Commentary

Comparative transcriptomics – model species lead the way

Plant biology has reached a stage where more and more molecular tools are being developed for species other than the general *reference* species like Arabidopsis or rice. Unfortunately this does not often include the construction of 'whole genome' microarrays. Although genome-wide expression analysis is very instructive in obtaining clues about (novel) genes that are active under the studied conditions, the cost of designing and producing microarrays for the wide variety of species with interesting traits are too great. That is also why, in recent years, several successful attempts have been made to carry out heterologous microarray hybridizations for studying the transcriptome of different non-model species. In this issue of *New Phytologist* Hammond *et al.* (pp. 239–260) present an improved method of using an Arabidopsis Affymetrix array to determine the transcript profile of two related *Thlaspi* species with contrasting metal accumulation and tolerance phenotypes.

'... it will be a true challenge to develop the bioinformatic tools to accomplish the design of gene-specific probes that are able to detect orthologues while distinguishing paralogues.'

Comparative transcriptomics in plant biology

The two main reasons for performing heterologous transcript profiling are: (1) to compare two closely related species with contrasting traits, and (2) to compare different tissues, stages or conditions in a species for which no specific arrays are available. These two objectives are often combined, as has also been done by Hammond *et al.* The need for comparative transcriptomics, which inherently involves heterologous microarray hybridization, is often fueled by the presence of interesting traits and properties in one species that are not found in any of the current model species. If the right conditions and tissues are chosen for sampling, and assuming that many of the basic processes in plants are similar, the differences found by comparative transcriptomics will include the detection of genes involved in the studied trait or property. Good examples of this are adaptive traits, such as physiological adaptation to continuous adverse environmental conditions (low or high light intensity, high altitude, low or high temperatures, extreme soil composition – pH, salt, heavy metals, nutrient deficiencies, regular flooding, etc.) or developmental adaptations (leaf or root hair density, leaf shape, flowering time, vernalization response, wax deposition, etc.). Other examples are differences in general plant architecture such as fruit or flower size, tuberization, etc.; differences in plant defense or differences in primary or secondary metabolite production. In order to obtain informative hybridization results, the two species under comparison should be closely related to allow for sufficient probe–cDNA cross-hybridization.

In particular, Arabidopsis arrays have been used for comparative transcriptomics in the past to compare Arabidopsis (*Arabidopsis thaliana*) to related Brassicaceae species such as *Arabidopsis halleri* (Becher *et al.*, 2004; Weber *et al.*, 2004), *Thlaspi caerulescens* (Hammond *et al.*, 2006; van de Mortel *et al.*, unpublished), *Thellungiella halophila* (Taji *et al.*, 2004; Gong *et al.*, 2005), *Brassica oleracea* (Hammond *et al.*, 2005) and *B. napus* (Li *et al.*, 2005), but also recently a tomato array has been successfully used to examine fruit ripening and development in tomato, eggplant and pepper (Moore *et al.*, 2005), and no doubt more such experiments will soon follow. Three of the Brassicaceae species were studied because of their special adaptive traits, such as Zn and/or Cd hyperaccumulation and hypertolerance of *A. halleri* and *T. caerulescens*, and salt and cold tolerance of *T. halophila*. An interesting general trend that emerged from the analysis of the adapted species was that many orthologues of genes that were induced by stress in Arabidopsis were more highly expressed in the adapted species, especially in the absence of the stressor.

As the Arabidopsis genome was the first plant genome to be fully sequenced, the largest variety of array platforms is available for this model species. In general, four different types can be distinguished: (1) spotted (full-length) cDNA microarrays; (2) spotted PCR-amplified gene-specific sequence tag (GST) or genomic amplicon arrays; (3) on-slide synthesized short oligonucleotide arrays; and (4) spotted or on-slide synthesized long oligonucleotide arrays (Rensink & Buell, 2005). Spotted cDNA microarrays are the least

sophisticated, containing a collection of clones from different cDNA libraries, which are PCR-amplified using vector specific primers. Short oligonucleotide arrays contain probes up to 25 bases in length. Long oligonucleotide arrays contain probes of between 50 and 70 bases. The spotted gene-specific fragment arrays contain unique segments of the gene which are amplified from genomic DNA (gDNA), or gDNA libraries using specific primers for each gene fragment. Oligo arrays are generally more technologically advanced and are therefore often only commercially available, while the spotted PCR fragment arrays are often developed within academia. Next to the Arabidopsis arrays there is a growing list of arrays for other species, mainly crops like barley, *Brassica*, *Citrus*, grape, lily, maize, potato, rice, soybean, sugar cane, tomato and wheat, but also for trees such as poplar, pine and spruce and for the legume model *Medicago truncatula* (Rensink & Buell, 2005; Huang *et al.*, 2006; Ralph *et al.*, 2006).

Considering that the successful use of heterologous microarray hybridization depends largely on the level of sequence similarity and considering that this is highest among members of the same plant families, the current range of arrays already covers many agronomically important plant families (Brassicaceae, Leguminosae, Graminae, Solanaceae). Of course it will still be possible that there is too much sequence diversity for members of the same family to permit heterologous microarray hybridization. Even if the average level of sequence identity is sufficient, it is important to note that the subset of probes may not hybridize efficiently due to lower sequence identity. In the *Thlaspi* interspecies comparison method described by Hammond *et al.*, the Affymetrix arrays used contained several 25-bp oligonucleotide probes per gene. *T. caerulescens* has an average coding region DNA identity with Arabidopsis of 88.5% (Rigola *et al.*, 2006). Upon heterologous hybridization, many probes did not perfectly match the orthologous *Thlaspi* sequence, resulting in a relatively low number of 'present' calls. After hybridization with *Thlaspi* gDNA, non-fitting probe pairs could be discarded without discarding the entire probe-set and thus still generate acceptable expression data. This method is only useful for microarray platforms that contain multiple probes for one gene. Moreover, when comparing two species, care should be taken to include the same probes in the final set for comparison. If not, using one probe for the gene in one species and another for the orthologous gene in the other can lead to false conclusions on expression levels. When using arrays with only one probe per gene, discarding probes that do not hybridize properly to the target cDNAs will result in fewer genes for which expression can be determined. This may be a disadvantage compared to short oligonucleotide arrays, but on the other hand, longer oligos permit less sequence conservation between species, so that less probes will be discarded compared to the short oligo arrays.

