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Habilitační práce

Stories of two life stages: reproduction modes, genome size, diversity and interactions among gametophytes and among sporophytes in ferns

Příběhy dvou životních fází: způsoby rozmnožování, velikost genomu, diverzita a interakce mezi gametofyty a mezi sporofyty u kapradin

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Appendix 1: Papers included to the habilitation

Paper 1: Hornych O. & Ekrt L. (2017): Spore abortion index (SAI) as a perspective tool of evaluation of spore fitness in ferns: An insight into sexual and apomictic species. – *Plant Systematics and Evolution* 303(4): 497–507.

Paper 2: Ekrt L. & Koutecký P. (2016): Between sexual and apomictic: unexpectedly variable sporogenesis and production of viable polyhaploids in the pentaploid fern of the *Dryopteris affinis* agg. (Dryopteridaceae). – *Annals of Botany* 117: 97–106.

Paper 3: Hornych O., Férová A., Hori K., Košnar J. & Ekrt L. (2022): Apomictic fern fathers: An experimental approach to the reproductive characteristics of sexual, apomict and hybrid fern gametophytes. – *American Journal of Botany* 109(4): 628–644.

Paper 4: Hornych O., Černochová L., Lisner A., Ekrt L. (2022): An experimental assessment of competitive interactions between sexual and apomictic fern gametophytes using Easy Leaf Area. – *Applications in Plant Sciences* 10: e11466.

Paper 5: Hornych O., Testo W., Sessa E., Watkins J., Company C., Pittermann J. & Ekrt L. (2021): Insights into the evolutionary history and widespread occurrence of antheridiogen systems in ferns. – *New Phytologist* 229: 607–619.

Paper 6: Hornych O., Černochová L., Košnar J. & Ekrt L. (2022): Biotic interactions between the gametophytes of wall rue (*Asplenium ruta-muraria*) and other fern species. – *International Journal of Plant Sciences* 183(1): 10–17.

- Paper 7: Liu H-M., Ekrt L., Koutecký P., Pellicer J., Hidalgo O., Marquardt J., Pustahija F., Ebihara A., Siljak-Yakovlev S., Gibby M., Leitch I. & Schneider H. (2019): Polyploidy does not control all: lineage-specific average chromosome length constraints genome size evolution in ferns. – *Journal of Systematics and Evolution* 57: 418–430.
- Paper 8: Ekrt L., Holubová R., Trávníček P. & Suda J. (2010): Species boundaries and frequency of hybridization in the *Dryopteris carthusiana* (Dryopteridaceae) complex: A taxonomic puzzle resolved using genome size data. – *American Journal of Botany* 97: 1208–1219.
- Paper 9: Kaplan Z., Danihelka J., Štěpánková J., Ekrt L., Chrtěk J. Jr., Zázvorka J., Grulich V., Řepka R., Prančl J., Ducháček M., Kúr P., Šumberová K. & Brůna J. (2016): Distributions of vascular plants in the Czech Republic. Part 2. – *Preslia* 88: 229–322.
- Paper 10: Hornych O., Ekrt L., Riedel F., Koutecký P. & Košnar J. (2019): Asymmetric hybridization in the Central European populations of the *Dryopteris carthusiana* group. – *American Journal of Botany* 106(11): 1477–1486.
- Paper 11: Ekrt L., Podroužek J., Hornych O., Košnar J. & Koutecký P. (2021): Cytotypes of bracken (*Pteridium aquilinum*) in Europe: widespread diploids and scattered triploids of likely multiple origin. – *Flora* 274: 151725.
- Paper 12: Hanušová K., Čertner M., Urfus T., Koutecký P., Košnar J., Rothfels C. J., Jarolímová V., Ptáček J., Ekrt L. (2019): Widespread co-occurrence of multiple ploidy levels in fragile ferns (*Cystopteris fragilis* complex; Cystopteridaceae) likely stems from similar ecology of cytotypes, their efficient dispersal and inter-ploidy hybridization. – *Annals of Botany* 123: 845–855.
- Paper 13: Ekrt L., Košnar J., Rothfels C., Hanušová K., Hornych O. & Urfus T. (2022). Cytogenetic, geographical, spore type and plastid haplotype data reveal cryptic patterns of species diversity in the cosmopolitan *Cystopteris fragilis* complex (Polypodiopsida: Cystopteridaceae). – *Botanical Journal of Linnean Society* 199: 728–739.

Appendix 2: Professional Curriculum vitae

Annotation

Ferns are primitive spore-bearing plants, which alongside lycophytes, represent the oldest lineage of vascular plants on Earth. They are the sister group to seed plants and currently the second most diversified group of vascular plants. Ferns used the diversification waves of angiosperms during late Cretaceous that formed terrestrial ecosystems on Earth to diversify themselves. In contrast to other land plants, the life cycle of ferns (and lycophytes) is unique. It requires two completely spatially and nutritionally independent generations of plants to complete itself. The leafy diploid sporophyte stage (frond) is bearing sporangia and the sexual haploid gametophyte stage, usually resembling a heart-shaped plantlet (prothallus). The alternation of those two generations greatly influences these plants. Ferns are the subject of this habilitation thesis. The research of this group focused on many aspects of their biology such as modes of reproduction, genome size, diversity, with special interest in interactions among gametophytes and among sporophytes. The thesis presents several thematic storylines. The first story focuses on the evaluation of fern spores formed by sexual, apomictic species and their hybrids (apo-sex hybrids). The special detailed view on the reproductive mechanisms of apo-sex hybrids surprisingly showed us evidence of both apomictic (unreduced spore and apogamous sporophyte formation) and sexual (regular spore formation) reproduction strategies present in one taxon. Furthermore, until now, it was believed, that apogamous sporophytes are generally considered to form earlier than sporophytes originating from the sexual process. Our comprehensive study proved that the apomictic reproduction was not necessarily leading to an earlier sporophyte formation and that the apomictic gametophytes are smaller in size than the sexual species. The apo-sex hybrids also tend to behave more like their apomictic parents but suffer from an early disadvantage in the form of lower spore germination rates. The second story explores the evolutionary significance of fern pheromones called antheridiogens. It was found that a pheromone system is widespread among ferns with several recognized different types. Surprisingly, apomictic species also respond to the pheromone system, despite its original function being the regulation of sexual reproduction. In addition to pheromones, ferns appear to produce exudates that may have suppressive or facilitative effects on younger gametophytes of various fern species. The final storyline reveals the many biosystematic adventures resulting from the distribution and interaction among sporophytes discovered using innovative methods in flow-cytometry. Through the application of genome size measurements, we were able to reveal genome multiplication, hybridization and evolution in target groups. This approach has been used to study aspects ranging from genome size evolution (*Asplenium*), species delimitation, population cytotype screening, detection of hybrids (*Dryopteris*, *Pteridium*) to large-scale cytogeographical studies (*Cystopteris*).

