Genetic analysis of plant root endosymbioses in the genus *Dryas* (Rosaceae)

Dissertation for the award of the doctorate in natural sciences at the Faculty of Biology of the Ludwig-Maximilians-University Munich; Genetics department

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Summary

The aim of this thesis was to analyse the symbiotic status of the genus *Dryas* and the genes determining symbiosis competence in this clade. Several plants form mutualistic associations with nitrogen-fixing bacteria and mycorrhizal fungi which increases nutrient availability to the plants. The nitrogen-fixing root nodule symbiosis (RNS) is phylogenetically restricted to four orders, the Fabales (*e.g.* Leguminosae *i.e.* Fabaceae), Fagales (*e.g.* Betulaceae, Casuarinaceae, Myricaceae), Cucurbitales (*e.g.* Coriariacreae, Datiscaceae) and Rosales (*e.g.* Elaeagnaceae, Rhamnaceae, and Rosaceae). During evolution, genes required for the arbuscular mycorrhizal (AM) symbiosis were co-opted for RNS. As result, all symbiotic hosts share a core set of orthologous genes. Interestingly all of the Rosaceae genera confirmed to contain nodulating species (*i.e. Cercocarpus*, *Chamaebatia*, *Dryas*, and *Purshia*) belong to a single subfamily, the Dryadoideae. The *Dryas* genus is particularly interesting from an evolutionary perspective because it contains closely related nodulating (*Dryas drummondii*) and non-nodulating (*Dryas octopetala*) species. The close phylogenetic relationship between these two species makes *Dryas* an ideal model genus to study the genetic basis of nodulation by whole genome comparison as well as by classical genetics techniques.

In order to make a step forward in the evolutionary studies of root symbioses in Rosaceae, I developed *Dryas* as a new model genus and completed the overview of its root symbioses. To set up and use *Dryas* as model genus, plant cultivation methods (from seed surface sterilisation to hydroponic systems), *Agrobacterium rhizogenes* transformation and DNA extractions methods were established. Through the development of genomic DNA extraction protocols, *de novo* whole genome sequencing was carried out for *D. drummondii* and *D. octopetala*. I discovered that *D. octopetala* does not form AM in the laboratory, and this result was confirmed by Prof. Didier Reinhardt on root material collected in the Swiss Alps. These results highlighted a previously unknown endosymbiosis-related polymorphic trait between *D. drummondii* and *D. octopetala*. Having access to their genomes and focusing our efforts for understanding the loss of root endosymbioses in *D. octopetala*, a targeted genomic comparison has been performed on orthologous genes known to be important for both endosymbioses.

This thesis contributes to establish *Dryas* spp. in a laboratory context and also describes for the first time an unexpected rare case of AM-symbiosis polymorphic trait within the same plant genus. Due to the closely relation between both *Dryas* spp., the whole genome sequencing of both species and the established methods, *Dryas* can now be considered as a model genus with significant value to study evolution of both root symbioses and Rosaceae.

Table of Contents

Abbreviations

ADP: Adenosine diphosphate
ALB: Aberrant localization of bacteria inside nodule
AM: Arbuscular mycorrhiza
ATP: Adenosine triphosphate
BAC: Bacterial artificial chromosome
BF: Bright field
BGI: Beijing Genomics Institute
bv.: Biovar
C: Carbon
ca.: circa ("around")
Ca: Calcium
$CaCO_3$: Calcium carbonate
CCaMK: Calcium-calmodulin-dependent kinase
cDNA: complementary DNA
CDS: Coding sequence
CERK: Chitin elicitor receptor kinase
CLE-RS: Clavata3/endosperm surrounding region root signal
CNGC: Cyclic nucleotide gated channel
CRISPR/Cas: Clustered regular interspaced short palindromic repeats / CRISPR associated
CsCl: Caesium chloride
CSG: Common symbiosis genes
CTAB: Cetyltrimethylammonium bromide
Cu: Copper
de novo: "from the beginning"
dH_2O: deionised water
DIP: Deletion/insertion polymorphism
DIP1: DELLA interacting protein 1
DMI: Does not make infection
DNA: Deoxyribonucleic acid
e.g.: *exempli gratia* ("for example")
ECM: Ectomycorrhiza
EDTA: Ethylenediaminetetraacetic acid
EF1-a : Elongation factor 1-alpha
EM: Ericoid mycorrhiza
ENOD: Early nodulin
EPR: Exopolysaccharide receptor
ERF: Ethylene response factor
ERN: ERF required for nodulation
EST: Expressed sequence tag
et al.: *et alia* ("and others")
etc.: *et cetera* ("and other similar things")
EtOH: Ethanol
EV: Empty vector
EVONOD: "Evolving nodules"
FaFaCuRo: Fabales, Fagales, Cucurbitales and Rosales
FAO: Food and agriculture organization
Fe: Iron
GA: Gibberellic acid
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
gDNA: genomic DNA
GC: Guanine + cytosine
glnII: Glutamine synthetase II
GPS: Global positioning system
GRAS: Gibberellic-acid insensitive, repressor of GA1 and SCARECROW
H: Hydrogen
H_2O: Dihydrogen monoxide (water)
H_2O_2: Hydrogen peroxide
$H_2PO_4^-$: Dihydrogen phosphate
i.e.: *id est* ("in other words")
in situ: "on site"
in vitro: "within the glass"
in vivo: "within the living"
IPD3: Interacting protein of DMI3
IPEN: International plant exchange network
IT: Infection thread
ITS: Internal transcribed spacer
JGI: Joint genome institute
K: Potassium
KH_2PO_4: Potassium dihydrogen phosphate
KNO_3: Potassium nitrate
O: Oxygen
OD_{600}: Optical density at a wavelength of 600 nm
Lb: Leghemoglobin
LCO: Lipo-chito-oligosaccharide
LRR: Leucine-rich-repeat
LysM: Lysin motif
LYK: Lysin motif domain-containing receptor-like kinases
MST: Monosaccharide transporter
Myc: Mycorrhizal (factor)
N: Nitrogen
N_2: Dinitrogen
NaCl: Sodium chloride
NaClO: Sodium hypochlorite (bleach)
NFP: Nod factor perception
NFR: Nod factor receptor
NF-Y: Nuclear factor-Y
NH_3: Ammonia
NH_4^+: Ammonium
nif: Nitrogen fixation gene
NIN: Nodule inception
NO_3^-: Nitrate
nod: Nodulation (genes and/ or factor)
NPL: Nodulation pectate lyase
NSP: Nodulation signalling pathway
NUP: Nucleoporins
P: Phosphorous
PCR: Polymerase chain reaction
PCW: Plant cell wall
P_i: Inorganic phosphate
PT4: Phosphate transporter 4
PVP: Polyvinylpyrrolidone
RAM: Reduced arbuscular mycorrhization
RH: Root hair
RIN: RNA integrity number
RLK: Receptor like kinase
RNA: Ribonucleic acid
RNAi: RNA *i*nterference
RNS: Root nodule symbiosis
RPG: Rhizobium-directed polar growth
rRNA: ribosomal RNA
s.l.: *sensu lato* ("in the broad sense")
ShRK: SYMRK homolog receptor kinase
SL: Strigolactones
SNP: Single nucleotide polymorphism
$S0_4^{2-}$: Sulphate
sp.: *species* (in singular)
spp.: *species pluralis* ("species in plural")
s.s.: *sensu stricto* ("in the strict sense")
STR: Stunted arbuscule
SYMRK: Symbiosis receptor kinase
TE: Transposable elements
TTSS: Type III secretion system
UV: Ultraviolet
WGA: Wheat germ agglutinin
Zn: Zinc