Challenges for comparative transcriptomics

Comparative transcriptomics can be a very rewarding tool for discovering new gene expression profiles in plant species in the absence of species-specific cDNA information or microarrays, on the condition that there are arrays from a sufficiently closely related species. It is clear that such is still not the case for many important families. Currently for instance, the Caryophyllaceae, Chenopodiaceae, Compositae, Rosaceae and Umbelliferae are not yet represented among the species for which microarrays are available. Even though for a growing number of species, including *T. caerulescens* (Plessl *et al.* 2005), spotted cDNA microarrays are being developed, the number of genes represented on such arrays are often limited and more information will probably be obtained by hybridizing to a heterologous, but genome-wide, microarray. Rather than trying to complete as many as possible of the species specific arrays, it will probably be more efficient to focus on the development of a family specific array. This may well be based on the transcriptome of one reference species, like Arabidopsis for the Brassicaceae, supplemented with probes representing genes not found in Arabidopsis but present in other Brassicaceae. The growing collection of Expressed Sequence Tags that are generated for very many different species would be an excellent source of information for the careful design of plant-family oriented gene-specific long oligonucleotide probes. This will not be easy and it will be a true challenge to develop the bioinformatic tools enabling the design of gene-specific probes that can detect orthologues while distinguishing paralogues, a problem that is in addition not solved in designing species-specific microarrays.

Another important item that should be dealt with to improve comparative transcriptomics is to be able to account for all possible transcripts that can be found in a plant cell. Even for Arabidopsis, of which the full genome sequence is known and for which gene expression has been studied by many groups all over the world, the recent use of whole-genome tiling arrays (WGAs) showed that there were still many regions with transcriptional activity although there was no gene annotated (Mockler & Ecker, 2005). WGAs contain non- or partially overlapping probes that are tiled to cover the entire genome. They are instructive for identifying rarely or lowly expressed genes or miRNAs that are hard to identify or predict otherwise, and for designing probes to add to the current arrays. Although very informative for plant genomics, including transcriptomics, the high costs associated with making such arrays makes it unlikely that this kind of array will soon be available for many gene families. Moreover, for these, model species will lead the way.

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Key words: comparative transcriptomics, model species, microarray.

Ozone – a significant threat to future world food production?

Air pollution and climate change are both recognised as significant threats to food production. Ozone was established as the most important regional pollutant in terms of its impact on agriculture in North America and Europe two decades ago, but more recently, it has become clear that global background concentrations of ozone are also increasing (Vingarzan, 2004). Assessing the impact of changes in both regional and global background ozone concentrations on food production in the context of other global atmospheric and climatic changes is a major challenge (Ashmore, 2005).

However, current assessments of the effects on crop yield from changes in ozone concentrations are based on experiments carried out in open-top chambers. These chambers modify environmental conditions such as temperature, evapotranspiration and irradiance, and hence there is uncertainty over how well they represent the real effects of ozone under field conditions. The study by Morgan *et al.* reported in this issue of *New Phytologist* (pp. 333–343) instead used free-air gas concentration enrichment (FACE) to increase ozone exposures under field conditions. This is the first large-scale study to use FACE to show the effects of ozone in reducing the yield of a major arable crop (soybean) under field conditions. Importantly, the study shows losses in soybean yield under field conditions that are at least as large as those predicted from chamber studies.

'… there is a need to consider plant adaptation strategies to increased ozone exposure alongside climate change.'

Global implications of ozone effects on crop yield

Morgan *et al.*'s study was carried out in North America. A number of models have now been developed to predict

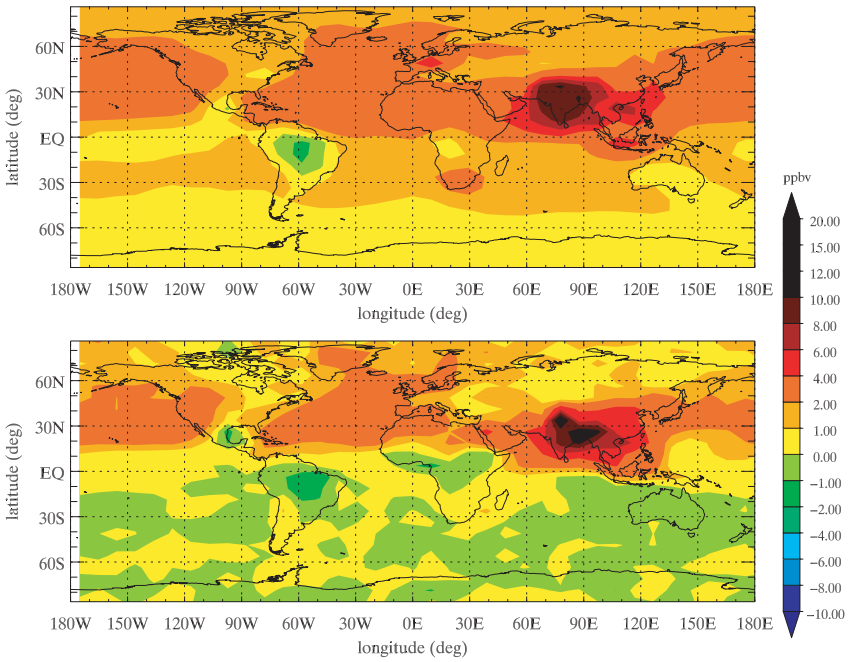

Fig. 1 Predicted differences in decadal annual mean surface ozone concentrations from the 1990s to the 2020s, for two global chemistry-transport models, under a 'Current Legislation' scenario. The upper diagram presents predictions for the TM3 model and the lower diagram presents predictions for the STOCHEM model. This figure is reproduced from fig. 11(a) of Dentener *et al*. (2005), with kind permission of Frank Dentener and David Stevenson.

future changes in global ozone concentrations, based on scenarios of precursor emissions and climate. These predictions can be linked to IPCC scenarios, so that the impacts of ozone can be considered in the context of wider global change; one example of recent predictions of change in ozone concentrations for the period 1990–2020 is shown in Fig. 1 (Dentener *et al.*, 2005). The results are based on a 'Current Legislation' scenario, which incorporates expected economic development and planned emission controls in individual countries.

The results predict increases in annual mean surface ozone concentrations in all major agricultural areas of the northern hemisphere. The modelled increases show a large spatial variation; they are low in the areas of North America where Morgan *et al.*'s study was conducted, but are high in south and east Asia. Morgan *et al.* reported that an increase of 13 p.p.b. in mean daytime ozone concentration caused a 20% decrease in soybean seed yield, and compared this to projected ozone concentration increases for the USA by 2050. However, the predictions of Dentener *et al.* (2005) indicate that this increase in ozone concentration could be reached as soon as 2020 in south Asia.

It is thus vital to consider the implications of these findings for food production outside North America and Europe. While evidence is limited, significant effects of air pollution on crop yield have been shown in Asia, Africa and Latin America (Emberson *et al.*, 2003). In Pakistan, field studies using a chemical protectant and open-top chambers (Wahid *et al.*, 2001) showed yield losses of about 50% in a local cultivar of soybean, at ozone levels comparable to those of Morgan *et al.* Although soybean is not a staple crop in south Asia, several other bean species are important, especially in India, as components of a largely vegetarian diet. In this context, effects on crop quality, which were not considered by Morgan *et al.*, may also be significant.