1 Introduction

1.1 Ferns – phylogeny, age and position in a plant tree of life

Ferns are a very diverse plant group growing in many habitats (e.g., terrestrial, arborescent, aquatic, epiphytic, epilithic), and, alongside lycophytes, represent the oldest lineages of vascular plants on Earth. Recent morphological and molecular phylogenetic analyses indicate that the extant vascular plant lineages have a basal dichotomy separating the lycophytes from euphyllophytes – representing ferns and seed plants. Therefore, ferns (referred to as monilophytes) are the sister group of seed plants (Kenrick & Crane 1997, Pryer et al. 2001, 2004). Fossil records date ferns back to the middle Devonian within 383–393 million years ago (mya) (Taylor et al. 2009). Recent divergence time estimates suggest they may be even older, possibly having first evolved as far back as 430 mya (Testo & Sundue 2016). However, despite the considerable age of the group as a whole, most of the earliest ferns have since gone extinct. The majority of living ferns arose from a much later diversification event occurring as recently as ca 70 mya. Molecular data and a reassessment of the fossil records show that polypod ferns (Polypodiales representing more than 80% of living fern species) diversified in the Cretaceous, after angiosperms. The diversification of polypod ferns was apparently caused by ecological responses to the previous diversification waves of angiosperms that formed terrestrial ecosystems on Earth (Schneider et al. 2004). Simply put, recent ferns (monilophytes) diversified in the shadow of angiosperms.

The extant ferns comprise four extant lineages: horsetails, a group composed of whisk ferns and ophioglossoid ferns, marattioid ferns and the most modern leptosporangiate ferns (Barker & Wolf 2010). Today, the group of leptosporangiate ferns with around 9000 living species is the second most diverse group of vascular plants on Earth, outnumbered only by angiosperms (Schuettpelz & Pryer 2007, PPG I 2016).

1.2 The fern life cycle as unique system of two independent life stages

In contrast to other land plants (mosses, seed plants) the life cycle of ferns and lycophytes is unique. It requires two completely spatially vicarious and nutritionally independent generations of plants to complete itself. The leafy stage (frond) bearing sporangia represents the diploid sporophyte and sexual generation, usually resembling a heart-shaped plantlet (prothallus), represents the haploid gametophyte.

In most ferns, the diploid frond (sporophyte) produces haploid spores via meiosis. Spore formation (sporogenesis) occurs in sporangia, usually on the underside of the frond. Each haploid spore grows into a tiny aboveground photosynthetic prothallus (all leptosporangiate ferns, several groups of eusporangiate ferns) or a underground heterotrophic prothallus (lycophytes, whisk ferns, ophioglossopsids). Each haploid prothallus can produce male gametes (spermatozoids) in antheridia and female gametes (eggs) in archegonia. Both free-swimming spermatozoids and sessile eggs may be produced on the same plantlet, possibly leading to self-fertilization. Such event is likely rare in nature, possibly due to the resultant complete homozygosity (Soltis & Soltis 1992, Sessa et al. 2016).

Fern sex can change over time and is environmentally determined, influenced by factors such as pheromones, nutrients, temperature, etc. A particularly interesting sex determinant is a

pheromone called antheridiogen. The main function of antheridiogen is to promote a precocious formation of antheridia, and, to an extent, enforce male unisexuality in younger gametophytes. This in turn promotes outcrossing by increasing the number of free-swimming spermatozooids around older (female) gametophytes. The young gametophyte retains its sensitivity to antheridiogens only until the onset of the meristic phase (the phase associated with archegonia formation) after which the gametophyte may be unable to form antheridia (Döpp 1950, Raghavan 1989). When water is present, sperm use their flagella to swim alongside a pheromonal trail to an egg and fertilize it. The fertilized egg is a diploid zygote formed by the combination of chromosome sets from the egg and sperm. The zygote grows via mitosis into the diploid sporophyte, completing the life cycle (Raghavan 1989).

1.3 Mechanisms of diversification and speciation in ferns

Three different speciation modes are recognized in extant ferns and lycophytes – primary, secondary and tertiary (Ranker & Haufler 2008).

The establishment of isolated populations, by means of rising geological barriers or dispersal events, followed by population evolutionary processes (drift, inbreeding, gene flow, metapopulation concepts etc.) is considered as primary speciation and is us a common pattern of diversification in ferns (Kato 1993). These diversification trends are similar to the vast majority of other organisms.

In ferns, as in the majority of land plants, whole genome duplication (polyploidization) is a major speciation force (Lavana 2020, Van de Peer et al. 2021). This phenomenon is usually connected with hybridization and sometimes called secondary speciation (Ranker & Haufler 2008).

Within land plants, polyploidization appears to be low or absent in liverworts, hornworts, cycads, and conifers, but is remarkably frequent in lycophytes, ferns (monilophytes), and angiosperms. Wood et al. (2009) estimated that of speciation events, 15% for flowering plants and 31% for ferns are associated with polyploidy. In ferns, systematic research of polyploidization was started by Manton (1950), who first reported on the intricacy of fern polyploid groups.

There are three types of genome multiplication rearrangement in ferns (autopolyploidy, allohomoploidy, allopolyploidy) (Ranker & Haufler 2008). Autopolyploidy involves the multiplication of one basic set of chromosomes. Autopolyploidy is in many ferns often associated with distinct reproductive strategies and increases the likelihood of reproductive barriers occurring, thus creating the potential for the development of spatial structure (Lovis 1964, Treweek et al. 2002). In some fern groups, it may serve as a frequent, rapid and important speciation mechanism, especially when accompanied by apomixis (Windham & Yatskievych 2003). During autopolyploidy, there is no mismatch between genetically different chromosome sets , so offspring fertility is high (Soltis et al. 2014, Jighly et al. 2018).