Units

n:	nano- (10^{-9})
µ:	micro- (10^{-6})
m:	milli- (10^{-3})
c:	centi- (10^{-2})
k:	kilo- (10^{3})
M:	Mega- (10^{6})
T:	Tera- (10^{12})

b(p):	base (pair)
Da:	Dalton
g:	gram
h:	hour
ha:	hectare
Hz:	Hertz
l:	litre
m:	meter
M:	molar
min.:	minute
pH:	"power of hydrogen"
sec.:	second
V:	Volt
v/v:	volume per volume
w/v:	weight per volume
x *g*:	times gravity
y(a):	year (ago)
°C:	degree Celsius
$\mu mol.m^{-2}.s^{-1}$:	Photosynthetic photon flux density (PPFD)
$:	US dollars

Species names and affiliations

Kingdom Plantae

Full scientific name	Abbreviation	Order	Common name
Agrimonia pilosa Ledeb.	*A. pilosa*	Rosales	Long ya cao
Allium schoenoprasum L.	*A. schoenoprasum*	Asparagales	Chive
Alnus glutinosa (L.) Gaertn.	*A. glutinosa*	Fagales	Common alder
Alnus rubra Bong.	*A. rubra*	Fagales	Red alder
Casuarina cunninghamiana Miq.	*C. cunninghamiana*	Fabales	River oak
Casuarina equisetifolia L., 1759	*C. equisetifolia*	Fabales	Australian pine
Casuarina glauca Sieber ex Spreng.	*C. glauca*	Fabales	Swamp oak
Ceanothus caeruleus Lag.	*C. caeruleus*	Rosales	-
Chaenomeles japonica (Thunb.) Lindl. ex Spach	*C. japonica*	Rosales	Maule's quince
Crataegus pinnatifida Bunge	*C. pinnatifida*	Rosales	Chinese hawthorn
Datisca glomerata (C.Presl) Baill.	*D. glomerata*	Cucurbitales	Durango root
Discaria trinervis (Gillies ex Hook. & Arn.) Reiche, 1897	*D. trinervis*	Rosales	Chacay
Dryas drummondii Richardson ex Hook.	*D. drummondii*	Rosales	Drummond's mountain-avens
Dryas integrifolia Vahl	*D. integrifolia*	Rosales	Entire leaf mountain-avens
Dryas octopetala L.	*D. octopetala*	Rosales	Eight-petal mountain-avens
Dryas x *lewinii* Rouleau	*D.* x *lewinii*	Rosales	-
Dryas x *sundermannii* Kellerer ex Sundermann	*D.* x *suendermannii*	Rosales	Sundermann's mountain-avens
Elaeagnus angustifolia L.	*E. angustifolia*	Rosales	Russian olive
Eriobotrya japonica (Thunb.) Lindl.	*E. japonica*	Rosales	Loquat
Exochorda racemosa (Lindl.) Rehder	*E. racemosa*	Rosales	Common pearlbush
Fragaria vesca L.	*F. vesca*	Rosales	Wild strawberry
Fragaria x *ananassa* (Weston) Duchesne ex Rozier	*F.* x *ananassa*	Rosales	Strawberry
Geum aleppicum Jacq.	*G. aleppicum*	Rosales	Common avens
Kerria japonica (L.) DC.	*K. japonica*	Rosales	Japanese kerria
Lotus corniculatus L. var. *japonicus* Regel	*L. japonicus*	Fabales	Bird's-foot trefoil
Malus domestica Borkh., nom. cons. prop.	*M. domestica*	Rosales	Apple
Malus pumila Mill., 1754, non auct.	*M. pumila*	Rosales	Paradise apple / Ping guo
Medicago truncatula Gaertn	*M. truncatula*	Fabales	Barrelclover
Muscari armeniacum Leichtlin ex Baker	*M. armeniacum*	Asparagales	Armenian grape hyacinth
Morella cerifera (L.) Small, 1903 / *Myrica cerifera* L., 1753	*M. cerifera*	Fagales	Candleberry
Myrica gale L.	*M. gale* (*M. scabies*)	Fagales	Bog-myrtle / Sweetgale
Oryza sativa L.	*O. sativa*	Poales	Rice
Oxalis corniculata L.	*O. corniculata*	Oxalidales	Creeping woodsorrel
Petunia x *hybrida* hort. ex E. Vilm., 1863	*P.* x *hybrida*	Solanales	Garden petunia
Phaseolus vulgaris L.	*P. vulgaris*	Fabales	French bean
Pisum sativum L.	*P. sativum*	Fabales	Garden pea
Populus trichocarpa Torr. & A. Gray	*P. trichocarpa*	Malpighiales	Western balsam poplar
Potentilla indica (Andrews) Th. Wolf / *Duchesnea indica* (Andrews) Teschem.	*P. indica / D. indica*	Rosales	Mock strawberry / Indian strawberry / False strawberry

Potentilla supina L.	*P. supina*	Rosales	-
Prunus armeniaca L.	*P. armeniaca*	Rosales	Apricot / Almond
Prunus avium (L.) L.	*P. avium*	Rosales	Sweet cherry
Prunus mume (Siebold) Siebold & Zucc.	*P. mume*	Rosales	Japanese apricot
Prunus persica (L.) Batsch	*P. persica*	Rosales	Peach
Prunus salicina Lindl.	*P. salicina*	Rosales	Japanese plum
Pyrus x *bretschneideri* Rehder	*P. bretschneideri*	Rosales	Chinese white pear
Pyrus communis L.	*P. communis*	Rosales	Pear
Rosa chinensis Jacq.	*R. chinensis*	Rosales	China rose
Rosa laevigata Michx.	*R. laevigata*	Rosales	Cherokee rose
Rubus plicatus Weihe & Nees	*R. plicatus*	Rosales	Blackberry
Rubus idaeus L.	*R. idaeus*	Rosales	European raspberry
Sesbania rostrata Bremek. & Oberm.	*S. rostrata*	Fabales	-
Solanum lycopersicum L.	*S. lycopersicum*	Solanales	Garden tomato
Spiraea thunbergii Siebold ex Blume	*S. thunbergii*	Rosales	-
Vicia sativa L.	*V. sativa*	Fabales	Spring vetch
Vitis vinifera L.	*V. vinifera*	Vitales	Wine grape
Zea mays L.	*Z. mays*	Poales	Maize

All the plant species mentioned in this thesis and the figure legends are listed here.