In assessing the wider implications for food production and security in regions with high projected increases in ozone concentration and increasing populations, it is important to note that soybean is among the most sensitive to ozone of the major crops. The effects on wheat and rice may be lower, although there is potential for significant yield reductions of major cereal crops in east and south Asia due to future ozone exposures (Emberson *et al.*, 2003; Wang & Mauzerall, 2004). Although maximum technically feasible reduction scenarios for precursor emissions have been identified which result in reductions in ozone exposure by the 2020s (e.g. Dentener *et al.*, 2005), in practice these scenarios are unlikely, and there is a need to consider adaptation strategies to increased ozone exposure alongside climate change. For example, in identifying and selecting genetic traits associated with increased tolerance of drought or high temperatures, ozone tolerance should also be considered.

Ozone studies in a broader environmental context: the importance of FACE

Ozone impacts on vegetation cannot be considered in isolation, because they interact with various other environmental factors including temperature, light, water, atmospheric CO_2, nutrients, pathogens and pests (Ashmore, 2005). The FACE study of Morgan *et al.* shows how an extreme climatic event (hailstorm) can affect yield loss due to elevated ozone.

FACE is very suitable for the study of such plant–climate and other interactions with ozone since it can closely approach natural field conditions. The large scale and long-term nature of the FACE experiments also facilitates studies on ecosystem level processes such as pathogen and pest outbreaks, intraspecific and interspecific competition, and carbon and nutrient cycling. The SoyFACE study, of which the work reported by Morgan *et al.* is part, involves several interacting factors, i.e. elevated CO_2, herbivory, drought and genotypic differences, which will yield a wealth of future information on ozone impacts to crops (e.g. Miyazaki *et al.*, 2004; Hamilton *et al.*, 2005).

The importance of such interactions is demonstrated by other ozone FACE studies in a young temperate tree ecosystem (Karnosky *et al.*, 2005) and a sub-alpine semi-natural grassland community (Volk *et al.*, 2006). In the AspenFACE experiment, elevated CO_2 reduced the effects of ozone on photosynthesis and the above-ground growth of trembling aspen (*Populus tremuloides*) (Karnosky *et al.*, 2003, 2005). This may have resulted from decreased stomatal conductance, an increase in detoxification capacity, or changes in other interacting factors. Carbon sequestration in soils at elevated CO_2 levels was also affected at elevated ozone in this experiment (Loya *et al.*, 2003). After 4 years of exposure to elevated ozone and CO_2, soil carbon formation was reduced by 50% compared to ambient ozone and elevated CO_2. These reductions most likely resulted from decreased plant litter inputs (Karnosky *et al.*, 2003) and the enhanced microbial respiration of recent carbon inputs.

Studies on pest and pathogen outbreaks are also facilitated by minimal impediment to movement to and from the plots. An early FACE study indicated that foliar pathogens differed in their response to sulphur dioxide in winter barley and winter wheat (McLeod, 1988). Infection of a foliar pathogen on trembling aspen increased under elevated ozone in the Aspen FACE experiment, probably due to the changes in leaf surface properties (Karnosky *et al.*, 2002). Elevated ozone also affected the performance of forest pests in the same experiment, which may be related to changes in plant chemistry or the abundance of natural enemies (Percy *et al.*, 2002; Karnosky *et al.*, 2003). Differential responses of tree genotypes and species were observed for photosynthesis and above-ground growth (Karnosky *et al.*, 2005), while a FACE study in an old regularly harvested grassland showed that the relative biomass contributions of functional groups (grasses, herbs and legumes) were affected by elevated ozone (Volk *et al.*, 2006). Cumulative ozone effects occurred over several years in both studies, emphasising the need for long-term studies, which are only possible with FACE, on whether ozone causes major shifts in species and genetic diversity in sensitive ecosystems.

The impacts of ozone need to be considered in combination with major global change factors. FACE is an excellent tool for improving such understanding, but it has some limitations. In contrast to open-top chambers, FACE systems cannot be used for ozone impact studies that include levels below ambient. Volk *et al.* (2003) suggested that the relatively high temporal fluctuations in the ratio of elevated ozone concentration to ambient under FACE conditions may influence biological responses to ozone. Significant spatial gradients can also occur within the large FACE plots, although this can be dealt with by careful experimental design including subsampling, subplots and the randomisation of plant genotypes and species (Karnosky *et al.*, 2003; Volk *et al.*, 2003; Morgan *et al.*, 2006).

Conclusions

Morgan *et al.* provide important new evidence of the effects of ozone on crop yield under field conditions. The impacts of ozone on future food security need to be considered as an important component of global change, especially in regions with rapid economic development. However, our knowledge both of the impacts of ozone in these regions and of its interactions with other elements of global change remains very limited.

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Key words: FACE, food security, global change, ozone, soybean.

Letters

On modelling Rubisco turnover: dynamics and applicability

We would like to thank Professors Hirel and Gallais for their largely positive commentary (Hirel & Gallais, 2006) that was inspired by our paper in the same issue of *New Phytologist* (Irving & Robinson, 2006). However, several points made by Hirel and Gallais misrepresent our views and we would like to clarify our position.

1. In the section 'The dual role of Rubisco', Hirel and Gallais write '… Irving and Robinson have questioned the role of Rubisco as a leaf storage protein'. That is not our view. In the first paragraph of our paper we acknowledge that Rubisco is a storage protein. Numerous studies have shown leaf Rubisco to be in excess at varying N availabilities, irradiances and developmental stages (Theobald *et al.*, 1998; Warren *et al.*, 2000; Murchie *et al.*, 2002). We unequivocally accept that Rubisco is an N store, but it is a dynamic store which operates at a whole-plant level (i.e. between leaves in a canopy), rather than solely as a static store involved in recycling N within single leaves.

2. Hirel and Gallais' statement 'The case of nongraminoid plants mentioned by Irving and Robinson, which apparently does not fit to the model …', in the section 'The dynamic model of Rubisco turnover: from theory to physiology and agronomy', is misleading. We hypothesize that our model will not be applicable to nongraminoid plants, but acknowledge that it has never been tested. It is possible that our model might be useful in modelling broadleaved plants, but we suggest that this is unlikely because of the differences in leaf-production mechanisms between these types of plant.