A likely infrequent and poorly studied mechanism of secondary speciation is allohomoploidy (Conant & Cooper-Driver 1980, Sigel 2016). This process comes up in diploid species which may be ecologically isolated, but not always reproductively isolated. Hybrid swarms may arise. Examples of allohomoploidy were found in the genus *Polystichum* in western North America

(Mullenniex et al. 1999) and tree ferns from the family Cyatheaceae (Conant & Cooper-Driver 1980).

The most widely studied speciation process in ferns is likely allopolyploidy. In this case, progenitors of hybrid-derived species are not interfertile and form sterile offspring when they cross (Manton 1950). Pteridophytes are generally prone to the formation of allopolyploid complexes (Haufler 2002, Fujiwara & Watano 2020).

The origin and persistence of polyploid ferns is enhanced by the empty niche space and greater tolerance to disturbances of polyploid ferns related to larger spore size is likely a selective advantage on islands, consistent with the number of high polyploids encountered among ferns on oceanic islands. Though recently originated polyploid ferns usually have identifiable progenitors with which they are partially sympatric, the rate of expansion of these ferns beyond the range of their progenitors varies (Barrington 2020).

Allopolyploidy is preceded by hybridization – the process of crossbreeding between genetically dissimilar parents to produce a hybrid (see below). It seems that barriers to development of hybrid zygotes are weak and field studies have demonstrated high frequency of vigorous but sterile hybrids in some complexes (Reichstein 1981, Petit et al. 1999). Primary interspecific hybrids are usually sterile due to unbalanced meiosis: pairing chromosomes (each coming from distinctly different genome) are not actually homologous, resulting in aborted spores. The polyploidization event restores the possibility of homologous chromosome pairing and proper spore formation. Possessing redundant copies of genes, gametophytes of polyploid species are more tolerant to selfing than their diploid progenitors. Thus, viable sporophytes may develop. However, even though allopolyploidy is generally accepted as a frequent mode of speciation in ferns, there are still many open questions about the so-called species complexes that involve allopolyploids (Ranker & Haufler 2008). Relatively high basal chromosome numbers and large genome sizes of extant ferns have probably been derived from lower ones by ancient (paleo)polyploidy (Walker 1979). Therefore, species recently representing diploids are likely these ancient polyploids whose ancestors became extinct. Although it was initially thought that paleopolyploidy is restricted to ferns only, it has recently been recognized in numerous families of angiosperms as well (Soltis et al. 2009).

Tertiary speciation, in other words polyploid genome reorganization is a major discovery of the past two decades. It results in a rapid reorganization of fern polyploid genomes (Soltis et al. 2003). This process could occur in genetically isolated populations whose separation is maintained by reciprocal gene silencing (Werth & Windham 1991). If the genome is highly polyploid, there can be a risks of aneuploidy caused by changes in cell structure and mistakes during meiosis (Comai 2005). During the following gene silencing, parts of the genome can be inactivated, even active alleles (Martínez de Alba et al. 2013).

1.4 Hybridization – interaction between the species

Despite the fact that a single gametophyte can form male antheridia and female archegonia at the same time and is capable of self-fertilization, most ferns seem to prefer outcrossing. In sexual fern species, if two different species meet, they are very often capable of crossing – hybridization. Fern hybrids (that arose from hybridization of sexual species) are mostly incapable of creating of functional spores (Wagner & Chen 1965).

The phenomenon of hybridization is well-known in ferns, new hybrids are constantly being described (Barrington et al. 1989, Sigel 2016). Many more species are allopolyploids, i.e., hybrids, that restored their fertility by genome duplication. Hybridization has profound positive and negative consequences for fern evolution.

In ferns, researchers presume that hybridization is frequent and the hybridization barriers are weak (Barrington et al. 1989, Sigel 2016). But, with a few exceptions (Testo et al. 2015), the nature of these barriers was not properly studied. Furthermore, there was no research quantitatively measuring the frequency of hybridization, hybridization rates, in natural populations. The much more complicated hybridization system could arise, when one species involved in hybridization is apomictic. The hybridization between fern sexuals and apomictic of different ploidy is one story discussed in this habilitation.

1.5 When it takes one to tango: aspects and consequences of apomixis in ferns

Apomixis in ferns is broadly defined simply as asexual reproduction (apo=without, mixis = mixing/sex). Apomixis encompasses both vegetative reproduction and asexual reproduction through the alternation of generations, including apospory, apogamy, and parthenogenesis (Grusz 2016, Albertini et al. 2019). However, apomixis is mostly used as a term describing an alteration of the typical fern sexual life cycle by a combination of two processes, diplospory (formation of diploid spores) and apogamy (formation of sporophytes without the merging of gametes). Apomictic ferns, by contrast to sexual ones, follow one of two alternative spore-generating pathways to yield chromosomally unreduced diplospores: either premeiotic endomitosis – PE; formerly known as Döpp-Manton sporogenesis (Döpp 1932, Manton 1950, Manton & Walker 1954) or meiotic first division restitution – MFDR; formerly known as Braithwaite sporogenesis (Braithwaite 1964). These diplospores, each having one full chromosome complement, then germinate. The resulting prothalli are capable of generating new sporophytes from somatic cells, which are usually located near the spot archegonia would be produced by sexually reproducing ferns. Rarely archegonia are observed in apomicts as well, but they are likely always abortive. In contrast, apomictic gametophytes have been known to make functional antheridia, but the viability of spermatozoids contained therein is largely dependent upon the pathway to diplospory undertaken in the parent, only PE yields viable spermatozoids. These processes are described in more details by Raghavan (1989) or Grusz (2016).

