and the length polymorphism was caused by adjacent indels (Table 1).

The frequency of length polymorphisms in the targeted microsatellites was only marginally higher than in the nontargeted flanking regions (i.e. 5.1% per base pair in the microsatellite region compared with 2.7% per base pair in the flanking region for our study) (Fig. S1).

We conclude that microsatellites of short length ($n \sim 5$ for di-, tri- and tetranucleotides, and $n \sim 10$ for mononucleotides), as investigated in the studies (Croll *et al.*, 2008a; Mathimaran *et al.*, 2008), seem not to enhance significantly the probability to find length polymorphisms of value for population genetic analysis. Nevertheless, as also stated in the accompanying letter (Croll *et al.*, 2008b), length polymorphisms that happen to occur within and around such short microsatellites may still be highly useful in genotyping.

Length polymorphisms such as those analysed here are useful to demonstrate genetic differences among *G. intraradices* isolates in general; it remains an ongoing debate whether markers in expressed sequences or in noncoding regions are of greater interest. Mutation rates vary across the genome, and it is generally assumed that noncoding regions evolve at a higher rate than coding regions, as a result of selective constraints on the transcripts and proteins encoded by the genes. On the other hand, markers in expressed parts of the genome, such as expressed sequence tag (EST)-derived markers, have advantages over nonexpressed markers as they could be both used for gene mapping as well as for population genetics. Moreover, EST-derived markers are believed to be more suitable for cross-species transferability (Varshney *et al.*, 2005; Ellis & Burke, 2007; Hisano *et al.*, 2007). For population genetics, 'neutral' markers not subject to selection are of particular interest, and markers derived from ESTs (Mathimaran *et al.*, 2008) may be less favourable in this respect. However, markers derived from a genome survey (Croll *et al.*, 2008a) may also be expressed. Moreover, nonexpressed parts of the genome can be under equally strong selection as expressed parts and we therefore suggest that 'neutrality', if required, has to be tested for each locus instead of relying on global assumptions.

Accidentally, two of the *bona fide* microsatellites selected in our study, namely *Glint09* and *Glint18* (Mathimaran *et al.*, 2008), were in a sequence previously studied, encoding a P-type II ATPase D (Corradi *et al.*, 2007). Analysis of each of these two loci displayed a clear single band in all our single-spore DNA preparations, indicating that it was represented by an allele (or alleles) of a single size in an individual spore of each strain analyzed. With respect to the locus of *Glint09*, this corresponded to a band of 107 bp, 115/116 bp or 121/122 bp (Mathimaran *et al.*, 2008). Experiments with DNA from mixed spores showed that two alleles of different size showed up as clear doublets with the appropriate size difference (data not shown). The size of the alleles found in individual spores matched the length variants (105/106, 114/115 and 121 bp) found combined either as two or three alleles in DNA preparations of mycelium from root-organ cultures of single strains in the previous study (Corradi *et al.*, 2007). We do not have an explanation for this difference, but we point out that different single spores of a given strain, subjected to whole-genome amplification (WGA), always yielded a unique band of constant length for a given polymorphic locus (Mathimaran *et al.*, 2008).

The ability to detect single alleles at a polymorphic locus in single spores is a clear advantage of the WGA method. Whole-genome amplification is particularly useful for detecting low-copy-number sequences from environmental samples (Gonzalez *et al.*, 2005) where standard polymerase chain reaction (PCR) methods are insufficient, and it has successfully been used to genotype powdery mildew (Fernandez-Ortuno *et al.*, 2007). Owing to the high-fidelity proof-reading function of Phi29 DNA polymerase, the WGA product is a highly accurate copy of the original genome (Dean *et al.*, 2002). Indeed, using this technique with four separate amplifications from single spores of two different isolates of *G. intraradices*, there was faithful amplification for all of the three loci tested (Mathimaran

et al., 2008). Thus, the WGA procedure greatly enhances opportunities to detect size polymorphisms at multiple loci in single spores.

The potential SSR markers identified by Mathimaran *et al.* (2008) have been deposited in a newly developed database for Glomus (http://glomus.vital-it.ch/), which is maintained by the Swiss Institute of Bioinformatics and is now accessible to scientists worldwide. In the future, this database will be upgraded to allow users to retrieve as well as to deposit useful length-polymorphic markers for tracing AMF. This is particularly important because large numbers of markers may soon be available from various AMF species, which need to be consolidated into a relational database for easy access of a particular marker locus, as in the case of databases for other eukaryotes (see e.g. the Swiss Vitis Microsatellite Database).

Interestingly, both studies reviewed here and in the accompanying letter (Croll *et al.*, 2008b) clearly show that all the loci characterized by length polymorphisms have a single size within a given isolate and thus are not heterogeneous in descendants of a single spore. This means that such length polymorphisms – whether caused by indels or microsatellite repeat polymorphisms – can be used to genotype AMF strains. This will be a great asset for future population genetic and ecological studies as well as for the re-identification and tracing of AMF strains of particular value used as biofertilizers in agriculture. Moreover, the absence of multiple alleles in a given strain suggests that AMF are essentially homokaryotic with a haploid genome rather than having an unusual heterokaryotic lifestyle, two contrasting hypotheses discussed recently in *New Phytologist* (Rosendahl, 2008; Young, 2008).

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Key words: arbuscular mycorrhizal fungi (AMF), *Glomus intraradices*, microsatellites, simple sequence repeats (SSRs), strain identification.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Sequence alignments for all 18 loci described by Mathimaran *et al.* (2008).

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