Insight into fern evolution: a mechanistic approach to main concepts and study techniques

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Abstract: Vicent, M.; Gabriel y Galán, J. Mª & Ainoüche, A. 2014. Insight into fern evolution: a mechanistic approach to main concepts and study techniques. *Bot. Complut.* 38: 7-24.

In this review, we report on the most important ideas and features in the field of fern evolution, with particular interest into the main evolutionary mechanisms that shaped the evolutionary history of their diversification. An exhaustive account for the bibliographical corpus on the matter falls beyond the space and extent of this work; instead, we will try to focus the different issues by including featured examples from both historical and recent approaches to the study of the evolutionary mechanisms in ferns.

Keywords: apomixis, hybridization, molecular techniques, morphology, phylogeny, phylogeography, polyploidy, speciation, species concept.

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En esta revisión, informamos sobre las ideas y características más importantes en el campo de la evolución de los helechos, con un interés particular en los principales mecanismos evolutivos que dieron forma a la historia evolutiva de su diversificación. Una relación exhaustiva de toda la bibliografía de este asunto va más allá del alcance de este trabajo, en su lugar, vamos a tratar de enfocar los diferentes temas con la inclusión de ejemplos destacados, tanto históricos como recientes, en el estudio de los mecanismos evolutivos de los helechos.

Palabras clave: apomixis, concepto de especie, especiación, filogenia, filogeografía, hibridación, morfología, poliploidía, técnicas moleculares.

FERNS: AN ANCIENT GROUP OF VASCULAR PLANTS, BIOLOGICAL AND GENETIC FEATURES

Ferns are a very divers group exhibiting various habits (*e.g.* aquatic, epiphytic, herbaceous, arborescents), and together with the group of lycophytes, represent the oldest lineages of vascular plants among the terrestrial plants. Despite its ancient origin, this lineage accounts with much more extant species (~11.000) than the more derived gymnosperms (<1.000 species).

In recent classifications of land plants, vascular plants have a basal dichotomy separating the lycophytes (vascular plants presenting lycophylls or leaves with an intercalary meristem, without leaf gaps and a single medium vein), from the euphyllophytes (vascular plants presenting euphylls or leaves with marginal or apical meristems and an associated leaf gap in the vascular stele and branched veins). This dichotomy occurred in the early-mid Devo-nian, ca. 400 million years ago (Stewart & Rothwell 1993, Kenrick & Crane 1997, Pryer *et al.* 2004).

Within the euphyllophytes, two significant clades diverged from a common ancestor thought to be within the extinct Trimerophytes group (Bateman *et al.* 1988, Stewart & Rothwell 1993): spermatophytes (seed plants comprising the extant gymnosperms and angiosperms and few extinct groups, being the Pteridosperms the most prominent one) and monilophytes (ferns *sensu stricto*). Lycophytes and monilophytes are all seed-free, and because of this, historically they have been considered together under various terms, such as: "pteridophytes" (ferns *sensu lato*) and "ferns and ferns allies" (Smith *et al.* 2006).

The monilophytes comprise four extant lineages (Fig. 1): leptosporangiate ferns, marattioid ferns, psilophytes (adder's tongue ferns, moonworts, and whisk ferns respectively), and horsetails (Smith *et al.* 2006, Barker & Wolf 2010). The group of Leptosporangiate ferns, with
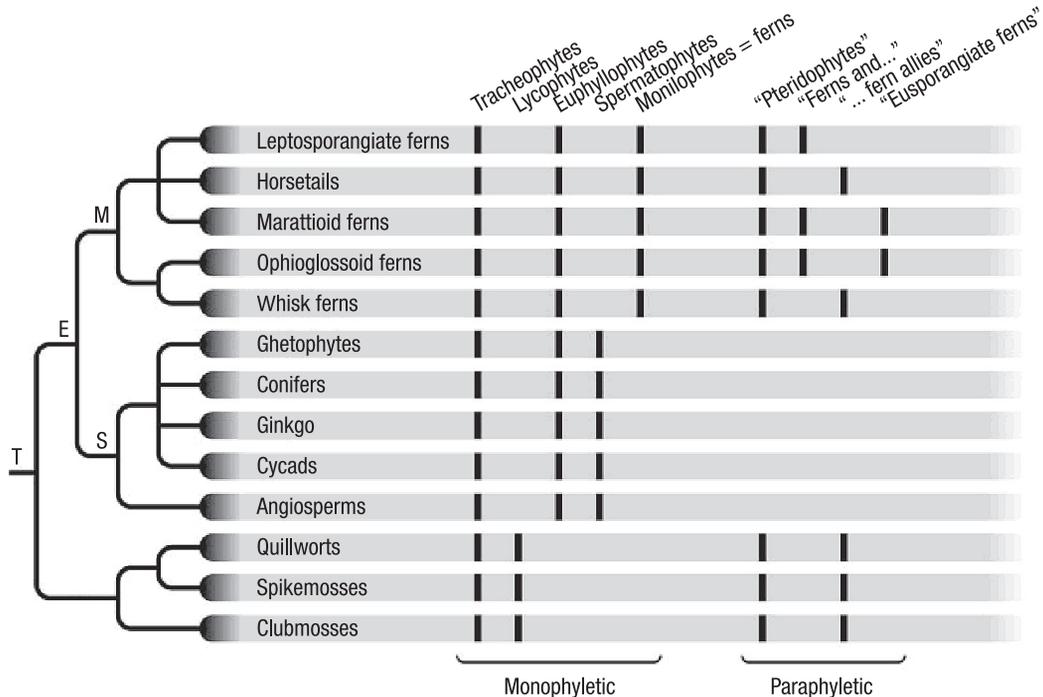
Fig. 1– Consensus tree showing relationships among the major lineages of vascular plants (from Pryer *et al.* 2004). Key clades are indicated on the tree: **T**: tracheophytes, **L**: lycophytes, **E**: Euphyllophytes, **S**: spermatophytes, **M**: monilophytes. A black band following a lineage name indicates membership of that lineage within a particular taxon listed across the top of the figure.

9.000 sp, is the second most successful lineage of vascular plants behind angiosperms (Schuettpelz & Pryer 2008). Accordingly, this review will focus on the monilophytes (ferns), with special interest into the group of leptosporangiate ferns. Regarding the sister relationship explained above, ferns are the sister group to all seed plants, and on account of this, they can be used as outgroup to establish the direction of evolutionary changes in comparative studies, for example comparing differences between gymnosperms and angiosperms (Barker & Wolf 2010).

From the biological and ecological point of view, ferns present two peculiarities that made them different from seed-plants: **(1)** Seed-plants and other plants produce two kinds of spores. However, most ferns are homosporous, producing one type of spore that, after germination, will produce a gametophyte. In spite of it, heterospory has appeared several times in plant evolution as in seed plants, some lineages of lycophytes (*e.g.* Selaginellales, Lepidodendrales-Isoetales), ferns (s.l. Calamitales, Salviniales), and even the pre-vascular rhyniophytoids have been proposed to be heterosporous (Taylor *et al.* 2009). **(2)** As compared to the other vascular plants, ferns exhibit a digenetic heteromorphous life cycle, including a free-living gametophyte. Thus, they present an alternation of generations

(Fig. 2): The mature diploid sporophyte produces haploid spores after meiosis; the spores germinate in a free-living, haploid and potentially bisexual gametophyte, and finally, the gametophyte produces the sex organs, and after fertilization the new sporophyte grows, via embryonary development, from the zygotic cell (Prada 2004); whereas female gametophytes of seed plants are nutritionally dependent on the sporophytes (Yadegari & Drews 2004).