In ferns, apomixis has mostly been confined to the largest leptosporangiate families Pteridaceae, Dryopteridaceae, Polypodiaceae and Aspleniaceae (Chao et al. 2010, Liu et al. 2012, Dyer et al. 2012, Guo & Liu 2013). It is estimated that about 10% of ferns are without capability of sexual reproduction (Walker 1979), but, apomixis was confirmed in about 3% of extant fern species (Liu et al. 2012). This is rather high number compared to less than 1% of angiosperms (Whitton et al. 2008).

2 Context of habilitation

2.1 The adventurous stories of fern spores and their intricate journey towards forming a green thalloid gametophyte

Fern spores are reproductive propagules capable of long-distance dispersal. Except for the small number of heterosporous species represented by water ferns, homosporous ferns usually reproduce by these tiny oval propagules tens of micrometers in size. Ferns produce a large number of spores ranging from several millions to hundreds of millions every year of its seasonal fertility (Moran 2004). Even homosporous ferns tend to vary in spore size, mainly because of polyploidy. Polyploid species tend to have larger spores compared to their diploid relatives (Barrington et al. 1989, Quintanilla & Escudero 2006, Barrington 2020). Spores are usually typical in their color and this also plays a crucial role in biology, distribution, germination and ecology of fern species. The majority of ferns have non-green (achlorophyllous), usually of brown or yellow spore color. Those spores usually germinate after a longer time (several weeks) and are much more resistant to environmental hazards. The taxa with green (chlorophyllous) spores are in a minority but this trait evolved multiple times and is spread throughout the fern phylogeny tree (Sundue et al. 2011). The presence of chlorophyll in the spore indicates its active metabolism and fundamentally changes spore characteristics. Green spores germinate within a couple of days and tend to result in faster growing gametophytes but have short life spans and die after average 48 days, compared to the tens of years achlorophyllous spores may remain viable for (Lloyd & Klekowski 1970). Sundue et al. (2011), first described so-called cryptochlorophyllous spores with olive color, both green and brown. They are protected by well-developed perispores but carry chlorophyll inside. The division of spore color should probably be viewed as a gradient rather than a dichotomy.

The shape of spores is mostly formed by the type of sporogenesis. As spores are the product of meiosis, four are produced at a time in a tetrad. Based on how the spores are oriented during sporogenesis in the tetrad, a scar is created at the point of contact of all four sister spores. The scar is either trilete (Y shaped), leading to a tetrahedral spore, or monolete (I shaped), resulting in an oval spore. The character of scar shape is highly conservative and rarely varies within family (Tryon & Lugardon, 1991).

Spores may also be of spherical shape with indistinct scar. This is caused by either 4 incomplete meiosis or cytokinesis cycles leading to polyploid (diplo)spores (Morzenti 1962). These aberrant spores can germinate and may serve as an important driver in the evolution of polyploidy in ferns.

Meiosis, through which spores generally form, is an important check point of genetic integrity as chromosomes have to form pairs. In contrast to angiosperms, in which some hybrids may successfully sexually reproduce (Rieseberg & Carney 1998), fern hybrids are unable to properly pair chromosomes during spore formation and their spores are mostly inviable, aborted. Aborted spores vary greatly in size, and are often very dark, shriveled, with a collapsed exospore. This may be very practical for researchers using aborted spores as an identification character (Wagner & Chen 1965). Fern hybrids are sterile and potentially reproductive dead-ends. Apo-sex hybrids (one parent apomictic, one sexual) are a peculiar exception to hybrid sterility. These apo-sex hybrids are generally considered to form 80–95% spores aborted

(Fraser-Jenkins 2007; **Paper 1, Paper 2**). A comprehensive comparison of sexual and apomictic taxa using extensive spore fitness data has been published (**Paper 1**). Based on a representative data set of 109 plants from 23 fern taxa, we accomplished the first robust analysis of spore fitness by examining spore abortion, calculating the ratio of aborted to all examined spores. We compared this trait for different fern reproductive types (sexual/apomicts/hybrids) and ploidy levels (diploid versus polyploid). The results confirmed the general assumption that shows higher spore abortion for apomictic taxa (18%) when compared to sexual taxa (3%). Furthermore, hybrids are characterized by having almost all spores aborted (99.8%) with the rare exception of an apo-sex hybrids. We found no significant difference in spore abortion between sexual taxa of various ploidy levels or between sexual taxa of target genera *Dryopteris* and *Asplenium*.

Fern apo-sex hybrids are also capable of producing viable reduced and unreduced spores, a characteristic rare among ferns (Windham 1983, Sigel et al. 2011, **Paper 2**). Like their apomictic parents, fern apo-sex hybrids can produce gametangia, which may be dysfunctional, and apogamously formed sporophytes (Walker 1962, Regalado Gabancho et al. 2010). Although the information is limited, our findings indicate a possible involvement of sexual as well as apomictic reproduction in apo-sex hybrids. A detailed study concerning the apo-sex pentaploid hybrid of *Dryopteris* × *critica*, a hybrid of triploid apomictic *D. borrieri* and tetraploid sexual *D. filix-mas* (**Paper 2**) was recently published. This partly fertile hybrid (both well-developed and aborted spores are present) surprisingly shows unstable sporogenesis with sexual and apomictic reproduction combined. While a standard number of spores per sporangium in sexual leptosporangiate ferns is 64 and in apomictic 32 (Regalado Gabancho et al. 2010, Dyer et al. 2012), the number of spores in this hybrid varied from ca 31 to 64. Within a single sporangium it was possible to detect the formation of either only aborted spores or various mixtures of aborted and well-developed reduced spores and unreduced diplospores. The spores germinated into viable gametophytes with two ploidy levels: pentaploid (5x, from unreduced spores) and half of that (ca 2.5x, from reduced spores). Moreover, 2–15 % of gametophytes (both 2.5x and 5x) formed a viable sporophyte of the same ploidy level due to apogamy. The existence of reduced viable spores and the occurrence of both types on one plant and even in one sporangium together is unexpected and novel. Moreover, both spore types are capable of viable next-generation sporophyte (F2) production, which has not been observed previously. In conclusion, the pentaploid hybrid is capable of autonomous reproduction (**Paper 2**).