Kingdom Fungi

Full scientific name	Abbreviation	Order	Common name
Agaricus bisporus (J.E. Lange) Imbach, 1946	*A. bisporus*	Agaricales	Button mushroom
Amanita muscaria (L.) Lam. (1783)	*A. muscaria*	Agaricales	Fly agaric
Geosiphon pyriformis (Kütz.) F. Wettst. (1915)	*G. pyriformis*	Archaeosporales	-
Gigaspora margarita W.N. Becker & I.R. Hall, 1976	*G. margarita*	Diversisporales	“AM fungus”
Gigaspora rosea T.H. Nicolson & N.C. Schenck	*G. rosea*	Diversisporales	“AM fungus”
Laccaria bicolor (Maire) P.D. Orton 1960	*L. bicolor*	Agaricales	Bicoloured deceiver
Penicillium roqueforti Thom (1906)	*P. roqueforti*	Eurotiales	-
Pisolithus microcarpus	*P. microcarpus*	Boletales	-
Postia placenta (Fr.) M.J. Larsen & Lombard, 1986	*P. placenta*	Polyporales	Brown rot fungus
Rhizophagus irregularis (Blaszk., Wubet, Renker & Buscot) C. Walker & A. Schuessler	*R. irregularis*	Glomerales	“AM fungus”
Serpula lacrymans (Wulfen) P. Karst. (1884)	*S. lacrymans*	Boletales	Dry rot fungus
Tuber melanosporum Mel28	*T. melanosporum*	Pezizales	Périgord black truffle

All the fungi species mentioned in this thesis and the figure legends are listed here.

1. Introduction

1.1. Root symbioses

1.1.1. Soil nitrogen and phosphorous

Plants requires sixteen essential elements for their growth and development. Three of them are derived from the atmosphere and water: carbon (C), hydrogen (H) and oxygen (O). The remaining thirteen essential elements are supplied from soil minerals and soil organic matter (Uchida 2000). Among those elements, nitrogen (N) and phosphorous (P) play crucial roles. Indeed, N is present in many vital organic compounds such as amino acids, vitamins and nucleic acids. By this, N is needed for all of the enzymatic reactions in a plant and is a major part of the chlorophyll molecule and is therefore necessary for photosynthesis. In photosynthesis and respiration, P plays a major role in energy storage and transfer as ADP and ATP (adenosine di- and triphosphate). P is an indispensable component of nucleic acids since the backbone of DNA and RNA is composed of triphosphate nucleotides. Another area where P plays a structural role is in cellular membranes (largely made up of phospholipids). P is also utilized for metabolic processes *e.g.* phosphorylation events (Schachtman et al. 1998). One of the most prominent mechanisms for the modulation of protein activity is reversible protein phosphorylation.

The N and P availability depends on climatic and edaphic factors including soil drainage, texture, temperature, aeration and the rate of mineralization from organic matter decomposition (Masclaux-Daubresse et al. 2010). They are the two most limiting elements in terrestrial ecosystems (Maathuis 2009; Menge et al. 2012).

The preferred form in which N is taken up depends on soil conditions and plant species (Miller and Cramer 2005). In general, plants adapted to low pH and reducing soil conditions tend to take up ammonium (NH_4^+). At higher pH and in more aerobic soils, nitrate (NO_3^-) is the predominant form. Around 80% of our atmosphere consists of N. However, the extremely stable dinitrogen (N_2) is not available to plants. Both free living and symbiotic microorganisms are capable of fixing atmospheric N_2 in the form of NH_4^+ that can be directly taken up by plants or converted into NO_3^- by nitrifying bacteria. Moreover, the rock incorporated N is slowly mineralized and both NO_3^- and NH_4^+ are highly mobile in the soil due to their water-solubility. So they can easily be drained away below the root zone (Maathuis 2009; Masclaux-Daubresse et al. 2010; Menge et al. 2012).

More than 90% of soil P is normally chemically fixed and cannot be used by plants. The inorganic P (P_i) released from the labile compartment, another part of insoluble P, can be taken up by plants. However, this release of the P_i from the labile fraction to soil solution is extremely slow and therefore P deficiency is widespread. The form in which P_i is found in

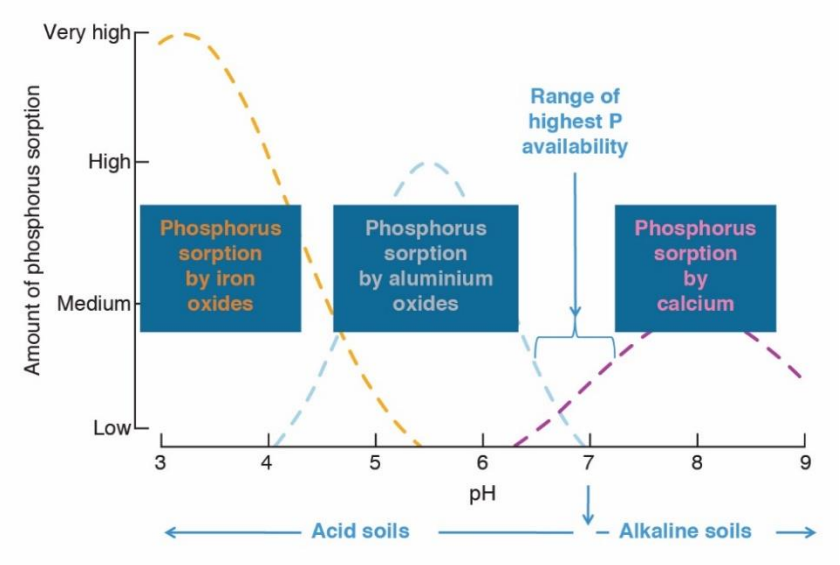


Figure 1 | Schematic representation of phosphorus status in soil.
The exact "availability of P" depends not only on pH, but also on concentrations of the sorbing minerals as well as other soil factors.
From Lambers et al. 2012

the soil solution is pH dependent. Dihydrogen phosphate ($H_2PO_4^-$), the form in which plants take up P_i, is almost exclusively the Pi-containing formulation found at typical soil solution pH.

P depletion due to prolonged weathering is the main cause of P limitation in ancient soils. Indeed, rainfall washes P out of rocks and subsequent run-off into groundwater and oceans where it is deposited as sediments on the seabed and slowly extracted back into the water (Schoumans et al. 2015). In young soils, N is widely thought to be the nutrient most limiting to plant growth. However, it is becoming increasingly clear that P limitation or N/P co-limitation of terrestrial primary production is widespread, even in young ecosystems (Wassen et al. 2005; Elser et al. 2007; Harpole et al. 2011). P limitation in young ecosystems may result from a variety of mechanisms (Vitousek et al. 2010). P can be limiting, even if total amounts of P in the soil are high, if it is poorly soluble. In particular, when it is strongly bound in complexes with aluminium, iron, calcium or allophane clay, as occurs when pH is either relatively high or low (Figure 1).

In order to overcome the soil N and P deficiency, humans have accelerated the natural circuits by an unsustainable N and P management. Over-use of N fertilizers, facilitated by the synthetic production of the Haber-Bosch process, resulted in a run-off into ground water and oceans causing water pollution and eutrophication (Howarth and Marino 2006; Howarth et al. 2006). P mining, excessive fertilizer application and effluent losses in cities caused a “terminal steady state” of P deficiency (Menge et al. 2012). Besides recycling and identifying new fertilizer production ways, researchers and farmers try to reduce the fertilizer input by breeding more effective crop varieties with reduced need for fertilizers and by finding ways to manage N and P application in a most efficient manner (Schoumans et al. 2015; Bonvin et al. 2015).

Plants have evolved a variety of mechanisms to overcome deficient nutrient concentrations in the soil. Indeed, nutrient acquisition from nutrient-impoverished soils frequently involves specialized root structures (*i.e.* cluster roots: Christmas-tree-like structures in roots with a dense packing of root hairs; these structures release carboxylates into the rhizosphere, thus solubilizing poorly available nutrients within the soil) or symbiotic structures (*e.g.* mycorrhizas, root nodules). Those last acquisition pathway called in association, with beneficial fungi or bacteria, will be detailed in the next sections.