Fern gametophytes are potentially bisexual, but it has been proved that in nature they are usually unisexual because of a pheromonal sex-determination system, the so-called antheridiogens (Schneller 2008). Even so, in the absence of a crossing fertilization, gametophytes may become bisexual and self-fertilize (Raghavan 1989, Shorina 2001, Prada *et al.* 2008). The alternation of generations has important evolutionary implications, since the two free-living organisms may often have distinct ecological requirements and experience separate selective forces (Soltis & Soltis 1990; Page, 2002; Farrar *et al.*, 2008). In this way ferns can provide unique research opportunities in the study of genes associated with only one of the two life-cycle stages (Barker & Wolf 2010).

Besides, ferns exhibit remarkable genomic features (in structure and size). It has been proved that ferns are
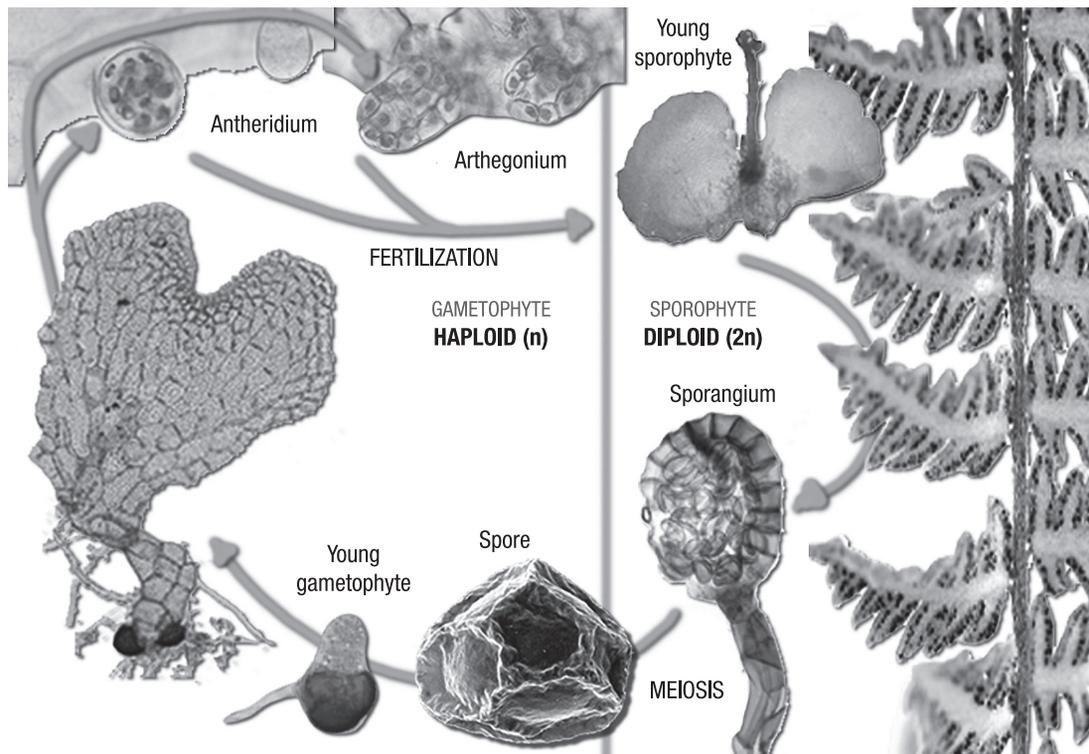
Fig. 2– Digenetic diplo-haplophasic, heteromorphous, homosporous life cycle of a fern. Original.

notorious for possessing the highest chromosome numbers recorded among living organisms. The following examples could help to visualize this fact: whereas the average gametic chromosome number for angiosperms is n = 15.99, the mean for all homosporous ferns is n = 55.27 (Soltis & Soltis, 1990). There are some surprising cases in which the gametic chromosome number range between n = 52 to 208 (*Psilotum* Sw.) or n = 104 to 208 (*Tmesipteris* Bernh.). But clearly the most amazing example is given by *Ophioglossum* L., which present n ~ 1400 (Khandelwal 1990).

High chromosome numbers in the ferns have been interpreted in two ways: the first is that it is the result of repeated polyploidy speciation events (with progenitor extinction); the second suggested that it is the result of selection for a greater number of linkage groups to reduce problems of homozygosity, in other words, they are primitively high (Soltis & Soltis 1990). Moreover, it was shown that these plants frequently experienced reticulate evolution (hybridization/allopolyploidization) along with their diversification (Haufler 2008, Dyer *et al.* 2012), which justifies the need to review the traditional systematics in most fern families (Barker & Wolf 2010).

RADIATION AND DIVERSIFICATION OF LEPTOSPORANGIATE FERNS

According to Schuettpelz and Pryer (2009), leptosporangiate ferns originated near the start of the Carboniferous period (359.2 Ma) and are thought to have experienced 3 successive radiations: an initial one in the Carboniferous, producing 6 now-extinct families; a second one in the late Paleozoic and early Mesozoic, that result in several families with extant representatives; and finally a third radiation beginning in the Cretaceous, primarily within what is now referred to as the "polypod" clade. Most divergences among extant lineages took place in the Cretaceous and Cenozoic. Thus, each day the hypothesis of a link between the diversification of leptosporangiate ferns and the Cretaceous rise of angiosperms is getting more reliable.

Schuettpelz and Pryer (2009) focus their study in epiphytic ferns, and they show that the increases in epiphytic fern diversification was almost 10 Ma later than the origin of modern rain forests, but coincide with the expansion of them. During the Paleocene/Eocene thermal maximum (PETM) the rise in temperature and precipi-

tation facilitated the expansion of angiosperm-dominated canopies, and with it, the number of available canopy niche space increase suddenly.

Remarkably, there appears to be no relationship between the PETM and the diversification curve for terrestrial ferns. It would be a result of the differences between terrestrial and epiphytic ferns, being that the epiphytic ferns are more sensitive to climatic conditions than the terrestrial ferns.

It is important to mention that the data presented above have been obtained using molecular clocks, but there are numerous articles that use as evidence on the origin and diversification of land plants dispersed spores and megafossils (Knoll & Niklas 1987, Stewart & Rothwell 1993, Kenrick & Crane 1997, Rothwell 1999, Taylor *et al.* 2009).

FERN PHYLOGENY

Since the introduction of genetic tools, fern phylogeny has experienced so huge advances that previous works are in perspective seen as unimportant. This is quite far from truth, as phylogenetics confirm, not infrequently, some of the major results previously proposed by morphological and paleobotanical data (Schneider *et al.* 2009, Gabriel y Galán *et al.* 2013). However, at the same time it is obvious that molecular techniques have allowed a very much better comprehension of taxa relationships, not only within families and genera, but also contributing novel findings at the higher levels (Hasebe *et al.* 1994, Pryer *et al.* 2004, Schuettpelz & Pryer 2007).

The scientific production that focuses on molecular fern phylogeny is enormous and it can't be properly covered in this work. To-date comprehensive synthesis and reviews can be found in Schuettpelz & Pryer (2008) and Pajarón & Pangua (2012). First phylogenetic hypothesis were done using one marker, the vast majority of times from the chloroplast genome (Hasebe *et al.* 1994). Many cpDNA markers have been largely used and several universal primers are found for them. A natural tendency in the technique was the increase in nucleotidic characters, so investigations included progressively a larger amount of markers (Rai & Graham 2010), and finally integrated datasets coming from different genomes (Windham *et al.* 2009). Consequently, primer design for fern nuclear markers is more recent (Ishikawa *et al.* 2002, Rothfels *et al.* 2013).