3. Our model was not designed explicitly for stressed plants; however, provided that the Rubisco concentration/content can be described by a log-normal curve, it should still be applicable to them. In our paper, we reanalysed data from Takeuchi *et al.* (2002). That study investigated the effect of ultraviolet B (UV-B) stress on rice genotypes. It seems likely that UV-B has dual effects: it can be involved in the generation of oxygen radicals, which modify the Rubisco large subunit such that it is more likely to be proteolytically degraded (Desimone *et al.*, 1996; Ishida *et al.*, 1998; Luo *et al.*, 2002); and UV-B appears to have an effect on Rubisco synthesis, which suggests that UV-B may have a role in degrading RNA transcripts. The shorter half-life of proteins in stressed plants would accelerate the loss of the ^{15}N label from the Rubisco pool, and this will be testable using our model.

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Endobacteria or bacterial endosymbionts? To be or not to be

A recent paper published by Partida-Martinez & Hertweck (2005) and comments on it by Sanders (2005) offer new ideas about the importance of endosymbiosis in fungi. This paper offers a good opportunity to discuss some of the functional aspects, structural features and phylogenetic relationships among the players in the endosymbiosis between fungi and bacteria. Analysis of the multiple interactions established by organisms from different kingdoms offers new keys for understanding the complexity of symbioses.

Symbiotic associations between endocellular bacteria and eukaryotic cells are widespread among animals (e.g. *Buchnera* and *Wolbachia* spp. in insects) and plants (e.g. *Nostoc* spp. with *Gunnera*; *Burkholderia* spp. with *Rubiaceae*; rhizobia with legumes), but relatively little is known about associations between bacteria and fungi (de Boer *et al.*, 2005; Artursson *et al.*, 2006). In the fungal kingdom, the presence of endocellular bacteria has been reported in some *Glomeromycota* species, both in arbuscular mycorrhizal (AM) fungi and *Geosiphon pyriforme*, as well as in the ectomycorrhizal basidiomycete *Laccaria bicolor* and *Tuber borchii* (Bianciotto *et al.*, 1996; Barbieri *et al.*, 2000; Schüßler & Kluge, 2001; Bertaux *et al.*, 2003; Bertaux *et al.*, 2005). A summary of the main biological features of these fungal/endobacterial couples is given in Table 1.

The AM fungi, which are themselves obligate plant symbionts, represent a specialized niche for rod-shaped bacteria, consistently found in many of the Gigasporaceae through all the steps of the fungal life cycle. On the basis of their ribosomal sequences, the endobacteria have been identified as a new bacterial taxon, *Candidatus* Glomeribacter gigasporarum (Bianciotto *et al.*, 2003).

Very recently a bacterium has been found living in association with a saprotrophic fungus, both forming a phytopathogenic alliance which leads to rice seedling blight disease (Partida-Martinez & Hertweck, 2005). The cause of seedling blight, an economically costly agricultural disease, had been thought to be caused by the rhizoxin released by some *Rhizopus* species. Partida-Martinez & Hertweck (2005) show that this toxin is synthesized by a β-proteobacterium belonging to the genus *Burkholderia*, which lives symbiotically inside the fungus. The authors were able to observe bacteria living inside toxin-producing *Rhizopus* strains by using laser microscopy, to isolate the microbes, and to demonstrate that they, not the fungus, produced the toxin.

We wish to respond to this interesting letter and the related commentary (Partida-Martinez & Hertweck, 2005; Sanders, 2005). We fully agree with the statement that the association between *Rhizopus* and *Burkholderia* sp. is an outstanding example of a bacterial–fungal symbiosis with a clear metabolic function, but it may be not the first one. The symbiosis between *G. pyriforme* and *Nostoc punctiforme* provides the association with the capacity (at least potentially) to reduce acetylene and to fix atmospheric nitrogen (Kluge *et al.*, 1992). We do not fully agree with the use of the term 'endosymbiosis'. The published confocal pictures show bacteria inside the *Rhizopus* hyphae, but do not provide any evidence of the cellular interactions between the bacteria and their fungal host. One of the landmarks of endosymbiosis is that both partners have to be alive. There are questions about the viability of

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Table 1 Fungal/endobacterial couples and their biological features

	Fungal/bacterial couples				
Features	*Gigaspora margarita*/*Ca*. G. gigasporarum	*Rhizopus microsporus*/ *Burkholderia* sp.	*Geosiphon pyriforme*/ *Nostoc punctiforme*	*Laccaria bicolor*/ *Paenibacillus* spp.	*Tuber borchii*/ CFB phylogroup
Fungal partner	Glomeromycota	Zygomycota	Glomeromycota	Basidiomycota	Ascomycota
Fungal isolate	BEG34	ATCC 62417	GEO1	S238N	ATCC 96540
Mycelium morphology	Coenocytic	Coenocytic	Coenocytic	Septate	Septate
Fungal structures hosting bacteria	Mycelium, spores, auxiliary cells	Mycelium	Bladder	Mycelium	Mycelium, ascocarp
Bacterial partner	β-proteobacteria	β-proteobacteria	Cyanobacteria	Firmicutes	Bacteroidetes
Bacteria genome size (MB)	1.4	nd	9.78	nd	nd
Bacterial status					
Free-living	No	nd	Yes	nd	nd
Culturable	No	Yes	Yes	Yes	No
Association type	Permanent	Cyclical	Cyclical	nd	nd
Bacteria surrounded by host membrane	Yes	nd	Yes	nd	nd
Metabolic capabilities of association	nd	Rhizoxin production	Higher photosynthetic activity, acetylene reduction (N_2 fixation?)	nd	nd

nd, Not determined; CFB, *Cytophaga-Flexibacter-Bacteroides*.
Ca. G. gigasporarum, *Candidatus* Glomeribacter gigasporarum.

the *Rhizopus* hyphae that harbour the endobacteria. When our group characterized the association between *Ca.* G. gigasporarum and its fungal host, we demonstrated that the bacterium lives and multiplies in the living mycelium, moving along the hyphae even when the fungus colonizes the plant roots. During all steps of this tripartite interaction, the bacterium is always inside a vacuole-like compartment surrounded by a membrane (Bianciotto *et al*., 1996) (Fig. 1). This is a well known feature in prokaryote/eukaryote endosymbioses: rhizobia in nodulated plants; *Buchnera* in insect cells; *Nostoc* inside *Geosiphon*; and plastids that originated as cyanobacterial symbionts are also surrounded by a membrane (Schüßler *et al*., 1996; Sessitsch *et al*., 2002; Moran *et al*., 2005; Okamoto & Inouye, 2005).