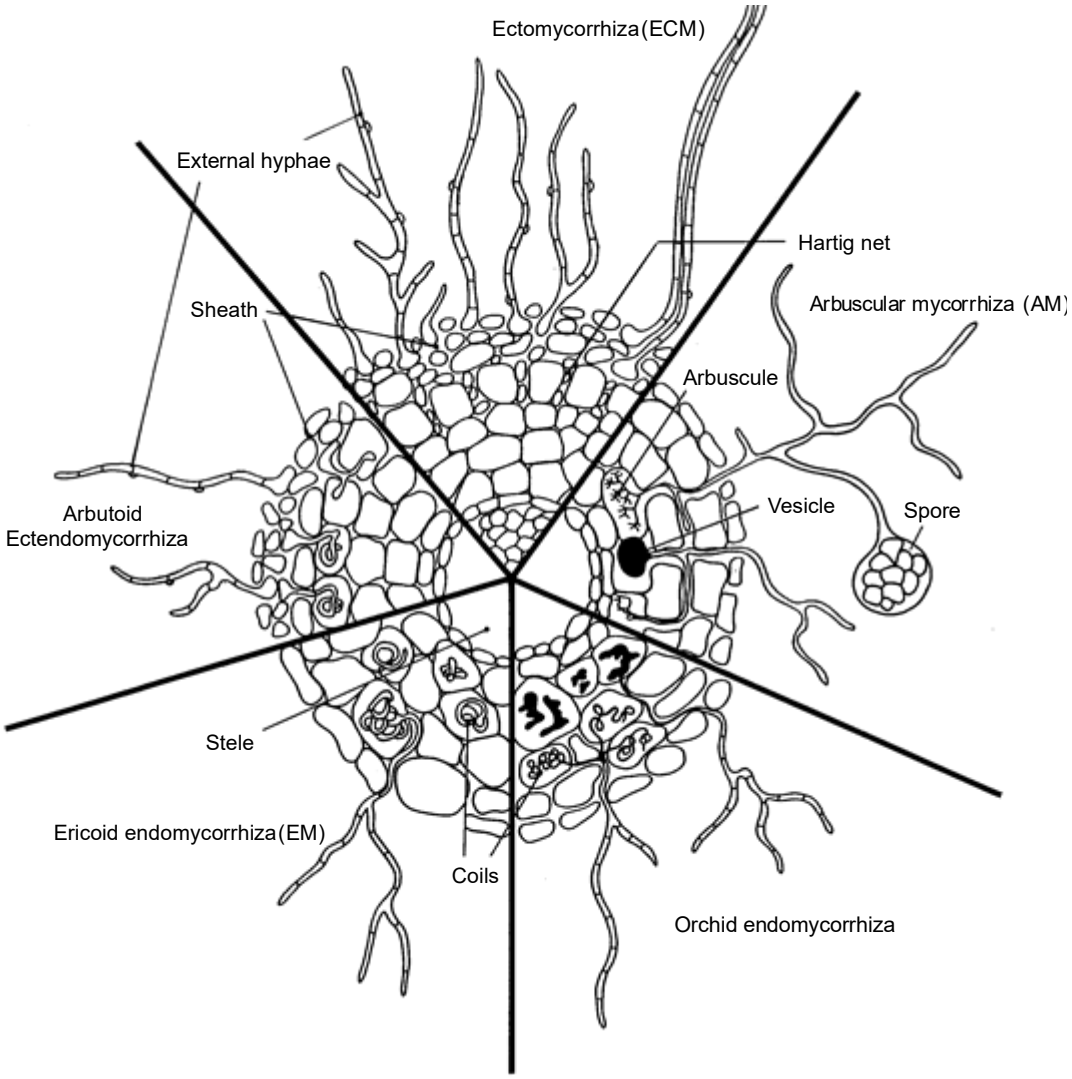


Figure 2 | Principal structural features of the five main types of mycorrhiza.
Modified from Selosse and Le Tacon 1998

1.1.2. Mycorrhizal symbioses

The mutualistic symbiotic relationship between plant roots and soil fungi, called mycorrhizal symbiosis, is one of the most prevalent associations within terrestrial ecosystems (Smith and Read 2008). Mycorrhizal symbiosis plays a key role in carbon bio sequestration, nutrient cycling, plant biodiversity and the productivity of natural and agricultural ecosystems. Indeed, mycorrhizal fungi form symbiosis with almost 90% of terrestrial plants (Wang and Qiu 2006), colonizing environments such as boreal, temperate and tropical forests as well as tundra, grasslands and many croplands (Read and Perez-Moreno 2003; Soudzilovskaia et al. 2015). In this interaction, fungal hyphae networks, specialized in the absorption of soil minerals and organic N, act as an extension of the root system, increasing plant uptake of essential nutrients such as P, N, sulphur, and water (Smith and Read 2008). Through this symbiosis, with mutualistic fungi, plant species are able to acquire metabolic capacities allowing them to use previously inaccessible ecological niches (Read and Perez-Moreno 2003). In addition, fungi protect their hosts against abiotic (metal pollution, drought) and biotic (pathogen infection) stresses (Smith and Read 2008). In return, the fungi obtain sugars derived from photosynthesis, as well as lipids (Garcia et al. 2016; Keymer and Gutjahr 2018).

Depending on the phylogenetic position of their partners and their symbiotic structures, several types of mycorrhizae are distinguished: ectendomycorrhiza, orchid, ericoid (EM), arbutoid, monotropoid and the two most common types, arbuscular mycorrhiza (AM) and ectomycorrhiza (ECM). In the ECM symbiosis, the fungal partner colonizes the intercellular spaces of the roots (apoplast), whereas during AM symbiosis, part of the hyphae develops inside the plant cells (Figure 2) (Smith and Read 2008).

Currently, more than one hundred genome sequencing projects of saprotrophic, parasitic or symbiotic fungal species have been completed or are in progress. These projects were made possible through the Broad Institute's "Fungal Genome Initiative" and Joint Genome Institute (JGI, from United States Department of Energy)’s programs. Regarding the symbiotic plant partners, several genomes has been sequenced; at least 19 tree genomes associating with ECM fungi (Neale et al. 2017). Regarding AM fungi host, several model plants could be quote such as *Medicago truncatula* (Tang et al. 2014), *Solanum lycopersicum* (Consortium et al. 2014) and *Oryza sativa* (Sakai et al. 2013).