It is expected in the next years that fern phylogeny continues to produce a high amount of publications. While the main branches of the tree are well known and generally assumed by the pteridological community, detailed information still lack in some of the terminal clades, due to a high cosmopolitan diversity and the incidence of hybrids and polyploids, among other facts. The *Pteridaceae* and some diverse eupolipoids families as *Aspleniaceae*, *Polypodiaceae*, *Blechnaceae* and *Dryopteridaceae* need a deep phylogenetic review.

DIVERGENT SPECIATION, BIOGEOGRAPHY AND PHYLOGEOGRAPHY OF FERNS

The establishment of isolate populations, by means of rise of geological barriers or dispersal events, followed by Darwinian processes of change over extended periods of time, is a common pattern of speciation and evolution in ferns, as it is in the vast majority of organisms (Haufler 2008). The final result is the divergence of the separated populations, which eventually will move through different evolutionary paths, originating new forms of diversity. Depending on the arrangement of the isolated populations, several modes of divergent speciation have been defined (allopatric, peripatric, etc).

Divergent speciation has been studied in ferns of various groups and from different geographical regions. To illustrate one of the typical divergent processes, we will mention the case of the allopatric origin of some *Polypodium* L. species. *Polypodium sybiricum* Sipliv. seems to have had a wide range through the North American mountains during glaciations, but appeared restricted to most northern areas during interglacial periods. It has been proposed that, with the retreat of ice to the north, some populations were maintained in southern refuges, which in term gave raise to new species, named as *P. amorphum* Suksd. and *P. appalachianum* Haufler & Windham (Haufler *et al.* 2000). It has been stated also that it is possible that the more complex ecological structure in the tropics could derive in more opportunities to ecological isolation and divergent speciation in ferns (Haufler *et al.* 2000).

Most of the modern studies on this matter come from flora comparisons with application of cladistic tools (Kato 1993), and from numerical similarities of genetic values, calculated from different sources of information, as proteins, isozymes or DNA sequences.

Moving to a broader perspective, the analysis of the allopatric speciation processes may lead to the understanding of biogeographical patterns of distribution in the ferns. Reports on such studies are known for long ago

Fig. 3– Fragment of an alignment of the trnG-trnR cpDNA marker, generated in an ongoing phylogeographical study on *Blechnum auriculatum* in South America. Numbers in the name of sequences identify the population (first) and the individual (second). Some polymorphic sites have been highlighted; those with nucleotides shared by various individuals have phylogeographical significance. Original.

(Tryon 1985, 1986; Barrington 1993). All of these classic works assumed that the ecological and historical factors affect species distributions, sometimes also in relation to taxonomy and groups relationships (Smith 1993).

Considered as a whole, ferns account for a particular feature in relation with its geographical distribution: many of the lineages are old in geological terms, at least when compared with angiosperms lineages. Thus, the understanding of the biogeography and evolution of the floras could need to move back many million of years, and call to vicariance processes to give reason of some ample distributions with disjunction mosaics. This is the case of the *Cyatheaceae* (Korall & Pryer, 2014), with a geographical ancestor that has been situated in the times of the Gondwana super-continent; the rupture of Gondwana in several units allowed to a common pool of *Cyatheaceae* species to evolve independently and give rise to the modern observed lineages along its trans-pacific disjunction. A similar pattern of geographical-evolutionary pattern is expected in some other widely distributed southern families, as the *Blechnaceae* (on-going studies).

Nevertheless, modern approaches in phylogeography and population genetics seem to conclude that, in many cases, vicariance is not a definite, reasonable argument to explain some observed patterns in fern distribution. Long-term dispersion, facilitated by means of small, low-weight dispersal units (spores), appears to elucidate better the distribution of some groups, as some *Taenitis* Willd. ex Schkuhr species in Asia (Kato 1993) and several groups of *Blechnum* L. in New Zealand/Australia (Shepherd *et al.* 2007). A similar conclusion has been extracted for animals and other plant groups (Sanmartin & Ronquist 2004).

The emergence of new, modern theories applied to biogeography (coalescence for example) and its integration

with the increasingly abundant phylogenetic information has extended the phylogeographic and population genetics techniques. Basically, they are based on the search of genetic polymorphisms (Fig. 3), the recognition of different haplotypes within and between populations, and the construction of networks that show the distribution and frequency of the haplotypes along the populations (Fig. 4). These techniques are gradually being applied to the ferns (Trewick *et al.* 2002, Hunt *et al.* 2009, Wang *et al.* 2011), typically with the use of chloroplast genome markers. During the last years, an increase of publications focusing
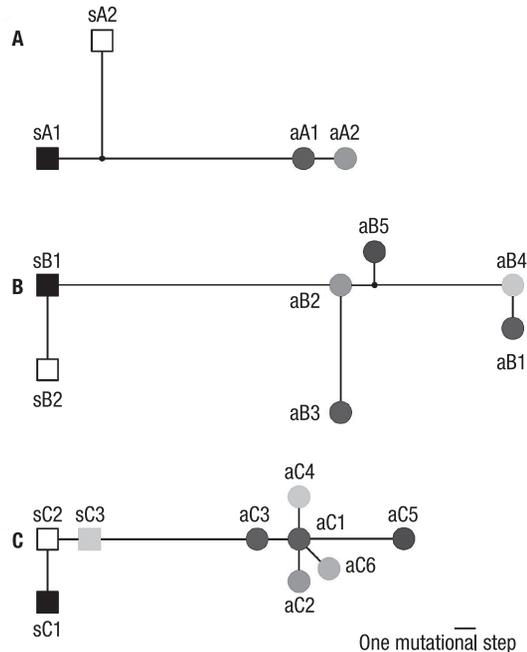

Fig. 4– Haplotype networks of several genes samples on various species of *Acrostichum* (from Zhang *et al.* 2013).

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fern phylogeography has been detected, and it is expected an intensification of the research in this matter.

HYBRIDIZATION, WITH SOME THOUGHTS AROUND THE SPECIES CONCEPT IN FERNS

Hybridization is an important evolutionary event, which is the merger of two divergent (homeologous) nuclear genomes (Hegarty & Hiscock 2005, Wissemann 2007, Ainouche *et al.* 2009). In other words, it is the result of the crossing between two different species. Some authors have called this process homoploid hybrid speciation, we will enter in this concept later (Soltis & Soltis 2009). All hybrids are characterized by a renovated reproductive interaction between populations or species just after a period of isolation. Hybrids are very common among ferns, probably because they lack mechanisms of pre-zygotic isolating barriers between species or, if so, these fail (Barrington *et al.* 1989, Pajarón & Pangua 2012). To give significance to this assertion, it is usually mentioned that these pre-zygotic mechanisms are extended within the angiosperms (pollinators, pollen recognition, complicated reproductive, floral strategies). Nevertheless, hybrids are also common within the angiosperms, very common in some lineages (Hegarty & Hiscock 2005). So, in despite that the absence of isolating pre-fertilization barriers could be an important fact, some other argument should be stated to give fully sense to the high incidence of hybridization in ferns. It is noteworthy the fact that some decades ago hybrids were thought to be rare organisms with few evolutionary possibilities, but actually they are considered as a potent evolutionary force, one of the most creative mechanisms in the evolution of biodiversity (Soltis *et al.* 2004).