Given that bacterial–fungal symbiosis still represents a largely unknown field, there are some interesting common points shared by the bacterial/fungal couples that have been investigated (Table 1). Excluding the *Geosiphon* case, as its colonization is limited to fungal bladders, both the fungal hosts *Rhizopus* and *Gigaspora* have a coenocytic mycelium, and the bacteria belong to the β-proteobacteria. However, some differences should also be highlighted. For instance, the two endobacteria are not as closely phylogenetically related as stated by Partida-Martinez & Hertweck (2005) and Sanders (2005). On the basis of 16S ribosomal sequences, in 2003 we moved the endobacteria of Gigasporaceae from *Burkholderia* to a new taxon (Bianciotto *et al*., 2003). To update and

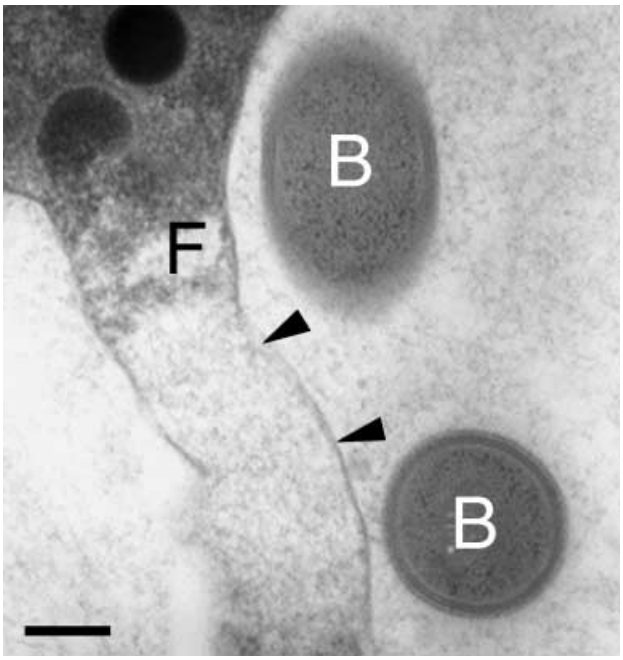

Fig. 1 Transmission electron image of *Ca*. Glomeribacter gigasporarum (B) living inside the cytoplasm of *Gigaspora margarita* (F). The bacteria are located in a vacuole-like compartment surrounded by membrane (arrowheads). Bar, 0.20 μm.

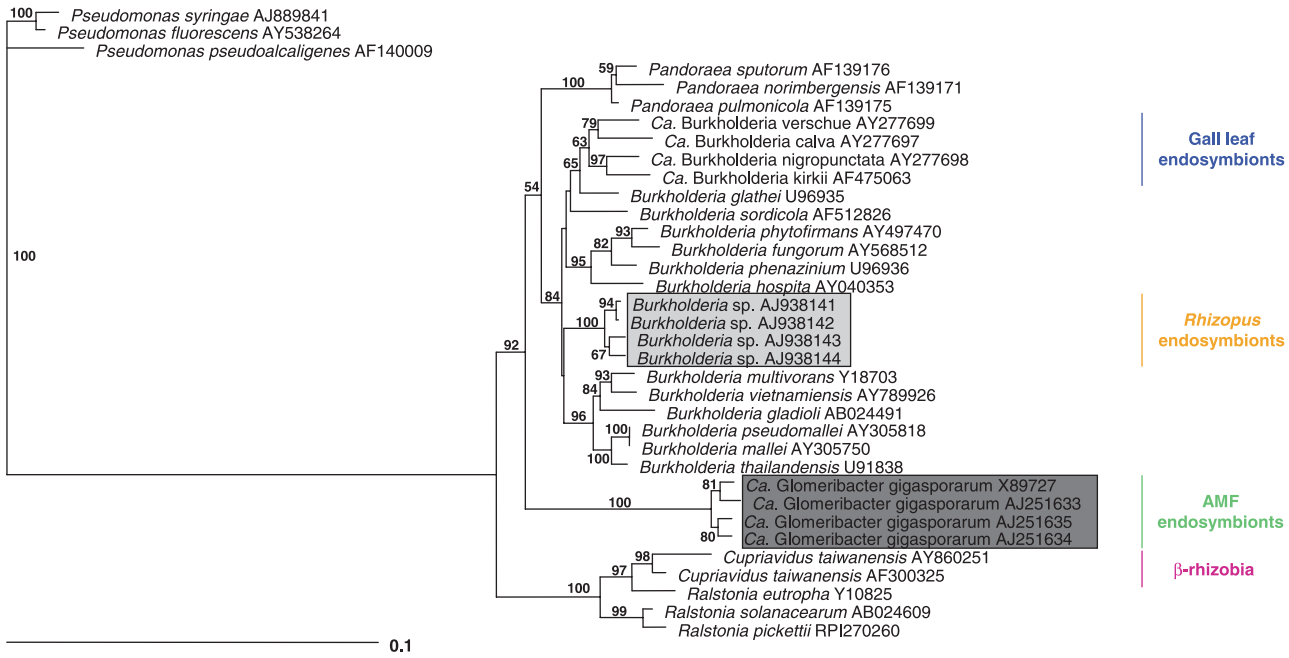

Fig. 2 Neighbour-joining tree obtained from the 16S rDNA sequences alignment of β-proteobacteria, including *Burkholderia* spp. and representatives of related genera. The *Burkholderia* isolates from *Rhizopus* and *Ca*. Glomeribacter gigasporarum are shaded grey. Specific symbiotic capabilities are indicated on the right. The Kimura two-parameter model was used for pairwise distance measurement. Bootstrap values >50% are indicated (100 replicates). *Pseudomonas syringae* (AJ889841), *Pseudomonas fluorescens* (AY538264) and *Pseudomonas pseudoalcaligenes* (AF140009) were used as outgroup taxa to root the tree. Bar, Kimura distance.

clarify the phylogenetic position of *Ca*. G. gigasporarum, we calculated a new inference based on 16S rDNA of several β-proteobacteria, including species able to establish transient or permanent associations with plants or fungi and free-living species (Coenye & Vandamme, 2003). Figure 2 shows that AM fungal endosymbionts cluster in a clade distinct from that including all the *Burkholderia* species, and that they are not related to the toxin producing *Burkholderia* isolates recovered from *Rhizopus*.

Finally, Partida-Martinez & Hertweck (2005) raise some ecologically important questions, such as the acquisition and transmission of the endobacteria. They were successful in their isolation and reintroduction into a symbiont-free host. Although they do not offer details on the reintroduction protocol, their results appear to suggest horizontal transmission, maybe based on a cyclical invasion. This is another clear difference from the unculturable endosymbiont of *Gigaspora*, where strict vertical transmission was demonstrated (Bianciotto *et al*., 2004).

There is no doubt that fungal–endobacterial interactions cover multiple aspects, from evolutionary biology to applied microbiology, and may offer unexpected tools to investigate key steps in the evolution of modern fungi.

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Key words: endosymbiosis, fungi, bacteria, *Candidatus* Glomeribacter gigasporarum, *Burkholderia* sp.