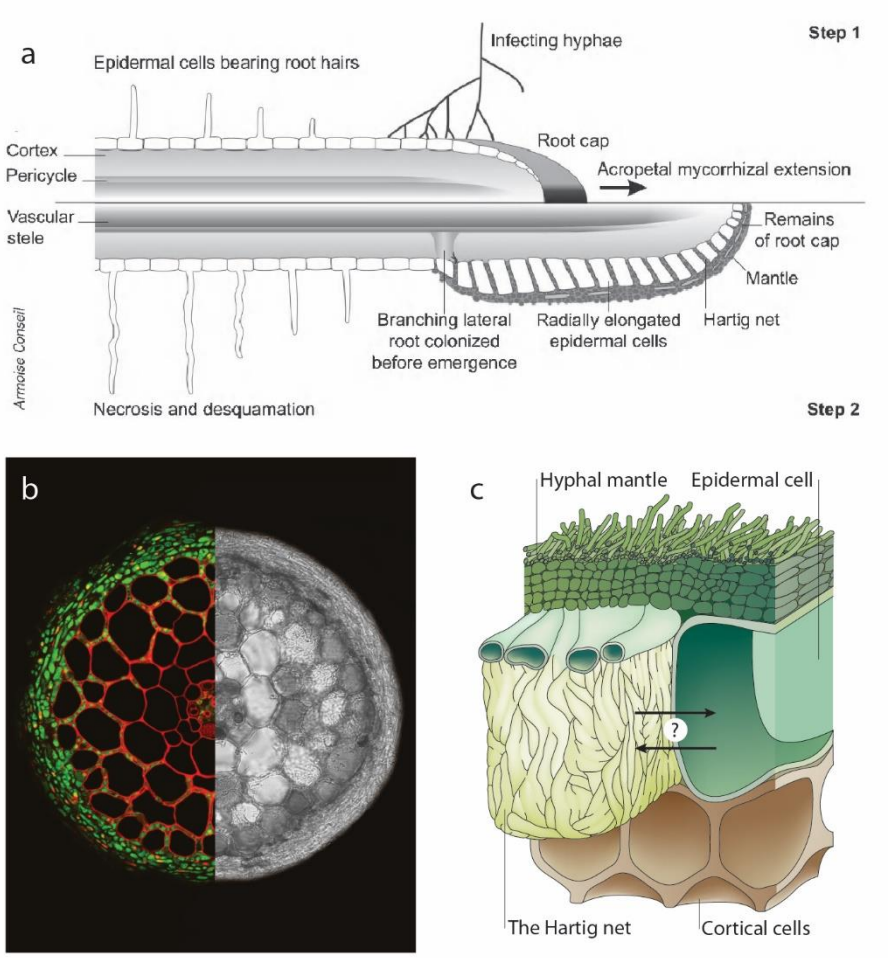


Figure 3 | Principal structural features of the ectomycorrhizal symbiosis.
(a) Dynamics of the colonization of the *Eucalyptus* root by the ectomycorrhizal fungus *Pisolithus microcarpus* during symbiosis development. The hyphae enter the root at the root cap and spread to reach the plant cells. The epidermal plant cells elongate radially and the Hartig network develops between these cells. (b) A transverse section through a rootlet of *Populus trichocarpa* that has been colonized by *Laccaria bicolor* (green). A dense mantle of fungal mycelium ensheaths the external surface of the rootlet, and intrusions of *L. bicolor* hyphae can be seen between the cell walls (red) of epidermal and cortical cells; these intrusions form the Hartig net. (c) A 3D reconstruction of the ectomycorrhizal interaction that shows the hyphal mantle covering the root surface, and the labyrinthine Hartig net.
(a) From Martin and Tunlid 2009. (b&c) From Martin et al. 2016

1.1.2.1. Ectomycorrhizal (ECM) symbiosis

1.1.2.1.1. Evolution and origin of the ECM symbiosis

ECM symbiosis evolution is quite recent. Berbee and Taylor (1993) suggest that ECM fungi first appeared *ca.* 130 million years ago (Mya). Phylogenetic and biogeographical studies support the diversification of symbioses between ECM fungi and conifers until about 50–60 Mya (Hibbett and Matheny 2009; Skrede et al. 2011); the fossil records of ECM roots date back to about 50 Mya (Lepage et al. 1997).

The ECM symbiosis mainly involves woody species representing a relatively small number of plants (about 8 000, 3% of seed plants, spread into 137 genera (Smith and Read 2008). However, these species are the dominant species of boreal, temperate, Mediterranean and some subtropical forests, so that this symbiosis can be considered as dominant in these ecosystems (Read and Perez-Moreno 2003). ECM fungi, whose number of species is estimated between 7 000 and 10 000, do not form a phylogenetically distinct group, but have appeared in Basidiomycetes and Ascomycetes; among saprotrophic, non-mycorrhizal fungi. The switch from saprotrophic to mycorrhizal nutrition modes probably happened convergently during fungal evolution, and in many independent lineages (Wang and Qiu 2006; Hibbett and Matheny 2009; Tedersoo et al. 2010; Skrede et al. 2011; Kohler et al. 2015).

1.1.2.1.2. Development of ECM symbiosis

The development of ECM symbiosis occurs through a series of well-characterized morphological events. ECM fungi are not obligate symbiote and can live independently of plant roots, as demonstrated by their growth capabilities in Petri dishes (Smith and Read 2008) and by the fact that they can live as facultative saprotrophs in soil (Martin and Nehls 2009).

Fungal hyphae emerge from spores or previously mycorrhizal roots and grow in the rhizosphere. The encounter and recognition between the two symbiotes involve the exchange of chemical signals. Only the outline of this signalling process is defined. In the vicinity of the root, the morphology of the fungi changes, especially with an increase in growth and an intense branching of the hyphae. Such a response is likely triggered by root exudates from the host, *e.g.* rutin and zeatin (Lagrange et al. 2001). This suggests that the fungus perceives the position of the host through the detection of host-derived signals. In return, the fungal hyphae, present in the rhizosphere, stimulates lateral root formation and root cell differentiation, through the action of secreted molecules, such as ethylene, auxin or hypaphorin, thereby providing a means of increasing the contact sites (Felten et al. 2009).

The fungal hyphae come into contact with the root surface, enter the region of the root cap, and propagate to living cortical cells (Figure 3 a; (Horan et al. 1988). This contact causes a change

of the fungi morphology. The apex of the hyphae swells forming a structure close to the infection structures of pathogens, appressoria. The plant also undergoes morphological changes such as radial elongation of cortical cells. The fungal hyphae then differentiate into different tissues.

The hyphae multiply and form a very dense sheath around the root, consisting of a series of thick layers, called the mantle (Figure 3 b&c). The hyphae of this structure are enclosed in a matrix of extracellular polysaccharides and proteins (Dexheimer and Pargney 1991). The main function of the mantle is the storage of nutrients.

From the mantle, the hyphae progress between the cortical cells within the apoplastic space, leading to the formation of a complex digital structure, like a labyrinthine, called the Hartig net (Figure 3 b&c) (Bonfante 2001). The hyphae of the Hartig net are coenocytic and contain numerous mitochondria, lipid bodies and a large endoplasmic reticulum, reflecting a very active metabolic status (Kottke and Oberwinkler 1987). The abundant membranes of this structure allow the exchange of nutrients and signals between plant and fungal cells. No penetration of hyphae into root cells could be observed in ECM fungi with the exception of senescent cells.

On the other hand, a network of extramatricial hyphae extends into the soil from the upper layers of the mantle to explore the soil and collect nutrients. These hyphae are sometimes organized into a root-shaped structure, called rhizomorphs. These structures are also capable of initiating primordia formation for the development of carpophores that will ensure the spread of sexually transmitted spores.