Fern hybrids are predominantly sterile, incapable of conducting normal meiosis (Fig. 5), although there are some exceptions (Whittier & Wagner 1961, Conant & Cooper-Driver 1980, Hegarty & Hiscock 2005). Many fern species are thought to be derived from hybrids, because morphological comparison with allied taxa generates evidences of intermediate, but they behave as a true and autonomous species. Hybridization has been demonstrated in ferns with great morphological variation, including *Asplenium* L., *Athyrium* Roth, *Polystichum* Roth, *Cheilanthes* Sw., *Pteris* L., and *Vandenboschia* Copel. (Ebihara *et al.* 2005, Chao *et al.* 2012). The origin of hybrids is a consequence of a return to sympatry and the breakdown of isolating mechanisms, not the action of natural selection and drift on allopatric populations. Fern hybrids are
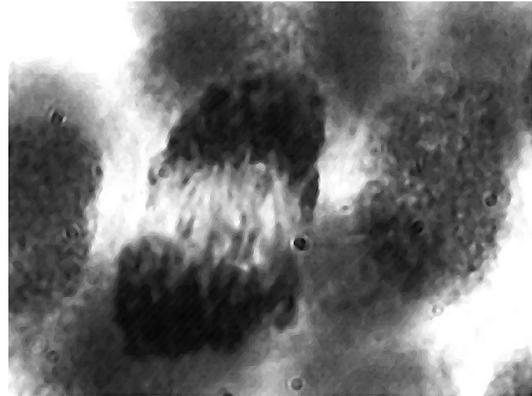
Fig. 5– Abnormal meiotic process in an *Asplenium* hybrid, showing smeared material and irregular segregation of chromosomes. Original.

responsible for many of the problems in species definition and systematics in the group. In some sense, they are not novelties, but combinations of previously existing entities. For these reasons there are authors (Wagner 1969, 1983) that suggested that hybrid species or nothospecies, should be recognized as qualitatively different from divergent species or orthospecies.

Morphological criteria do not provide sufficient basis for robust hypotheses about the origin and evolution of hybrid species; although morphological criteria related to spore abortion have been used to document fern hybrids (Wagner *et al.* 1986). At the same time, evidence from chromosome number and pairing behaviour have provided criteria for recognizing hybrid species and distinguishing them from their progenitor species, such as for instance the classical Manton's work (Manton 1950).

Also, biochemical markers (phenolic compounds and isozymes) were frequently used to discriminate populations of hybrids from phylogenetically patristic intermediates, which can be similar to hybrid taxa (Endler 1977). Interpretation of phenolic data was relatively easy, because hybrids sum marker compounds characteristic of their progenitor species, for example in some *Asplenium* complexes (Smith & Levin 1963). However, work on phenolics was not always adequate to solve problems in a number of reticulate complexes: chromatographic patterns were not clear, status as homologous character was uncertain or there wasn't enough variability in these secondary compounds to provide sufficient species-specific markers (Barrington *et al.* 1989).

Isozyme markers allowed much more accumulation of genetic insights to understand the mating system and consequences of outcrossing as a main mechanism favou-
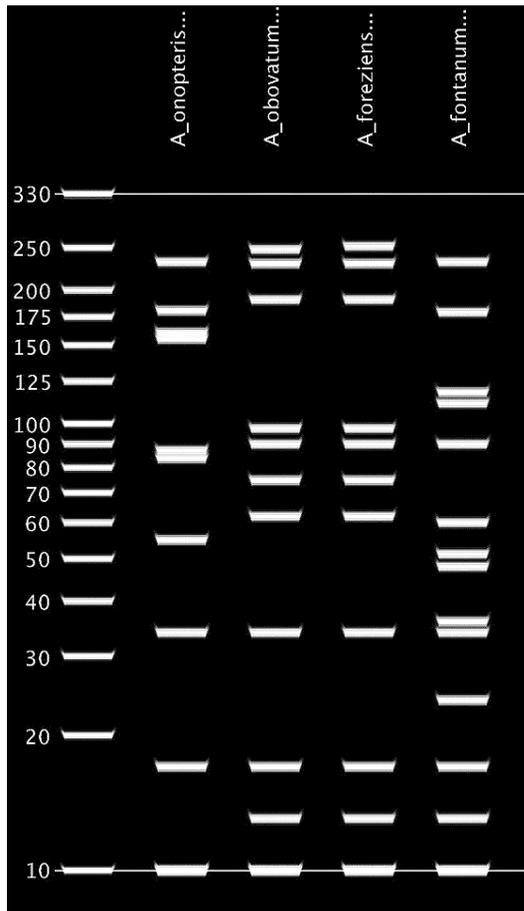
Fig. 6– In silico gel of an enzimatic digestion (RFLP) of the chloroplast genome marker trnG-trnR, in three species of an *Asplenium* complex: two diploids *A. obovatum* and *A. fontanum* and the allotetraploid derivative of both, *A. foreziense* (*A. onopteris* was included as diploid control). The pattern of band polymorphisms allows stating that *A. obovatum* acted as mother, since the chloroplast is maternally inherited in *Asplenium* (Vogel *et al.* 1998). Original.

ring intergametophytic mating in the main homosporous fern lineages, in contrast to previous inbreeding assumptions (Soltis & Soltis 1990, Jiménez *et al.* 2009, Yatabe *et al.* 2009). In the decade of 1980 morphological discrimination of hybrids was improved with quantitative analysis of morphometric characters. Multivariate statistical analyses provide even better criteria for distinguishing hybrids, because they allow simultaneous unbiased consideration of numerous characters.

Thus the progressive development of the morphological, cytological, and biochemical criteria, allowed

improvement in the perception of hybrids in the diverse fern group (Barrington *et al.* 1986). However, only the recent development of molecular phylogenetic analyses, using nuclear and plastid DNA markers, including RFLP (restriction fragment length polymorphisms) and *in situ* hybridization techniques, provided more accurate information allowing elucidation of multiple hybridization events which affected diploid and/or polyploid taxa in various fern families, depicting complex reticulated evolutionary patterns of relationships among taxa (see below the section on Reticulate Evolution). Thus, plastid markers allow us to observe maternal heredity and to create trees in which only the maternal phylogenetic relationships are shown (Fig. 6), while nuclear markers allow us to observe biparental heredity, which means that in the plant complexes with hybrids, we will be able to differentiate two haplotypes for each hybrid, being possible to establish both parental. Combining the information obtained with both plastid and nuclear markers makes easier to figure out the relationships within a complex group.