The interaction and possible hybridization between sexual and apomictic are very challenging and still not fully explained topics in general. Our recent study (**Paper 3**) presents the most thorough comparison of gametangial development in sexual and apomictic ferns to date. We cultivated the spores of 43 apomicts, 7 apo-sex hybrids, and 16 sexuals, and measured their development (germination, lateral meristem formation, sexual expression, production of sporophytes) in vitro over 16 weeks. All three examined groups (sexuals / apo-sex hybrids / apomicts) formed antheridia but differed in overall gametophyte development. Sexuals created archegonia (86% of viable samples), but no sporophytes. Apomicts occasionally created nonfunctional archegonia (8%) but usually produced apogamous sporophytes (75%). Until now, it was believed that apogamous sporophytes are generally considered to form earlier than

sporophytes originating from the sexual process (Whittier 1968, Huang et al. 2006, Regalado Gabancho et al. 2010, Haufler et al. 2016). Only a single previous paper indicated the opposite trend (Laird & Sheffield 1986). So, despite expectations, in our study, the apomictic reproduction was not leading to the earlier sporophyte formation (**Paper 3**). Apo-sex hybrids in this study had lower germination rates than their parental species, indicating a genomic imbalance (chromosomal incompatibilities during meiosis) caused by the hybridization event and leading to post-zygotic hybridization barriers. This effect is multiplied by their considerably higher spore abortion rate (**Paper 1, Paper 2**). Thus, of the ca 10% apparently viable spores, about a half fail to germinate. All germinated apo-sex hybrids produced antheridia (archegonia were absent) and abundant apogamous sporophytes. This is supported by other studies (Walker 1962, Regalado Gabancho et al. 2010). Finally, we can say, that apo-sex hybrids tend to behave more like their apomictic parents than the sexual ones but suffer from an early disadvantage in the form of lower germination rates (**Paper 3**).

We also looked at the problem from an alternative viewpoint. The presence of fern gametophytes has been demonstrated to affect the size and sexual expression of other gametophytes in populations based on the resulting gametophyte density and the release of pheromones into the habitat. When sown at high densities, fern gametophytes tend to be small and male, or may even completely lack gametangia, while at lower densities, gametophytes are larger and female or hermaphroditic (Huang et al. 2004, DeSoto et al. 2008). However, at very low densities or in single-spore cultures, gametophyte growth may be retarded or abnormal (Dyer 1979). Very little is known about the interactions between the gametophytes of multiple species at the same stage/age occurring at densities permitting the formation of both types of gametangia. There are several possible outcomes of such interactions. First, the gametophytes in mixed-species populations may grow at the same rate as in monoculture. Second, overyielding may occur, leading to gametophytes of at least one species growing faster in the presence of another species, as has been observed in angiosperm sporophytes (Turnbull et al. 2013, Wright et al. 2017). Third, one or more species may underyield (grow smaller) due to competition for resources or chemical allelopathy (Rünk et al. 2004, Testo & Watkins 2013, Cheng & Cheng 2015). To address this lack of information, we cultivated three fern species of the *Dryopteris filix-mas* complex (Dryopteridaceae). *Dryopteris filix-mas* is a sexually reproducing tetraploid with diploid gametophytes, while *D. affinis* and *D. borreri* are apomictic and gametophytes have the same ploidy level as sporophytes (**Paper 4**). The gametophytes of the three species in our study differed significantly in their growth capabilities, as expressed by their total cover area in monocultures. The diploid gametophytes of the sexual tetraploid *D. filix-mas* grew largest. In comparison, the apomictic gametophytes were smaller, ordered by ploidy level. Interestingly, the apomicts were smaller than the sexual species overall, which seems to go against the generally presumed faster growth of apomictic gametophytes (Walker 1962, Regalado Gabancho et al. 2010) and also support this trend in our previous study (**Paper 3**). In general, the performance of the tested species in mixtures was dependent on the competitor species identity, indicating the importance of competition between gametophytes. Our innovative method based on software Easy Leaf Area, which evaluate the numbers of red and green pixels that are automatically compared to calculate the green area in a defined region,

can be used for a rapid assessment of fern gametophyte cover in large gametophyte populations (**Paper 4**).

It is well known that most ferns seem to use special pheromones – antheridiogens (Schneller 2008). There is an ongoing discussion about whether antheridiogens primarily slow down growth, which prompts antheridial formation, or vice versa (Näf, 1956; Korpelainen, 1994; Quintanilla et al., 2007). Nevertheless, antheridiogens mediate interactions between gametophytes of different developmental stages or ages. But this is another story presented in the next chapter.

2.2 The secret life of fern gametophytes: An exploration of fern pheromones and their evolutionary significance

Gametophytes of homosporous ferns can be bisexual and are theoretically capable of gametophytic selfing, the fusion of two gametes originating from a single gametophyte via mitosis (Hauffler et al. 2016). As the gametophyte grows from a spore originating via a single meiotic event, the sporophyte arising from such event is completely homozygous (Klekowski & Lloyd 1968). Thus, the recent homosporous lineages maintain their genetic diversity by mechanisms that reduce the rate of gametophytic selfing. Fern gametophytes often use a dynamic system controlling sex expression via pheromones called antheridiogens (Schneller 2008). Antheridiogens (AG) were discovered and subsequently described in 1950 during experiments with *in vitro* gametophyte cultivation (Döpp 1950, Näf et al. 1975). Antheridiogen production and response vary considerably among fern taxa and the system involves complex inter- and intraspecific interactions. Moreover, later studies indicated that various types of AGs occur across fern phylogeny (Schneller et al. 1990). The primary function of AGs is the stimulation of precocious formation of antheridia. When a gametophyte of an AG-responsive species grows in the absence of this pheromone, it first develops archegonia. However, right before the gametophyte reaches the archegoniate phase, it begins exuding AGs into its environment. At the same time, the gametophyte loses the ability to respond to the pheromone. Younger or slow-growing asexual gametophytes in the immediate surroundings of the first gametophyte respond to the AGs by halting growth and forming antheridia (i.e., becoming male). The population ends up composed of a few larger female gametophytes and many smaller male gametophytes (Döpp 1950, Näf et al. 1975, Tryon & Vitale 1977). As fern sperm are flagellated and need to swim through water to reach archegonia, a greater abundance of sperm due to the AG system may help overcome the limitations of dry environments. Likewise, AG leads to a greater number of unisexual gametophytes, therefore limiting self-fertilization and facilitating outcrossing, the exchange of gametes between gametophytes, thus maintaining heterozygosity in fern populations (Schedlbauer & Klekowski 1972). Through the AG system, homosporous ferns gain these advantages, which are usually afforded to heterosporous plants because of their pre-determined sexes and the consequent inability to undergo the extreme form of selfing found in homosporous plants. Additionally, larger gametophytes may be able to pheromonally suppress sporophyte formation in smaller gametophytes, leading to reduced competition. On the other hand, smaller gametophytes may use the system to contribute to the next generation despite being unable to form archegonia or support young sporophytes due to unfavorable conditions (Willson 1981).