Meetings

A remarkable moment in Australian biogeography

The evolution of Australia over the last 25 million years: the consequences of aridification and ice-age cycles. A special session of the Combined Australian Entomological Society's 36th AGM and Scientific Conference, 7th Invertebrate Biodiversity Conference, and Society of Australian Systematic Biologists and Conference. Australian National University, Canberra, Australia, December 2005

To borrow a Churchillian metaphor, Australian biogeography is a riddle wrapped in a mystery inside an enigma. There can be no doubt that a key driver of the evolution of the unique Australian biota was the development of quintessentially Australian dry and infertile landscapes. However, the rub is that there is vanishingly little specific information about when during the Cainozoic (Tertiary and Quaternary Periods), and how, the humid Gondwanic rainforests were transformed into the modern fire- and drought-adapted Australian biota. A recent meeting in Canberra entitled 'The evolution of Australia over the last 25 million years: the consequences of aridification and ice-age cycles' marks an important milestone in Australian biogeography. This meeting was facilitated by the Australian Research Council's Environmental Futures Network (http://nesuab.ees.adelaide.edu.au/page/default.asp?site=1). For the first time, researchers working on Australian palaeoecology and geomorphology and the relationships and phylogeography of plant, vertebrate and invertebrate groups were able to meet, compare data and recognize their remarkably convergent perspectives.

'The lack of geological rejuvenation of the landscape, and attendant soil infertility, is thought to have been of critical importance in preadapting the Australian flora to aridity.'

Aridification during the Tertiary

Jim Bowler (University of Melbourne, Australia) and John Chappell (Australian National University (ANU), Canberra, Australia) provided sketches of the geomorphology of Australia emphasizing the progressive and intensifying aridification during the Tertiary. Humid landscapes with truly great lakes and rivers were transformed into arid lands with deeply weathered plateaux capped with laterite, desiccated river channels (palaeo-rivers), deflated lake beds and massive sandy and stony deserts. Bowler dramatically illustrated this by showing that the channel of the Murray River (which drains south-east Australia's largest catchment) currently meanders sinuously within its former riverbed – signifying a decline in river discharge by orders of magnitude. Chappell noted that the transportation of sand at the height of Quaternary aridity has buried landscape features to create a modern landscape with apparently isolated hills and ranges. Perhaps the most compelling evidence of late-Tertiary landscape aridification is the remarkable diversity and endemicity of a range of freshwater invertebrate groups now adapted to living in subterranean groundwater throughout inland Australia (known as stygofauna) (Leys *et al.*, 2003). Steve Cooper (South Australian Museum (SAM), Adelaide, Australia) and Remko Leijs *et al.* (also from SAM) reported that molecular phylogenetic studies date the transition from surface to subterranean water by the stygofauna to about 5 million years ago (mya). Clearly, understanding Australian biogeographical patterns demands an understanding of the history of Tertiary geomorphology. In particular, many speakers, beginning with Bowler and including a number of biologists, recognized the period from 10 to 7 mya (Upper Pliocene–Lower Miocene) as critical to our understanding of the development of modern Australian ecosystems. This cooler, drier period (for which Bowler coined the term 'Bob Hill discontinuity') is sandwiched between a sequence of warm, humid environments in the early Miocene and late Pliocene. Importantly, this period is poorly represented in the fossil record, yet many arid-zone plant and animal lineages apparently date to this time window (Leys *et al.*, 2003).

The lack of geological rejuvenation of the landscape, and attendant soil infertility, are thought to have been of critical importance in preadapting the Australian flora to aridity. Using the macrofossil record from south-eastern Australia, Bob Hill (University of Adelaide, Adelaide, Australia) traced systematic changes during the Tertiary to the abundance, distribution and protection of stomata in sclerophyll lineages such as *Banksia*, *Callitris* and *Casuarina*, which formerly occurred in rainforests. There have been dramatic changes in plant community composition throughout the Tertiary and into the first half of the Quaternary. David Bowman (Charles Darwin University, Darwin, Australia) posited that adaptation to fire, characteristic of the Australian flora, possibly originated from the monsoon tropics where ignition sources from lightning storms mark the transition from dry to wet seasons. Such a strongly seasonal climate, thought to have developed in the Tertiary, may have also preadapted taxa to aridification (Bowman & Prior, 2005). Bowman argued that the widespread landscape burning by late-Pleistocene human colonists was insignificant in the evolution of fire-adapted flora, but may have contributed to the extinction of megafauna by changing the vegetation structure. In this context, it must be acknowledged that the general lack of macrofossils of fire- (and drought)-adapted biomes is a serious impediment to understanding the evolution of Australian flora. However, the advent of molecular techniques to underpin phylogenetic and phylogeographical analyses has provided new insights into evolutionary processes and patterns of key groups (Crisp *et al.*, 2004).

Using molecular techniques, Pauline Ladiges *et al.* (University of Melbourne) traced the radiation of eucalypts from ancestral and now relictual rainforest lineages thought to extend back to the early Cretaceous (Ladiges *et al.*, 2003). They interpret the biogeographical distribution of modern eucalypts as reflecting basic splits in the lineage that preceded Tertiary aridity, thereby highlighting the great antiquity of the eucalypts. Likewise, Daniel Murphy *et al.* (Royal Botanic Gardens, Melbourne, Australia) presented DNA evidence for the antiquity of the species-rich and monophyletic Australian *Acacia*, which are almost entirely phylodenous. Murphy *et al.* found higher sequence variation in a relatively species-poor, arid-zone *Acacia* clade compared with a species-rich clade from the humid east coast (Fig. 1). They interpreted this as more recent radiation in the humid zone and compared with the more ancestral arid zone taxa.

Interactions

Perhaps the most compelling evidence for the antiquity of the modern drought-adapted flora concerns the radiation and diversification of gall-forming insects that parasitize trees by inducing the host to grow specialized structures to provide food, shelter and a stable microclimate (Fig. 2). It is probably no coincidence that Australia has the highest diversity of gall-forming insects in the world, given that galls are a superb adaptation to aridity (Gullan *et al.*, 2005). Lyn Cook (ANU) and Penny Gullan (University of California, Davis, USA) reported the evolutionary congruence of molecular phylogenies between the highly diverse gall-forming scale insects with their equally diverse Myrtaceaous host tree species. Such parallel evolution is thought to reach deep into the Cainozoic, tracking the radiation and diversification of eucalypts. Michael McLeish (Flinders University, Adelaide, Australia) and Tom Chapman (also Flinders University) reported host specialization among gall thrips and arid-zone *Acacia* species. Their phylogenetic analysis suggests that the