One of the most exciting challenges when dealing with hybrids occurs when one (or both) of the parentals has disappeared from nature or it has never been collected. In *Spartina* Schreb. (a grass) all the recognized species range from tetraploids to dodecaploid originated from hybridizations and polyploidy, and no diploid species is known (Ainouche *et al.* 2009). How can be reconstructed the genomes/species that originated all the descent lineages? A notable case in the ferns is that of "*Dryopteris semicordata* (Sw.) Kuntze". This name have been used to refer to an unknown species, never collected in nature, proposed to support the origins of a notable amount of species (Sessa *et al.* 2012a) (Fig. 7). After a long time of discussion, and only when reliable molecular information has allowed to built a robust relationships hypothesis, this elusive "plant" has been reconstructed extrapolating likely character states from extant species (Stein *et al.* 2010)

Even when putative parentals seem to be well established by application of one or more of the above cited techniques, the ultimate, unequivocal and certain proof should come from the verification that a sexual contact is possible between gametophytes of both species. In nature, this evidence is almost impossible to achieve, but there are some striking in vitro experiments that have developed artificial hybrids by cultivating female gametophytes of one species close to male gametophytes of the other species (Herrero *et al.* 2001, Yatabe *et al.* 2009). This information was used along other datasets to confirm the involvement of the analysed plants in forming the hybrids.
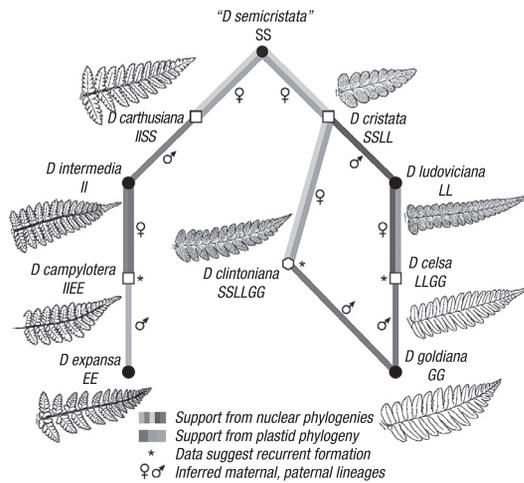
Fig. 7– Scheme of one of the proposed hypothesis of relation-
ships within a *Dryopteris* complex: "*D. semicristata*" is an
unknown species proposed as origin of the lineages (Sessa
et al. 2012a).

Fig. 8– Polyploid incidence and speciation frequencies acros
major groups of vascular plants. Polyploid speciation
frequencies are the fractions of branching events that were
accompanied by a ploidy shift across the studied phylogenetic
trees for each group. The speciation frequencies reported
here are based on an irreversible model of polyploid evo-
lution. A binomial standard error follows each incidence and
frequency estimate. The Higher Monocots are represented
by Arecales, Commelinales, Poales, Proteales, Zingiberales;
the Basal Monocots by Alismatales, Asparagales, Dios-
coreales, Liliales, Pandanales from Wood *et al.* (2009).

POLYPLOIDY

Polyploidy is a common mechanism of speciation in
plants (Soltis & Soltis 1999, Haufler 2008). It consist in the
multiplication of homologues chromosomes above the two
paired sets, in other words, an organism is polyploid if it
present more than two homologous sets of chromosomes.

There are two different forms of polyploidy (Haufler
2008):

— *Autopolyploid*: polyploid originated by the
multiplication of one basic set of chromosomes, *i.e.* which
results from the duplication of the whole genome of one
species. The evolution of autopolyploids in many ferns,
which is often associated with distinctive reproductive
strategies, increases the likelihood of reproductive barriers
and thus the potential for the development of spatial
structure (Trewick *et al.* 2002).

— *Allopolyploid*: polyploid originated though hybri-
dization between two different species followed by chro-
mosome doubling. In that way, fertility may be restored in
the polyploid after chromosome doubling of the sterile
hybrid (Barrington *et al.* 1989, Vogel *et al.* 1999).

Polyploids can be also divided in paleopolyploids and
neopolyploids. According to Ainouche *et al.* (2009):
"Newly formed polyploids (neopolyploids) provide the
unique opportunity to compare a new species to its parental
as the parent species are easier to identify than for older
polyploids (paleopolyploids) where the progenitors may
have become extinct". To study this kind of cases would

allow us to make significant scientific advances in the
understanding of evolution. Also, It has been suggested
that hybridization combined with polyploidy (allopoly-
ploidy) confers an immediate ecological aptitude to invade
new habitats, which increase the importance of their study
(Ainouche *et al.* 2009).

As underlined in the introduction, polyploidy has been
recognized as an important phenomenon in vascular plants,
and several lines of evidence indicate that most, if not all,
plant species ultimately have a polyploid ancestry (Wood
et al. 2009). Detection of repeated polyploidization depends
on distinguishing between neo- and paleopolyploids
(Barrington *et al.* 1989). Neopolyploids are documented
recent allopolyploids with chromosome numbers that are
multiples of the lowest number presently known for their
evolutionary group; whereas, paleopolyploids are assumed
to be ancient allopolyploids that have the lowest known
chromosome numbers for their evolutionary group. By
evaluating the frequency of polyploidy speciation in
vascular plants, Wood *et al.* (2009) established that 31% of
fern speciation events are accompanied by ploidy increase,
as compared to only the 15% in angiosperms (Fig. 8).

Moreover, ferns with the lowest chromosome numbers
for their genus have so far consistently shown diploid gene
expression, *i.e.* displaying a genetic diploid behaviour
based on isozymes studies despite the high chromosome
numbers (Soltis & Soltis 1990). The origin of such high
chromosome number was a matter of debate and two

hypotheses were proposed, as it was said before: one suggesting that homosporous ferns are true polyploids, resulting from repeated cycles of polyploidy, followed by a process of diploidization with a massive gene silencing; while the other, suggested that they are true diploids having achieved high chromosome numbers via other mechanisms (Barrington *et al.* 1989, Soltis & Soltis 1990). Although much is now known on the origin and evolution of seed plant polyploidy genomes, the question remains open for ferns and still need to be tested and elucidated using novel molecular approaches.

PLOIDY LEVEL

The knowledge of the ploidy level, beyond the value that presents itself, is an essential piece of information for evaluating evolutionary processes in the organisms. The ploidy level could be acquired by means of several techniques. Morphology may aid, because sizes of various structures could change with ploidy (Stebbins 1950). Thus, in ferns, the size of spores and stomata guard cells can often be employed to infer differences in ploidy levels among closely related species (Barrington *et al.* 1986, Huang *et al.* 2006). Spore measurements were successfully employed in several recent studies on the evolution of apomixis in ferns despite the occurrence of aborted spores, which may add some error to the estimate range (Beck *et al.* 2010, Huang *et al.* 2011, Sigel *et al.* 2011). In any case, this is a very cheap and fast technique, assumed that some previous information is available.

Genome size can be estimated by propidium iodide flow cytometry (Obermayer *et al.* 2002), and, if a calibration data is known for the genome size of a diploid related species, inference of ploidy level can then be done. This technique needs pieces of living tissues, generally leaves, which is limiting if information is needed from species far away collected. Although to a much less extent, flow cytometry has been applied also to determine genome size and ploidy level in gametophytes (Bellefroid *et al.* 2010), for example, to test the ploidy status of aposporous gametophytes of *Platycerum bifurcatum* (Cav.) C. Chr. (Ambrožič-Dolinšek *et al.* 2002).

ONE ORIGIN VS MULTIPLE ORIGINS
OF HYBRIDS AND POLYPLOIDS

In due time, hybridization was completely assumed by the scientific community as an extended process within plants, far from the initial conception of isolate cases. But during a more or less long period, the view of the phenomenon tacitly implied that a concrete event was difficult and of improbable occurrence; in other words, hybrids and its polyploid derivatives are common but each of them is formed only once because is highly improbable that the event could occur twice. Nowadays, the picture is quite different: many studies derived in the comprehension that most hybrids are continuously formed de novo and that random genomes from the parental populations can be mixed repeatedly. This implies that it is possible to observe plants with different morphologies (which have eventually received even different names) that actually respond to several events of hybridization from the same parental populations (Hegarty & Hiscock 2005). In conclusion, current research shows that most hybrids and polyploids have been originated multiple times (they are recurrent) and fewer times are product of a single event (Soltis & Soltis 1999, Wissemann 2007). Furthermore, this feature greatly multiplies the already powerful potential of the hybridization in the creation of biodiversity.