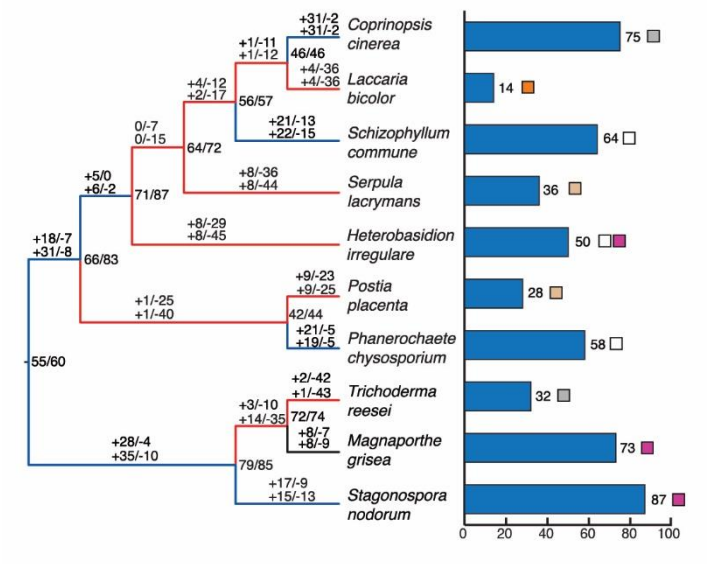


Figure 4 | Patterns of gene duplication and loss in 12 lignocellulose-active CAZy gene families in Agaricomycetes.
The histogram indicates the number of copies in each genome. Red, blue, and black branches indicate lineages with net expansions, net contractions, or no change in copy number, respectively. Numbers at nodes and along branches indicate estimated copy numbers for ancestral species and ranges of gains and losses, respectively.
From Eastwood et al. 2011

1.1.2.1.3. Genomes of ECM fungi

In the database of JGI Mycorrhizal Genomics Initiative project, sixty ECM genomes are already sequenced, including sixteen ECM species genomes which are published (Nordberg et al. 2014). Those genomes present an average of 17 580 predicted genes (with a maximum of 23 130 for *Laccaria bicolor* and a minimum of 7 496 for *Tuber melanosporum*).

The two first published genomic sequences of ECM fungi were *L. bicolor* (Martin et al. 2008a) and *T. melanosporum* (Martin et al. 2010). Their analysis identified several molecular features related to mycorrhizal symbiosis. Compared to most fungal genomes, these two fungi have relatively large genomes, 65 and 125 Mbp, respectively. This is mainly explained by a significant proliferation of transposable elements (TE) which represent approximately 20% and 60% of the *L. bicolor* and *T. melanosporum* genomes, respectively (Martin et al. 2008a; Martin et al. 2010). These TE are distributed uniformly across both genomes. By allowing genomic rearrangements, they could have played a fundamental role in the plasticity and evolution of symbiotic genomes.

The comparison of genomes of *L. bicolor* and *T. melanosporum* with those of saprotrophic and pathogenic fungi reveals massive losses of genes encoding plant cell wall (PCW) polymers degrading enzymes such as cellulose, lignin, pectin and xylan (Figure 4) (Martin et al. 2008a; Martin et al. 2010). Similarly, ECM fungi of the genus *Amanita* have also lost several genes encoding cellulases (Nagendran et al. 2009). Adaptation to the symbiotic lifestyle would therefore lead to convergent losses of enzymes acting on PCW, probably to avoid the onset of defence reactions of the plant. Interestingly, the genomes of two brown rot, *Postia placenta* (Martinez et al. 2009) and *Serpula lacrymans* (Eastwood et al. 2011) also have a reduced repertoire of cellulases acting on PCW. Recent phylogenetic analyses indicate that the ancestor of Agaricomycotina was probably a saprotrophic fungus (Hibbett and Matheny 2009). The loss of the enzymatic machinery of degradation of cellulose and lignin, from a white rot ancestor, would have led to the appearance of brown rot fungi. The loss of these enzymes would have facilitated the interaction between the saprotrophic fungi, colonizing the litter, and the roots without damaging them, giving rise to mycorrhizal symbiosis (Eastwood et al. 2011).

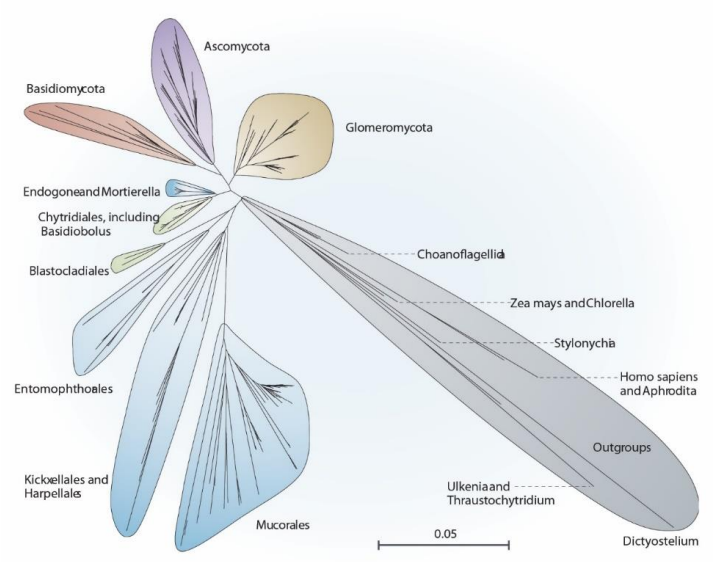


Figure 5 | Arbuscular mycorrhiza fungi form an independent phylum, the Glomeromycota.
A phylogenetic tree showing the Glomeromycota in relation to other main fungal lineages: the Ascomycota and Basidiomycota and the non-monophyletic Chytridiomycota (green) and Zygomycota (blue) All AM fungi belongs to Glomeromycota clade and excepted *Geosiphon pyriformis*, all tested members of the Glomeromycota form AM.
From Parniske 2008 modified from Schüßler et al. 2001.

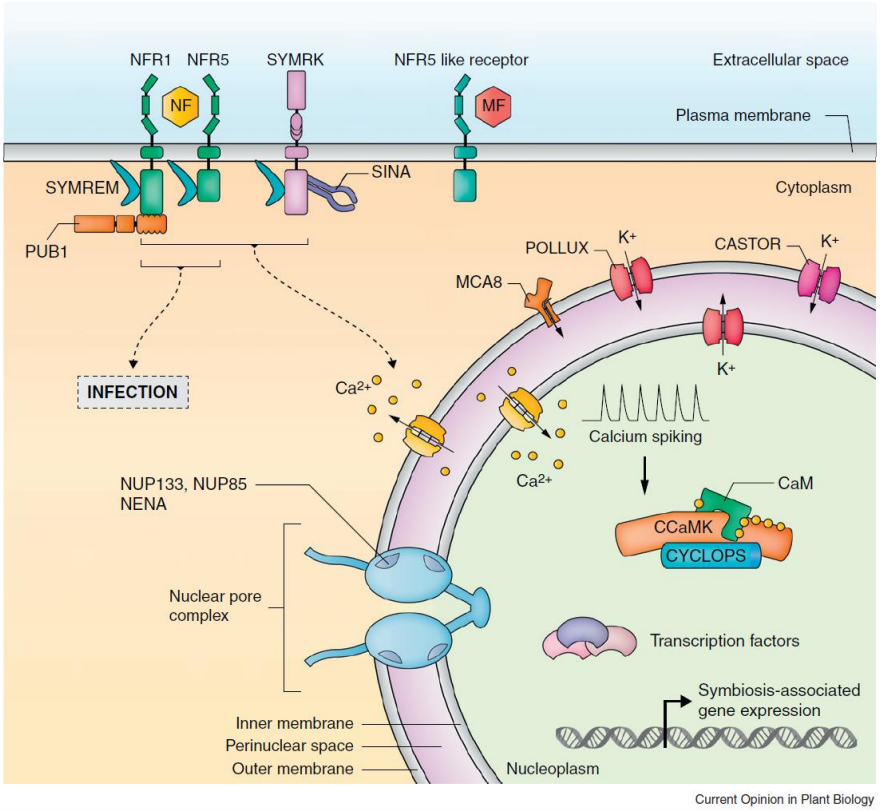


Figure 6 | Common symbiosis signalling components for arbuscular mycorrhiza and root-nodule symbiosis.
Perception of AM fungal or rhizobia-derived signals triggers early signal transduction. The symbiosis receptor kinase SYMRK acts upstream of the Nod factor and Myc factor-induced calcium signatures that occur in and around the nucleus. Perinuclear calcium spiking involves the release of calcium through calcium channels. The potassium-permeable channels CASTOR and POLLUX might compensate for the resulting charge imbalance. The nucleoporins NUP85 and NUP133 are required for calcium spiking. The calcium–calmodulin-dependent protein kinase (CCaMK) forms a complex with CYCLOPS, a phosphorylation substrate. Together with calmodulin, this complex might decode the symbiotic calcium signatures. *Lotus japonicus* protein nomenclature is used.
From Singh and Parniske 2012.