Despite the apparently widespread occurrence of AG systems in ferns and their potentially large evolutionary significance via their effects on population structure and mating behavior, our understanding of their evolution and distribution across the fern phylogeny was limited until recently. The meta-analysis of 88 published papers on 208 fern species in total together with new data from cultivation experiments was presented (**Paper 5**). Using this large dataset, we showed that the AG system is widespread among ferns. About two-thirds (64.5%) of all tested species responded positively to AGs. Several AG types are well-established by now, but others still require thorough testing to determine their scope, distinctness, and features. The majority (66.7%) of apomictic species surveyed to date respond to AG. The consequences of this may play an important role in survival and competition among fern gametophytes in nature as well as interactions between apomictic and sexually reproducing taxa. This study also suggests that there is no difference between diploids (67.0%) and polyploids (68.1%) in response to AGs, so the pheromonal system may be advantageous even to species which are predominantly selfers (**Paper 5**).

Apart from the precocious formation of antheridia, AGs also enable spores to germinate in darkness, bypassing the usual requirement of light (Weinberg & Voeller 1969, Haufler & Welling 1994). Such germinated spores grow into small belowground gametophytes with a few antheridia (Schneller et al. 1990). The sperm released from these antheridia then navigate toward open archegonia (Lopez-Smith & Renzaglia 2008) and may fertilize the antheridiogen-releasing gametophytes aboveground. Thus, AGs effectively allow ferns to mine the otherwise dormant spore bank for genetic diversity (Schneller 1988).

There is still a large potential for studying new types of AGs systems and their interactions with biotic or abiotic factors. One of these still-unresolved potential AG types was reported in a small calcicole fern *Asplenium ruta-muraria*. It was initially described as a unique AG system in *A. ruta-muraria* due to the species' lack of response to gibberellins (Schneller 1995), which are similar to Schizaeales-type antheridiogens. Within our study (**Paper 6**) we cultivated the spores of multiple fern species, including representatives of three established antheridiogen types and various related and unrelated members of Aspleniaceae. Specifically, the effect of older meristic gametophytes on the sexual expression of younger gametophytes was observed. Our tested individuals of *A. ruta-muraria* decidedly did not employ any system resembling antheridiogens as observed in Schizaeales and Polypodiales. The exudates of female gametophytes did not preclude the formation of archegonia among younger gametophytes or stimulate germination in darkness. Surprisingly, the exudates of *A. ruta-muraria* may have suppressive or facilitative effects on younger gametophytes of various fern species. The mechanics and properties of these effects have been poorly explored until now and may have evolutionary significance in the subsequent development of sporophytes.

2.3 The story of how fern sporophytes are affected by genome size: delimitation, distribution, diversification and hybridization in the shadow of genome multiplication

As mentioned in the introduction, one of the most important mechanism of plant diversification is genome multiplication – polyploidy (Haufler 2002). The quickest and most powerful tool used to evaluate and determine the plant genome size or estimate the DNA ploidy level is flow-

cytometry (FCM). The evolution of plant genomes is dynamic, encompassing a range of genomic processes including multiple rounds of whole genome duplication (WGD), polyploidization, chromosomal rearrangements and the turnover and evolution of repetitive DNA (Wood et al. 2009). This is mirrored in the tremendous variation in nuclear genome sizes across land plants (ca 11,850-fold). This has crucial implications for flow cytometric applications both with respect to technical issues, e.g., a series of internal standards of different genome size is required (Loureiro et al. 2021, Čertner et al. 2022). In recent years, an increasing degree of attention has focused on the contrasting genomic profiles and evolutionary processes underlying different lineages of land plants, and the consequences of these differences on their diversification and mechanisms driving genome size evolution (Leitch & Leitch 2012, Landis et al. 2018, Fang et al. 2022, Fujiwara et al. 2023).

Although ferns and flowering plants could share some of the pathways involved in diploidizing the genome, they differ in their response to the additional chromosomes and DNA arising from WGD (Clark et al. 2016). The distribution of genome sizes in angiosperms is biased towards small genomes (median = 1.7 pg/1C) but is more normally distributed with medium-sized genomes in ferns (median = 11.4 pg/1C) (Leitch & Leitch 2013, Suda et al. 2015, Pellicer et al. 2018). Ferns are also typically characterized by higher chromosome numbers (mean $2n = 121.0$; $2n$ range = 18–1440) compared to angiosperms (mean $2n = 15.99$; $2n$ range = 4–640) and genome size and chromosome number ($2n$) are often correlated in ferns but not in angiosperms (Barker 2009, Haufler 2014, Clark et al. 2016). Such differences at the molecular level could also lead to the higher contribution of hybrid speciation to extant fern diversity (Wood et al. 2009, Mayrose et al. 2011, Soltis et al. 2015) and evolutionary potential (Vanneste et al. 2015, Clark et al. 2016) compared to other land plant lineages. This suggests that genome size evolution in ferns is not only shaped by repeated cycles of polyploidy but also by other mechanisms. In our study (**Paper 7**), we aimed to explore whether the evolution of fern genome sizes is not only shaped by chromosome number changes arising from polyploidy but also by constraints on the average amount of DNA per chromosome. To test these relationships, we used 147 accessions belonging to 54 taxa of genus *Asplenium*. This genus is suitable for such analysis because of a highly conserved base chromosome number and a high frequency of polyploidy. We surprisingly revealed that genome size varied substantially between diploid species, resulting in overlapping genome sizes among diploid and tetraploid species of genus *Asplenium*. The observed additive pattern indicates the absence of genome downsizing following polyploidy. The genome size of diploids varied non-randomly and we found evidence for clade-specific trends towards larger or smaller genomes. The 578-fold range of fern genome sizes have arisen not only from repeated cycles of polyploidy but also through clade-specific constraints governing the accumulation and/or elimination of DNA (**Paper 7**).