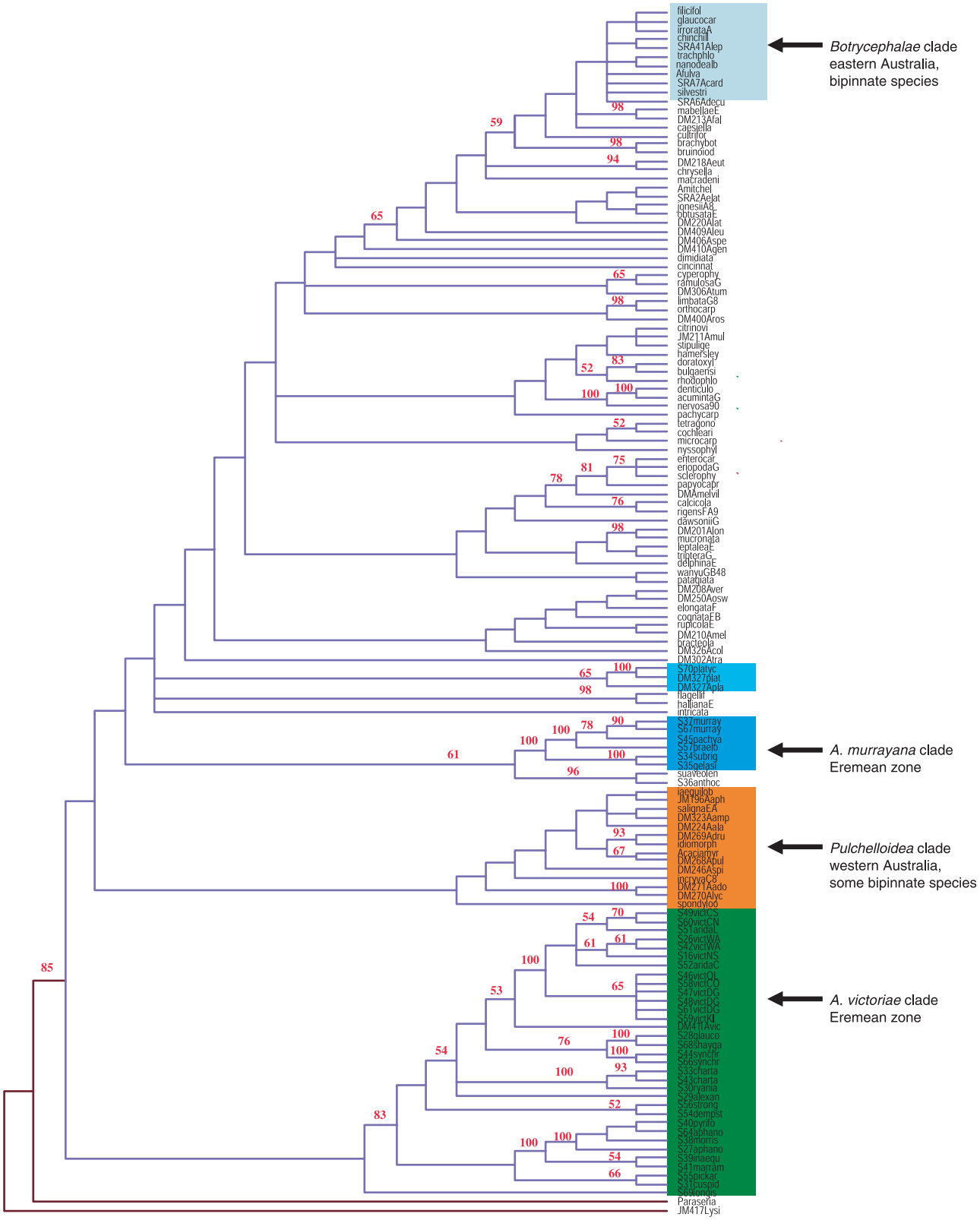

Fig. 1 Cladogram of Australian *Acacia* (*Acacia* subgenus *Phyllodineae*), based on internal and external transcribed spacer nrDNA sequence data, showing the relative ages of four clades. Note bipinnate-leaved species are polyphyletic, with the eastern Australian *Botrycephalae* group being relatively younger than the *Pulchelloidea* clade. The *Acacia victoriae* clade from semiarid and arid regions of Australia is relatively old (an early node). (D.J. Murphy, P.Y. Ladiges and G.K. Brown, personal communication.)

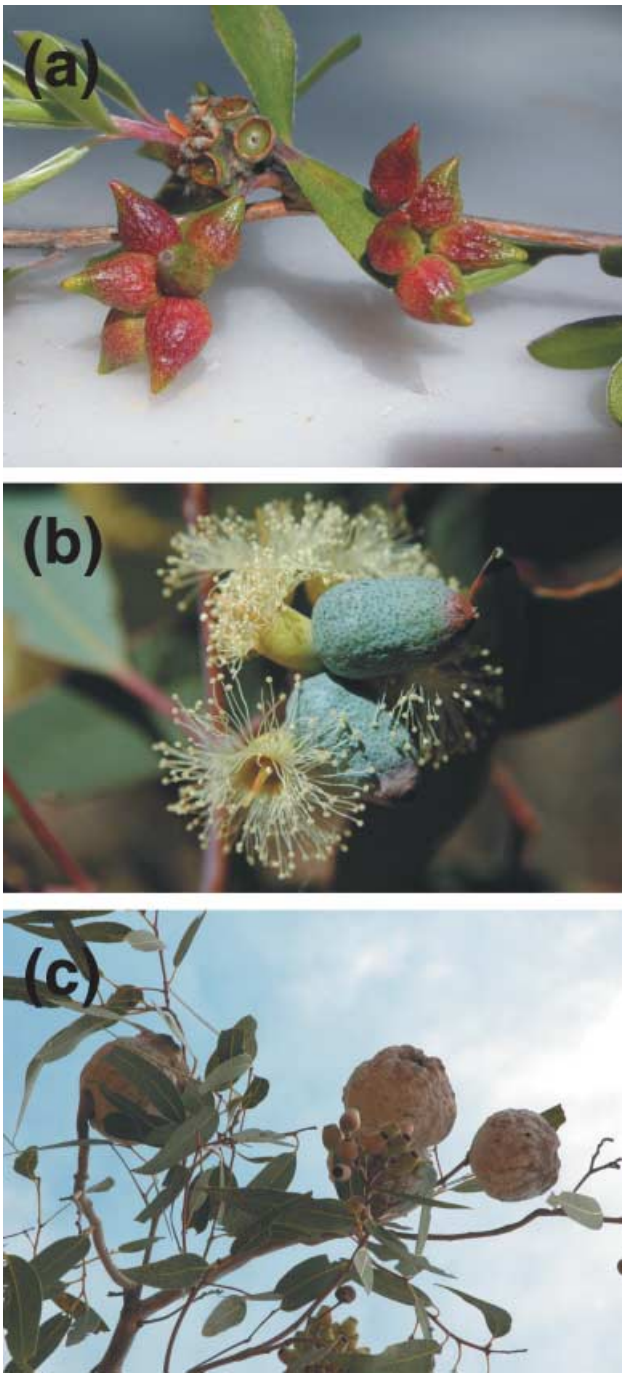

Fig. 2 Galls induced by females of Myrtaceae-feeding eriococcid scale insects in Australia. (a) Red galls of *Eremococcus* sp. on leaves of *Agonis marginata*. Male offspring develop within the maternal gall whereas females disperse. (b) Two galls of *Apiomorpha malleeacola* on a fruit of *Eucalyptus socialis*. Males induce separate, tube-shaped galls (not shown). (c) Three galls of *Cystococcus pomiformis* on *Corymbia* sp. These galls are commonly known as bloodwood apples or bush coconuts, and both the insect and the white flesh inside the gall are edible. Sons are produced first and develop within the maternal gall. When the males near maturity, daughters are produced. The tiny immature females climb onto the abdomen of adult males and are carried from the gall by their winged brothers. Photos (a,b) courtesy of Mike Crisp; (c) courtesy of Lyn Cook.