Multiple origins have been assessed calculating levels of heterozygosity, from observations of isozymes most times (Vogel *et al.* 1999). But direct comparisons of DNA, typically mixing nuclear and chloroplastic data to assess both biparental and uniparental heredity, have produced the vast majority of findings. Phylogenies constructed with multiple accessions of diploid parentals and allotetraploids can show multiple origins if several accessions of a polyploid appear more closely related to separate accession from one or more of the parental (Soltis & Soltis 1999). Nevertheless, differences in sequences (multiple genotypes) can also be obtained in a single origin polyploid by introgression with a progenitor or by microevolutionary changes post polyploidization (Sessa *et al.* 2012a).

Recurrent processes of hybridization have been elucidated in several complexes of ferns, for example in *Polystichum* (Stein 1990), *Asplenium* (Werth *et al.* 1985, Perrie *et al.* 2010, Hunt *et al.* 2011), *Astrolepis* D. M. Benham & Windham (Beck *et al.* 2012), *Acrorumohra* (H. Itô) H. Itô (Chang *et al.* 2009) and *Pteris* (Chao *et al.* 2012). In some *Dryopteris* Adans. complexes studies are inconclusive and, for many allopolyploids, the question remains unresolved (Jiménez *et al.* 2009, Stein *et al.* 2010, Sessa *et al.* 2012a).

RETICULATE EVOLUTION

As previously has been said, hybrids and polyploids are merged in the evolution of the organisms, in fact there

are authors that consider both concepts as a whole, as different kinds of hybrid speciation. Soltis & Soltis included under the name of hybrid speciation two phenomena: the homoploid hybrid speciation (what we have called hybrid, the result of the crossing between two different species resulting in another organism that maintains the same ploidy level as its parentals) and allopolyploidy (even autopolyploidy when considering that autopolyploidy may typically involve hybridization between populations of the same species) (Soltis & Soltis 2009). Either considering hybridization and polyploidy as different processes, either considering both different kind of hybrid speciation, it is clear that both phenomena are changing our way to understand the evolution. Recently the term reticulate evolution has been created to comprise this new way to understand evolution.

Reticulate evolution is increasingly being recognized as a fundamental process in the evolutionary histories of organisms. Phenomena such as horizontal gene transfer, hybridization, and polyploidy are changing the conventional image of evolution as a bifurcating process, to evolutionary scenarios that are more properly represented as networks than as trees. Solving the relationships among taxa in complexes that have experienced these phenomena presents a unique challenge for systematists, and an opportunity to increase our understanding of phylogenetic conflict and the historical processes it represents (Sessa *et al.* 2012a, and references therein).

Reticulate evolution appears to be particularly common in plants, and ferns in particular have long been noted for their tendency towards reticulation, frequently as a result of inter-specific hybridization and polyploidy (Pajarón & Pangua 2012, Sessa *et al.* 2012b). Many leptosporangiate groups are known to evolve via reticulate evolution and to form cryptic complexes, involving all of the cited processes as hybridization, polyploidy and apogamy. Reticulate processes have a particularly high impact in some families, for example, among others, *Pteridaceae*, *Dryopteridaceae*, *Aspleniaceae* and *Polypodiaceae*. Recent works are studying, among others, the following genera: *Cheilanthes* (Grusz *et al.* 2009), *Astrolepis* (Beck *et al.* 2010, Beck *et al.* 2012), *Asplenium* (Regalado *et al.* 2010, Dyer *et al.* 2012, Vicent 2013), *Polypodium* (Haufler *et al.* 1995, Hunt *et al.* 2011), *Polystichum* (Barrington 1990, Little & Barrington 2002), *Dryopteris* (Juslén *et al.* 2011, Sessa *et al.* 2012a), *Vandenboschia* (Ebihara *et al.* 2005) and *Pteris (*Chao *et al.* 2012)*. The pattern of reticulate relationships among members of the *Asplenium monanthes* L. complex inferred from phylogenetic analyses of nuclear and plastid
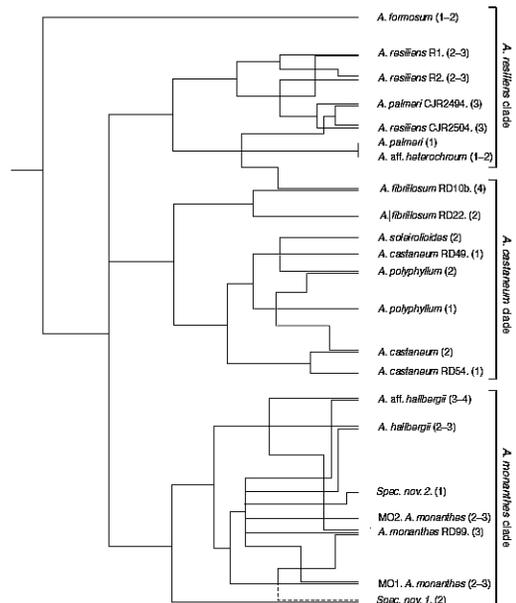
Fig. 9– A reticulation network illustrating the evolutionary history of the *Asplenium monanthes* complex. This summarizes a hybridization network performed in Splits Tree and illustrates hypothetical hybrid relationships observable by comparison between nuclear and plastid trees. From Dyer *et a*l. (2012).

DNA sequences is presented as an example in Fig. 9 (Dyer *et al.* 2012).

APOGAMY, WITH SOME MORE THOUGHTS AROUND THE SPECIES CONCEPT IN FERNS

Apogamy is a very common phenomenon in the ferns (Dyer *et al.* 2012), with high incidence in some lineages as for example *Aspleniaceae*, *Dryopteridaceae* and *Pteridaceae*. The biology and cytology of apogamy is largely known, and it has attracted researches' interest since long ago (Woronin 1907, Braitwaite 1964, Raghavan 1989, Regalado *et al.* 2010). A review, even cursory, on the studies of apogamy in ferns falls outside the scope of this work (and it would require a space at least similar). Nevertheless, the study of apogamy in a evolutionary context is important, because in many cases (for example, complexes of *Asplenium*, *Dryopteris*, *Polystichum* and *Cystopteris* Bernh.) it is related to processes of hybridization and polyploidy (Bicknell & Koltunow 2004). Thus, it seems that for some ferns variation is related to reproduction, life cycle and ploidy level (Wagner 1963,
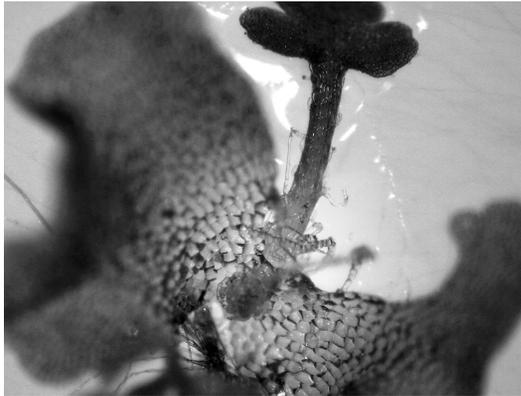
Fig. 10– Apogamous sporophyte of *Argyrochosma nivea* var *nivea*, associated to a proliferating cell area under the gametophyte notch, with long, glandular hairs. Very few individuals have been observed that form archegonia, whilst antheridia have never been developed. However, there are three varieties of this species, apparently linked by hybridization and polyploidy. Original.