The application of FCM is very helpful for the development of plant biosystematics. Resolving genome size/DNA ploidy level of many intricate and cryptic groups of plants, including ferns, was a significant step forward (Bureš et al. 2003, Ebihara et al. 2005, Ekrt et al. 2009, Dyer et al. 2013, Henry et al. 2014, Dauphin et al. 2016). We applied flow-cytometry with a combination of multivariate morphometrics to one of the most taxonomically intricate fern group of Central Europe – *Dryopteris carthusiana* group (**Paper 8**). This conspicuous and very common component of temperate woodlands is typical for its overall phenotypic

similarity, great plasticity and the incidence of interspecific hybridization, which has resulted in continuous disputes concerning species circumscription and delimitation. The group consists of one diploid *D. expansa* and two allotetraploids *D. carthusiana* and *D. dilatata*. Our flow cytometric measurements of 858 plants from 85 localities revealed unique genome sizes in all species and hybrids, allowing their easy and reliable identification for subsequent morphometric multivariate analyses. Even the two tetraploids markedly differ in their genome size (21% difference). This study (**Paper 8**) showed that genome size data may help to resolve taxonomic complexities in this important component of the temperate fern flora and that multivariate analyses may resolve species boundaries. This study together with other thorough examinations of the genus *Dryopteris* (Ekrt et al. 2007, 2009, 2010, 2013) led to a chorological compilation of distribution of all *Dryopteris* species in the Czech Republic (**Paper 9**). Based on a thorough revision of 40 public herbarium collection including the largest ones (e.g., BRNM, BRNU, CB, HR, MP, OL, OLM, PR, PRC, ROZ) the distribution pattern of individual species in the Czech Republic was processed. All revised data from herbarium vouchers used for mapping were georeferenced, and entered into the Pladias database (Wild et al. 2019) and geographically sorted according to the traditionally used CEBA (Central European Basic Area) grid template of Niklfeld (1997). During the following years, based on herbarium revisions and a review of literature, the distribution maps of all fern and lycophytes of the Czech Republic were published (Ekrt in Kaplan et al. 2016, 2017a, 2017b, 2018a, 2018b, 2019, 2020).

Going back to **Paper 8**, a very interesting finding resulted from the study. We found a very varied frequency of particular hybrid taxa that depended primarily on the evolutionary relationships, reproductive biology, and co-occurrence of progenitors. However, the dataset was not designed to prove this, and evidence was insufficient. Therefore, a follow-up study (**Paper 10**) was conducted to focus on this aspect. This also represents the most thorough investigation of hybridization rates in natural populations of ferns. The *Dryopteris carthusiana* group was also chosen as it forms three different hybrid combination and is frequently available in mixed populations. A total 40 mixed (co-occurrence of at least two species) population were sampled and all mature plants in a continuous area containing about 100 individuals were collected and the genome size of all studied individuals (about 3962 plants) was determined using flow cytometry. We found hybrids in 85% of populations. The triploid *D. ×ambroseae* (*D. expansa* × *D. dilatata*) occurred in every population that included both parent species and is most abundant when the parent species are equally abundant. By contrast, tetraploid *D. ×deweveri* (*D. carthusiana* × *D. dilatata*) was rare (15 individuals total) and triploid *D. ×sarvelae* (*D. carthusiana* × *D. expansa*) was completely absent in this study. The parentage of hybrid taxa is considerably asymmetric. Overall, we can say that hybridization rates differ greatly even among closely related fern taxa. In contrast to angiosperms, our data suggest that hybridization rates are highest in balanced parent populations, supporting the notion that some ferns possess very weak barriers to hybridization.

The other application of flow-cytometry and genome size measurement was focusing on the experimental interspecific cytotype variability of the fern *Pteridium aquilinum* (**Paper 11**). In Europe, *P. aquilinum* is considered an aggressive colonizer and a weed, growing in woods, pastures, abandoned fields and various other disturbed habitats. This success is likely due to a combination of sexual and clonal reproduction (Page 1976). The taxon is considered diploid

with a notable exception of the triploids occasionally found in the British Isles (Sheffield et al. 1993). Unlike in most even-ploidy cytotypes, fern triploids are often apomictic or infertile, due to genetic imbalance. An interesting opportunity to study these phenomena has emerged with the discovery of this triploid bracken, a triploid that is fertile but not apomictic. To study the cytotype variation of the species, we analyzed 1456 randomly sampled fronds from 135 population. The diploid cytotype of *P. aquilinum* is dominant in continental Europe with 121 entirely diploid populations found, but we also found 9 mixed and 5 entirely triploid populations. Triploids were also revealed in Norway, Sweden, Germany, Switzerland, the Czech Republic, and Austria, but are rare. There seems to be no obvious geographical patterns in the distribution of cytotypes, so we additionally tested genetic differences (9 microsatellite regions) between ploidy levels as well. It was found that the triploid cytotype is rare and likely originated multiple times from the diploids and relies on clonal and possibly limited sexual reproduction to maintain itself. However, diploids and triploids are often genetically different within a population, indicating that the triploids may also migrate between populations, because 21.4% of triploid plants found were fertile (**Paper 11**).