1.1.2.2. Arbuscular mycorrhizal (AM) symbiosis

1.1.2.2.1. Evolution and origin of the AM

AM symbiosis is probably the most common terrestrial symbiosis. Indeed, it is associated with more than 80% of current land plant species, mainly herbaceous species, including important crop species, such as wheat, rice, corn and soybean (Smith and Read 2008). However, there are only about 160 AM symbiosis species of fungi, all belonging to the phylum Glomeromycetes, a monophyletic clade distinct to the Ascomycetes and Basidiomycetes ones (Figure 5) (Schüßler et al. 2001). Fossil data provide proof of the existence of this symbiosis more than 400 Mya, coinciding with the appearance of the first terrestrial plants (Figure 18 (Selosse and Le Tacon 1998). As Delaux et al. (2015) have shown ancestral of land plants, algae, were preadapted for symbiosis. It has therefore been suggested that AM fungi may have played a crucial role in the colonization of the terrestrial environment by plants. This could explain the quasi-ubiquitous distribution of this AM symbiosis in the plant kingdom as well as in ecosystems.

1.1.2.2.2. Development of AM symbiosis

AM fungi are obligate biotrophs that depend on their association with plant roots to complete their life cycle. The colonization of roots follows a series of distinct stages. AM fungi exist in soil as spores. Fungal development begins with the germination of the hyphae from the spores, which explore the soil in search of a host root. In the absence of a host, growth of hyphae is limited by the amount of carbon and lipid stored in spores that feed the hyphae (Bécard and Piché 1989; Bago et al. 2000). The hyphae then stop their growth and retract their cytoplasmic mass into the spore, which returns to dormancy (Bécard et al. 2004). Successive cycles of spore germination can occur in AM fungi. Mycelium perception of the host plant is via short-range, rapidly degraded root exudates, which have been identified as strigolactones (Akiyama et al. 2005). These compounds induce a pre-symbiotic fungal reaction characterized by continuous hyphal growth, increased physiological and mitochondrial activity, and abundant hyphae branching, thereby increasing the chances of encounter with the host. In return, germinated spores produce diffusible signals, called Myc factors, such as lipochitooligosaccharides (LCOs), which are perceived by plant roots even in the absence of physical contact with the fungus (Maillet et al. 2011). These signals, which stimulate root growth and branching, activate in the plant a signalling pathway involving common symbiosis genes (CSG) to those triggered by the symbiosis between rhizobial bacteria and legume plants, Figure 6 (Kistner et al. 2005). As AM symbiosis occurred before the nitrogen-fixing rhizobial symbiosis, the mycorrhizal signal transduction pathway may have been recruited by nitrogen-fixing bacteria (Kistner and Parniske 2002).

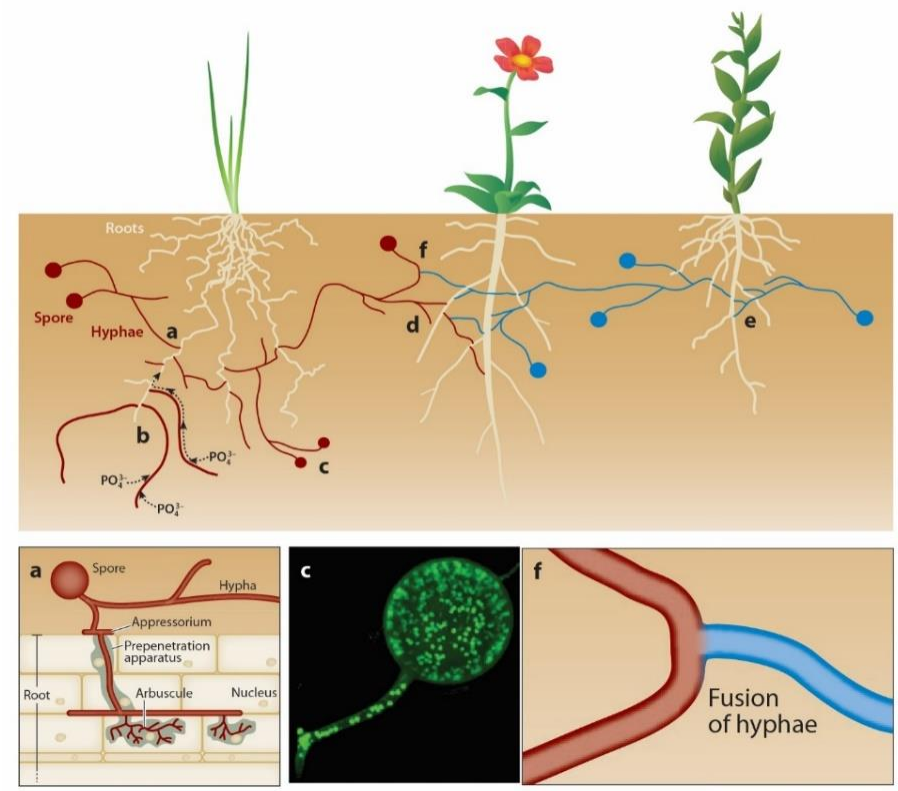


Figure 7 | The arbuscular mycorrhizal fungi lifestyle.
(a) Germinated fungal spores have to find a plant root in order to survive, but it is also possible that they could connect directly to existing fungal hyphal networks. The fungus penetrates the plant root, after forming an appressorium, then colonizes the cortex of the plant root and develops arbuscules which allow the transfer of nutrients between the two partners. (b) The fungus then produces new hyphae that grow out from the root and can absorb nutrients and transport them back to the plant. (c) Hyphae can produce new spores. Inset (c) shows a spore containing many nuclei labelled with a fluorescent dye. (d) Hyphae can continue to grow through the soil and colonize new plants, thereby creating the hyphal network. (e) Other genetically different individuals may colonize adjacent plants or even the same plant. (f) Hyphae of genetically different individuals can fuse.
From Sanders and Croll 2010.

After the first physical contact between the hyphae and the plant, the fungus forms an appressorium or hyphopodium on the surface of the root by which it colonizes the intercellular space of the root cortex (Genre et al. 2005). The plant cell forms a subcellular structure called the prepenetration apparatus that predetermines the path of hypha growth through the plant cell (Figure 7 a). It is only after the differentiation of this cytoplasmic bridge that the fungal hypha enters the host cell (Genre et al. 2005).