Murakami & Iwatsuki 1983, Regalado *et al.* 2010, Liu *et al.* 2012).

The case of the *Asplenium auritum* Sw. complex is well known. Cytological studies revealed the existence of tetraploids of *A. auritum*, and octoploids of *A. monodon* Liebm. (Walker 1966). Also, the hybrid between them (*A. auritum* x *A. monodon*) has been described. In addition, *A. auritum* has a normal sexual reproduction and life cycle, but *A. monodon* and the hybrid of both present an apogamous way of reproduction, *i.e.* via formation of embryos without fecundation, and reconstituting a diploid meiotic mother cell during sporogenesis. As it was stated by Morzenti (1969), apogamous species usually have higher chromosome number than the sexual species related, which is reflected in spore sizes, since cell size is proved to be correlated with ploidy level in ferns (Barrington *et al.* 1989). The striking fact is that, apparently, the growth of vegetative and reproductive cells due to a higher ploidy level and the capacity of avoiding a bigametic mating system, give the apogamous hybrid a greater ability to survive and spread in nature (Regalado *et al.* 2010). Furthermore, it has been stated that "a significant element in the extent of the potential range of a new species is the adaptation inherited from the parental species" (Tryon 1985). Thus, apogamy could be one of these prominent adaptive elements: it has been proved to be inherited and to give apogamous derivatives a wider range in nature (Morzenti 1969, Liu *et al.* 2012).

Sometimes, apogamy with formation of gametangia has been documented (Migliaro & Gabriel y Galán 2012), and has been said to be facultative/induced, *i.e.,* the plant could either experience sexual contacts or, due to some external facts, produce apogamous sporophytes; however, some apogamous ferns are known to produce no gametangia at all, or gametangia failed to produce viable, normal gametes, so the apogamy is obligated (Raghavan 1989). And when hybrids are formed between apogamous species or apogamous and sexual species (Regalado *et al.* 2010), a striking enigma arises: how could the hybrids be formed if gametes are never developed? A notable example could be found in *Argyrochosma nivea* (Poir.) Windham, an Andean fern from the *Pteridaceae* (Fig. 10). Three varieties have been described attending to the sole character of the presence/absence and colour of abaxial farina. Some studies point out that some individuals could have emerged by means of hybridization (Sigel *et al.* 2011). But further studies in the biology of the reproduction showed that, from the many cultures made of spores coming from several individuals of the three varieties, it should be accepted that two of the three varieties lack the possibility of forming gametangia (both archegonia and antheridia), thus the apogamy is totally obligated (Gabriel y Galán 2011, and further personal observations). If no sexual contacts are possible, neither the variety nor the subspecies traditional categories fit for these populations. Further research is needed to get solution on how this ferns evolved from a common ancestor and which are the biological relationships within them. Meanwhile, two stances are possible: one, understand that the obligated apogamy without gametangia imposes a biological barrier to the populations for crossing, so different species have to be assumed to exist; second, refer as varieties the different extreme entities of the biological complex pools of organisms, in which the occurrence of apogamy, hybridization and polyploidy obscures the proper elucidation of its nature (Barrington *et al.* 1989, Gastony & Windham 1989, Haufler 2008).

FERN GENOMICS

Genomics is to date one of the most promising areas to study organismal evolution at the molecular level. The so-called next-generation sequencing (NGS) allows achieving huge amount of DNA data, comparatively much more than with the Sanger techniques, including the possibility of sequencing the whole genome (WGS) of an organism in a quite simple way. From the whole genome

Fig. 11– Complete annotated genome of *Adiantum capillus-veneris* (from GenBank, National Center for Biotechnology Information, USA).

data it is possible to separately assembly the genomes of organelles as plastids and mitochondria, without a previous need of isolating them, which is a time- and resources-consuming technique (Jansen *et al.* 2005, Nock *et al.* 2011). The term plastome refers to the (complete) genetic information of the plastid (Fig. 11). Many methods are widely used today, provided by several companies, including 454, Illumina, Heliscope, SOLID, and some others, which permits rendering long reads and deep coverage. But as technology is moving quickly, other sequencing strategies are expected to appear. NGS has been accompanied by a continuous price reduction (also rendering better quality) and the development of bioinformatics applications for reading rescue, assembly and annotation (Ratan 2009, Kumar & Baxter 2010). Along with the availability of massive DNA data, the advance in RNA manipulation and inverse transcription has derived in the increasing publication of complete transcriptomes of organisms, *i.e* collections of RNA data (Wang *et al.* 2009). The transcriptome shows some striking features: it represents the genes that are being actively expressed, and it could be subjected to variation of external, environmental significance.

With all this information increasingly available, the genomic era has reached to the plants, allowing a profound comprehension of evolutionary, biological, ecological and developmental issues (Rokas & Abbot 2009). But, in parallel to what happens with many other aspects of the

ferns, genomics has only begun to slowly surface this world, and compared with other plant groups there is a clear lack of knowledge. As for the time of writing, it seems that no fern nuclear genome has been sequenced yet. Nevertheless, it could be interesting to point out that the complete genome of the lycophyte *Selaginella moellendorffii* Hieron. has been published (Banks *et al.* 2011). The following Table 1 shows the to-date 11 fern and 4 lycophyte plastomes that have been sequenced and deposited in GenBank (National Center for Biotechnology Information, USA).

Regarding RNA, there is an ongoing huge collaborative project, called *One Thousand Plant Project*, which has the objective of obtaining the transcriptome of one thousand plants. Approximately 75 accessions correspond to ferns. The complete list with additional information can be explored in onekp.com. This project has created its own data repository, which can be blasted and accessed. In GenBank there are also transcriptomes and WGS projects for other few species.

The transcriptome is an invaluable piece of information to know which genes are expressed at any time related to key developmental events of the plants, as spore germination, prothaliall stage, developing and mature organs, sporangia maturation, etc. (Salmi *et al.* 2005, Banks *et al.* 2011, Der *et al.* 2011). Furthermore, comparing transcriptomes trough a lineage allows to understand which genes have been evolutionary activated or silenced. Also, transcriptome information has allowed to search and enlarge the gene sets of potential phylogenetic interest (Rothfels *et al.* 2013).

Whole genomes comparisons (comparative genomics) are the path to detect gene rearrangements and other large-scale mutations, for example chromosomes or genes inversions, transpositions, deletions, duplications, losses, etc. In ferns, this has been done by comparing plastomes (Gao *et al.* 2009, Karol *et al.* 2010, Wolf *et al.* 2011), because no nuclear sequences are yet available. Also, a similar research could be made with only small fragments of DNA (Gao *et al.* 2011), which logically renders less volume of information. All this research is showing a notable amount of chloroplast DNA re-arrangement and change through the different lineages of ferns, in consonance with the known fact that cpDNA is moderately conservative, more than nuclear genome but less than mitochondrial genome (Wolfe *et al.* 1987, Wolf *et al.* 2011).