The intensive and long-term use of flow cytometry in fern biosystematics has finally led us to the challenge of producing the largest cytogeographic study ever performed on ferns. We focused on the interspecific cytotype variability of the polyploid *Cystopteris fragilis* complex (**Paper 12**). The collection of 5518 individuals from 449 localities across four continents and subsequent analyses of genome size took us five years (thanks to the great help of many colleagues). We revealed five different ploidy levels (2x, 4x, 5x, 6x, 8x) and three species with intraspecific ploidy-level variation: *C. fragilis*, *C. alpina* and *C. diaphana*. Two predominant *C. fragilis* cytotypes, tetraploids and hexaploids, co-occur over most of Europe in a diffuse, mosaic-like pattern. Within this contact zone, 40 % of populations were mixed-ploidy and most also contained pentaploid hybrids. Despite our application of possible ecological preferences, field-recorded parameters and database-extracted climate data, the environmental conditions were only observed to have a limited effect on the distribution of the predominant cytotypes. Flow-cytometric analyses revealed subtle but highly significant differences in monoploid genome size (Cx value) between *C. fragilis* tetraploids and hexaploids. Specifically, the monoploid genome of hexaploids is on average 2.6 % smaller than that of tetraploids. This might be a consequence of ‘genome downsizing’, a process of systematic DNA loss, which is known to commonly accompany polyploidy (Leitch & Bennett 2004). Based on this study, we can confirm, that both the ploidy level diversity and the frequency of mixed-ploidy populations observed here suggest that, in this respect, ferns can match the well-documented patterns in angiosperms (Kolář et al. 2017). While ploidy coexistence in *C. fragilis* is not driven by environmental factors, it could be facilitated by the perennial life-form of the species, its reproductive modes and the efficient wind dispersal of spores (**Paper 12**).

A follow-up research on *Cystopteris fragilis* complex was built on prior knowledge of the genome size/ploidy level of the plants under study (**Paper 12**) and was focused particularly on the phylogenetic pattern among the members of the complex worldwide, based on phylogenetic analyses of plastid DNA among main cytotypes and spore character (**Paper 13**). We selected a representative set of 87 *C. fragilis* samples reflecting the ploidy level (4x, 5x, 6x) and spore type (spiny/rugose) variability of the group found in Northern Hemisphere populations for

sequencing of two plastid loci (*rbcL*, *trnG-R*). The spore character was evaluated in this study as the production of rugose instead of regular spiny spores, is sometimes associated with a potential species called *C. dickieana*. Our plastid DNA analyses revealed two haplotype lineages, which we label the hemifragilis and reevesiana clades, based on their potential relationship to the two presumed diploid parents of *C. fragilis*. The tetraploid, pentaploid and hexaploid accessions did not form monophyletic groups and they were each distributed in both major clades of the *C. fragilis* complex. Rugose spores were rarer overall (26% of samples), but five times more prevalent in the hemifragilis clade and therefore should not be associated with a single name. It is evident, that this study is limited by using only plastid DNA data (**Paper 13**) but further reveals a great genotypic and cytotypic complexity present in this taxonomic complex. The proper delimitation and understanding of *C. fragilis* still remains a great challenge.

3 Future perspectives

In many aspects, ferns as vascular spore-bearing plants have been neglected in comparison to angiosperms. Therefore, there are numerous remaining challenges that show the future directions we can take. Some of the studies presented raise questions for further research of ferns and, possibly, lycophytes. Future research will have to include both the gametophytic and the sporophytic stages. Many other stories may still be unfolding that we will no doubt hear about in the future.

Spore abortion in ferns was until now studied only in temperate leptosporangiate ferns (Quintanilla & Escudero 2006, Paper 1). Eusporangiate ferns as well as lycopods were still not tested. Within ferns, there is an extensive opportunities concerning other regions (arid, tropical) where examining spore abortion will be challenging. Furthermore, the comparison between plants of different habitats corresponding with optimal vs. sub-optimal conditions is still understudied.

Focusing on the gametophyte, its unique system of sex determination and ensuing population demographic control also deserves more interest in the future. More than seventy years after the discovery of antheridiogens by Walter Döpp (Döpp 1950) the vast majority of fern species (98%) remains unexplored. It may be expected that large, species-rich families such as Athyriaceae, Thelypteridaceae, Polypodiaceae and Cyatheaceae, with few, if any, species tested, should be the subject of future inquiries, as should eusporangiate fern lineages or even lycopods. We are now beginning to understand how antheridiogens operate on the molecular level but many questions about their distribution and evolution remain unanswered. Hopefully, the presented comprehensive dataset (Paper 5) can provide a starting point for fern researchers to learn whether their species of interest use this intriguing system of pheromonal control over sexual determination.

Further questions arise about possible gametophyte competition during their development. Of great importance possible are the understudied allelopathic influences that were discovered during the study of *Asplenium ruta-muraria* (Paper 6). The exudates of female gametophytes did not preclude the formation of archegonia among younger gametophytes or stimulate germination in darkness. However, they may have suppressive or facilitative effects on younger gametophytes of various fern species. The mechanics and properties of these effects have been

poorly explored until now, may have evolutionary significance and deserve further study in ferns.

During our study on the formation of fern polyhaploids (Paper 2), we suggested that the aposex hybrids might be of certain evolutionary potential, particularly if their polyhaploid offspring are capable of producing viable spores and crossing with sexual species. Many other interesting research topics were raised based on those data, such as the incidence of polyhaploid formation in other fern groups or ploidies, genetic variation of polyhaploid offspring, and especially the occurrence and fertility of polyhaploid descendant from tetraploid hybrids.

Despite continued study focusing on the dynamics of genome evolution and genome size diversity, we still measured the genome size of only about 5.1% of the fern species (Fujiwara et al. 2023). More FCM measurements are needed for a better overview of genome size variability particularly in the still poorly understood fern and lycophyte groups. Our research group is very active in this field. We continuously excerpt published data on genome size of ferns and lycophytes and also obtain genome sizes of still not measured taxa. We believe we will create the largest database of genome size of ferns and lycophytes used for further analyses.

Members of our research group also participate in the Fern and lycophyte genome databank and silica archive (FerDA) project coordinated by L. Eklert. We are creating the Europe's largest genebank for ferns and lycophytes via a gradual collection (and storing it as silica-dried) of fern and lycophyte material during various field excursions to many European countries. We were inspired by a similar project by our colleagues from the Duke University – Fern labs Database (<https://fernlab.biology.duke.edu/>). The silica-dried samples stored within FerDA are precisely georeferenced, documented by herbarium vouchers and usually of known genome size or ploidy level. This project is still in progress, but several thousands of samples are ready to be used for possible phylogenetic studies in the future.

4 References

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