parallel evolution of gall thrips and *Acacia* occurred during the late Tertiary aridification. Sonja Scheffer *et al.* (US Department of Agriculture, Beltsville, MD, USA) reported the truly extraordinary case where *Fergusinina* flies and *Fergusobia* nematodes have coevolved a unique and obligate mutualism to parasitize myrtaceous trees. Fly oviposition occurs simultaneously with the deposition of juvenile nematodes, which induce gall formation on the host tree. The fly lavae and nematodes grow and feed inside the gall. When fully developed, nematodes enter the female fly larvae, where they remain until being deposited at the next oviposition site by the ensuing adult. In general, the flies and nematodes show host specificity for plant species from at least six genera (*Eucalyptus*, *Melaleuca*, *Corymbia*, *Syzygium*, *Metrosideros* and *Angophora*). Christine Lambkin (CSIRO Entomology, Canberra, Australia) and David Yeates *et al.* (CSIRO Entomology) reported on the timing of divergences in the species-rich, schlerophyll forest-fly family Therevidae, finding that the basic splits in the family predated Tertiary aridification, but that the major species-generating cladogenesis occurred during the most recent third of the Tertiary.

Physiologically, many Australian arid-zone mammals show the most extreme adaptations to aridification in comparison with other arid zones around the world. Ken Aplin (CSIRO Sustainable Ecosystems, Canberra) showed that many of the characteristic allochthonous arid-zone groups arose in the late Miocene and early Pliocene (at the time of the 'Bob Hill discontinuity'), with novel adaptations to life in open (nonrainforest) habitats, and often without clear links to fossil lineages of earlier times. The marsupial mole is an exception, being of extremely old, isolated and endemic arid-zone specialist lineage.

Plant diversification

A recurrent feature of Australian phylogeography is strong local-to-regional-scale genetic diversification that appears to reflect a pattern of 'cut-and-cut-again' of existing populations (Fig. 1). This was well illustrated by Mike Crisp (ANU) and Lyn Cook, who explored the evolutionary effect of the Nullabor Plain that divides the south-eastern from the south-western temperate floras. They concluded that the numerous groups of allopatric sister taxa are the result of vicariant speciation associated with the formation of Nullabor bio-geographical barrier(s) during the Tertiary, with divergences clustered in the period from 7 to 9 mya, and again at 2.5 mya. Margaret Byrne (Department of Conservation and Land Management (CALM), Perth, Australia) has found that three widespread trees species in south-western Western Australia, in different genera (*Acacia*, *Eucalyptus* and *Santalum*), all showed substantial and spatially congruent genetic (cpDNA) divergence. This phylogeographical pattern probably reflects past restriction to refugia during episodes of aridity during the Quaternary. Gay McKinnon *et al.* (University of Tasmania,

Hobart, Australia) showed that the molecular phylogeography of *Eucalyptus* and *Nothofagus* in south-eastern Australia reflects the genetic impact of Pleistocene refugia. Paul Sunnucks's (Monash University, Melbourne, Australia) and Dave Rowell's (ANU) laboratories have focused on phylogeographical patterns in log-inhabiting, low-vagility invertebrates in a temperate, montane forest in south-eastern Australia. Their combined studies have shown remarkably high genetic divergences over very small distances, underlining the great stability of the forest habitats and microhabitat preferences of the organisms (Garrick *et al.*, 2004). Phylogeographical patterns are consistent with repeated contraction into restricted refugia during the Quaternary glacial cycles of aridity. Sunnucks was led to generalize that 'if it doesn't fly, it is a species complex'.

Teasing out phylogeographical patterns is highly problematic in most Australian environments because, unlike the atypical humid zone, there is an absence of reliable environmental archives such as pollen. Further, modelling palaeoclimates is difficult because of subdued topography and associated gentle rainfall and temperature gradients. Indeed, often the best evidence for environmental change is the phylogenetic patterns themselves. Given the inevitable reliance on molecular data, Craig Moritz's (University of California, Berkeley, USA) injunction to build phylogenies based on many, rather than single, genes is important and timely.

In sum, an emerging view from the meeting was that Australian ecosystems have been dramatically affected by increasing aridification on geological timescales right up to the present, and there was agreement concerning the great antiquity of the characteristically Australian plant and animal groups. This is reflected in deep divergences in molecular phylogenies, with many species lineages pushing well into the Pliocene; tightly coevolved mutualisms between plants and animals; many novel adaptations to escape the effects of aridification (stygobionts and gall-formers); and specific adaptations to soil infertility, drought stress and fire. The deepest divergences in characteristic arid-zone taxa often pre-date Tertiary aridification, suggesting biogeographical structuring in the mesic ecosystems of early Tertiary Australia. More recent phylogeographical patterns reflect numerous cycles of aridity throughout the Quaternary ice ages that created refugia and caused vicariant speciation or significant genetic differences among populations of widespread species.

The challenge for Australian biogeography is to move beyond these broad-brush generalizations by uncovering regional and continental phylogeographical patterns; assessing the phylogenic congruence among different taxa, particularly plants and animals; and integrating these data to make a coherent whole. To achieve this geological and biological knowledge, gaps must be filled. Fundamentally, the Tertiary fossil record for the northern and western half of the continent awaits discovery and documentation. A much better appreciation of the paleoclimates of the Late Miocene and earliest Pliocene (7–10 mya) is required because this period is emerging as critical in establishing the phylogenetic composition of the modern Australian biota. To help resolve inconsistencies between fossil and molecular chronologies, phylogeneticists need to refine methods of estimating divergence times. At the broadest scale, we need to move from phylogenies of individual lineages to phylogenies that encompass lineages characteristic of entire biomes, thereby bridging the gap between historical and ecological approaches to biogeography.

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Key words: Australia, aridity, biogeography, geomorphology, molecular phylogenies, plant, animal co-evolution, Tertiary Period.