Using multi-technique approaches, including massive data genomics and transcriptomes, geneticists and plant biologists have ascertained that plants use the hybridi-

Table 1
Fern and lycophyte plastomes sequenced to date (from various sources, including GenBank, National Center for Biotechnology Information, USA)

Species	Base Pairs	Genes	Associated reference
Ferns			
Adiantum capillus-veneris	150,568	130	Wolf *et al.* (2003)
Alsophila spinulosa	156,661	133	Gao *et al.* (2009)
Angiopteris evecta	153,901	141	Roper *et al.* (2007)
Cheilanthes lindheimeri	155,770	Not specified	Wolf *et al.* (2011)
Equisetum arvense	133,309	132	Karol *et al.* (2010)
Equisetum hyemale	131,760	132	Grewe *et al.* (2013)
Lygodium japonicum	157,260	131	Gao *et al.* (2013)
Marsilea crenata	151,628	129	Gao *et al.* (2013)
Ophioglossum californicum	138,270	130	Grewe *et al.* (2013)
Psilotum nudum	138,829	150	Wakasugi *et al.* (1998)
Pteridium aquilinum	152,362	Not specified	Der *et al.* (2011)
Lycophytes			
Huperzia lucidula	154,373	129	Wolf *et al.* (2005)
Isoetes flaccida	145,303	136	Karol *et al.* (2010)
Selaginella moellendorfii	143,780	Not specified	Banks *et al.* (2011)
Selaginella uncinata	144,170	Not specified	Tsuji *et al.* (2007)

zation and polyplodization processes to rearrange the genomic information (Kashkush *et al.* 2002, Paun *et al.* 2007, Hegarty & Hiscock 2008). Two main mechanisms have been cited in relation to polyploidy.

First, gene silencing appears as a widely used mechanism that can lead to great changes in genome expression upon hybrids and polyploids formation. When gene losses don't occur, the silencing of genes is very probably caused by epigenetic means (especially by methylation), because mutations have not time enough to accumulate (Soltis & Soltis 2000, Adams & Wendel 2005, Paun *et al.* 2007). Whilst the epigenetics is being fundamentally studied in model flowering plants, only very few model ferns exists so, again, this evolutionary important mechanisms are less known: in both *Ceratopteris* Brongn. and *Adiantum* L., suppression of gene expression mediated by epigenetic controls as methylation have been demonstrated (Rutherford *et al.* 2004, Tsuboi *et al.* 2012). As far as we know, it is still pending the study of these gene silencing mechanisms in the formation of fern polyploids.

The second striking feature associated with the quick and extend rearrangement of a recently formed polyploidy nuclei is constituted by the existence of transposable elements. Because polyploids have multiple copies of all genes, they could avoid the deleterious nature of these transposons. The conclusion is that polyploids could experience a quick and higher restructuring of their genome (Soltis & Soltis 1999).

Finally, we would like to point out that other aspects of fern genome characteristics, like genome size and C-value, curves of gene duplication, Ks values etc. and it relationships to evolution have been reviewed recently by Nazakato *et al.* (2008).

THE RENAISSANCE OF MORPHOLOGY
IN EVOLUTIONARY RESEARCH

Before the molecular age, phylogeny and evolution of ferns were approached using morphological characters, including anatomical and ultrastructural data. For many years, pteridologists have produced a huge amount of information, which was used for many purposes. And some times, explicit analyses on character evolution and phyletic relationships were included (Bower 1914).

Evolutionary morphology was almost completely obscured with the emergence of genetics applied to the
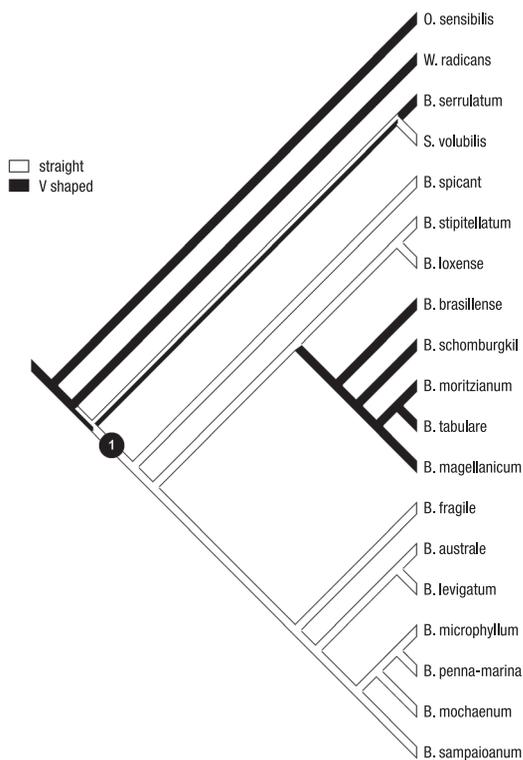
Fig. 12– Xylem form evolution in the genus *Blechnum* from an ongoing study of character evolution in the family. Two states have been defined (straight and V-shaped); the analysis software calculated the most parsimonious history over a previously known phylogenetic tree (Gabriel y Galán *et al.* 2013). Original.

organismal level. For decades, phylogenetics was seen as the ultimate frontier to understand taxa relationships and evolution, and thus morphological studies were abandoned out of the strictly taxonomic descriptions and proposals. Nowadays, the two worlds, morphological and molecular, have smoothly converged (Schneider *et al.* 2009). Although the phylogenetic picture has not been totally drawn, there is a growing and renewed interest in knowing what has been the evolution of morphological characters (Schneider 2013, Vasco *et al.* 2013).

Most of the current studies on morphology are done based on a reliable molecular phylogeny constructed for a particular group. Cladistic approaches are possible to study how morphological character states change through the branches of the phylogenetic tree. As a result, researchers can propose hypothesis on how morphological characters have evolved in the group (Fig. 12). Usually, this method is applied to the study

of one to some characters, at the genus level (Labiak *et al.* 2010, Sigel *et al.* 2011). Also, comparing the velocity of changes of the morphological and molecular characters, some conclusions can be made about the dynamics of the evolution (Sundue & Rothfels 2014).

CONCLUSIONS AND PERSPECTIVES

In this review, we report on the most important features that characterize the ferns, and the main evolutionary mechanisms, which shaped the evolutionary history of their diversification.

Thus, despite their early origin, ferns still represent an important and diverse group of extant vascular plants that is more successful among vascular plants than the gymnosperms of less ancient origin. As compared to the other vascular plants (seed plants), ferns exhibit specific features, such as a unique digenetic heteromorphous life cycle. Moreover, these plants possess the highest chromosome numbers recorded among living organisms, as consequence of high levels of polyploidy, and for having frequently experienced reticulate evolution along with their diversification. Together with hybridization and polyploidy, apogamy has a significant role in the evolutionary history of ferns. Molecular techniques have shown to be important in the study of fern evolution, but it was shown too, that a return to morphology is needed, to fully understand all the mechanisms involved in the history of ferns.

There are many unanswered questions that will lead to future researches as: the high chromosome number in ferns and its origin; the relationship between closely related ferns with geographical disjunctions; the relationship between their geographical distribution and their evolutionary history; morphological character evolution; the need to reconsider some paraphyletic taxa; etc.

In this context there is a great need to re-examine phylogeny and systematics in most fern families using modern molecular phylogenetic approaches together with other techniques in order to best understand their evolutionary history, along with their morphological and molecular characters evolution.

Finally, it is important to change our vision of evolution and to assimilate that evolution is better seen as a complex network than as a linear tree. The understanding of this fact is absolutely necessary to use the correct techniques, ask the correct questions and mostly important to study correctly the fern evolutionary history.

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