Then, the fungus crosses the outer cell layers, propagates longitudinally in the inner cortex and forms dichotomous branched hyphae within the cortical cells, called arbuscules (Figure 7 a). These elaborate structures remain separated from the cytoplasm of plant cells by an extension of the plasma membrane of the host, the periarbuscular membrane. This membrane follows the contours of the branches of the hyphae, leading to an increase in the surface of the plasma membrane. Arbuscules are considered to be the main nutrient exchange site between the fungus and the plant (Paszkowski 2006; Bonfante and Genre 2010). Arbuscules are ephemeral structures with an estimated lifespan of 4-5 days. The fungal structures are then degraded, and the host cell recovers its old organization. The cortical cells are then able to allow a new fungal penetration and the formation of arbuscules (Paszkowski 2006; Bonfante and Genre 2010).

The fungus also develops extra radicular mycelium that extends out of the root. This mycelium acquires the nutrients in the soil that will be transferred to the plant. The life cycle of AM fungi is supplemented by extraradical mycelial spore formation, which may enter another colonization process (Figure 7).

1.1.2.2.3. Genomes of AM fungi

Glomeromycetes are coenocytic organisms, their spores and hyphae contain hundreds of nuclei within the same cytoplasm, Figure 7 c (Jany and Pawlowska 2010). In addition, there is great genetic variability within the same spore. This variability has been observed within ribosomal DNA sequences (Sanders et al. 1995; Clapp et al. 2001; Rodriguez et al. 2004) and, later, in protein coding regions such as β-tubulin and H^+/ATPase (Corradi et al. 2004; Corradi et al. 2009). This polymorphism could come from a population of genetically different nuclei within the same spore (Kuhn et al. 2001), or from a population of genetically identical nuclei characterized by intranuclear polymorphism (Pawlowska and Taylor 2004).

Currently, only few genomic AM fungal sequences are available. The size of these genomes, generally estimated by flow cytometry, is very variable depending on the species, ranging from 15 Mbp (*Rhizophagus irregularis*) to more than 700 Mbp (*Gigaspora margarita*) per nucleus (Hijri and Sanders 2004). In 2004, *R. irregularis* was selected by JGI as candidate for the first AM genome sequencing project (Martin et al. 2004). Although a considerable amount of

sequences has been acquired, the assembly of these sequences had proved problematic. Indeed, the size of the assembled genome was several times greater than the size estimated by the amount of DNA per nucleus (Martin et al. 2008b). However, the first complete genome of *R. irregularis* was published by Tisserant et al. (2013) and improved and completed with other *R. irregularis* strains by Chen et al. (2018). Due to the lack of large-scale genomic and transcriptomic data available for the fungal partner during long time, AM symbiosis has been mainly studied from the perspective of the plant partner.

1.1.2.3. Nutrient exchange during mycorrhizal symbioses

Nutrient exchanges between the plant and the fungus are at the heart of the mycorrhizal symbiosis. Indeed, in soils of natural ecosystems, essential nutrients for plant growth are fixed in the organic layers of the soil. They are therefore only available in limited quantities for plants. This makes them ecologically dependent on their fungal partner (Smith and Read 2008). On the other hand, AM fungi, which are obligate biotrophs, are entirely dependent on the host plant for carbon. In contrast, ECM fungi are not dependent on their host for their carbon requirement. However, since simple sugars are rare in forest soils, the symbiotic way of life allows them to gain privileged access to the sugars present in large quantities in root exudates (Smith and Read 2008). Host plant growth is enhanced by mycorrhizal infection via increases in the absorbing surface area, by mobilizing sparingly available nutrient sources, or by excretion of chelating compounds or ectoenzymes. Mycorrhizal infection may also protect roots from soil pathogens (Smith and Read 2008) and thereby increase root growth and nutrient acquisition of the host root. For these beneficial effects, mycorrhizal plants allocate between 10 and 20% of the net photosynthates to the fungus for formation, maintenance and function of mycorrhizal structures (Jakobsen and Rosendahl 1990). Invasion of plant tissue by fungal mycelium and the establishment of a stable relationship result from coordinated developmental programs in both partners that cannot be reproduced in the absence of the other. Indeed, both partners must activate the transporters and the necessary anabolic and catabolic enzymes.

Since decades, by combined stable isotope labelling and RNA-sequencing approaches, several nutrient exchanges between the plant host and fungi symbiont were characterised. It was described for different mycorrhizal symbioses the capacity for the external hyphae to take up and deliver nutrients to the plant for several nutrients: such as P (AM, EM, ECM), NH_4^+ (AM, EM, ECM), $N0_3^-$ (ECM), potassium (K; AM, ECM), calcium (Ca; AM, EM, ECM), sulphate ($S0_4^{2-}$; AM), copper (Cu; AM), zinc (Zn; AM) and iron (Fe; EM). For example, it was quantified, in experimental chambers, that the AM external hyphae can deliver up to 80% of plant P (Li et al. 1991a), 25% of plant N (Ames et al. 1983), 10% of plant K (George et al. 1992), 25% of plant Zn

(Kothari et al. 1991) and 60% of plant Cu (Li et al. 1991b). In exchange plant furnish photosynthates to the fungi partner in the form of sugar and lipids (Laczko et al. 2004; Keymer and Gutjahr 2018). From both partners, several transporters were identified to play a role during symbiosis. In the transporters so far identified some are involved in sugar (*e.g.* for ECM *Am*MST1 (Nehls et al. 1998)), amino acid (*e.g.* in *Amanita muscaria* (Nehls et al. 1999)), lipids, N, P, K, $SO4^{2-}$, metal ions, Zn … (for an overview of transporter in AM see the review of Wang et al. (2017).

1.1.3. Bacteria symbiosis focus on root nodule symbioses (RNS)

During evolution, some bacteria and archaebacteria evolved in order to be able to convert N_2 into NH_3 (ammonia) by the process termed biological nitrogen fixation, these diazotrophs are either free-living or form symbiotic associations with some organisms; *e.g.* termites, protozoa (French et al. 1976; Sapountzis et al. 2016).

Some cyanobacteria can establish symbiotic associations with plants of different branches such as pteridophytes (symbiosis between *Azolla*, aquatic fern, and *Anabaena*), tropical gymnosperms of the cycad family (*Cycas*-Nostoc symbiosis) or Angiosperms (*Gunnera*-Nostoc (Rai et al. 2007)). Symbiosis with cyanobacteria does not necessarily lead to the formation of visible specialized structures, but rather to the hijack of existing organs. This is the case for the *Azolla-Anabaena* interaction where the nitrogen-fixing microorganism is housed in a leaf cavity (Lechno-Yossef and Nierzwicki-Bauer 2002). Other symbioses are characterized by the formation of specialized organs; Cycobacteria-*Cycas* interaction leads, for example, to the formation of coralloid roots in the plant partner (Costa et al. 1999; Costa and Lindblad 2002).

However, the main nitrogen-fixation symbiosis involving a microorganism and a plant host are endosymbiosis with the ability to form a new organ called nodule, which is usually on roots. In some legumes such as *Aeschynomene*, *Cassia*, *Parkinsonia* (Prin et al. 1991) and *Sesbania rostrata* (Dreyfus et al. 1988), shoot nodules can also be observed. The establishment of this organ, developed *de novo* and optional for the survival of the plant, requires a program of organogenesis induced by the presence of the symbiont (Kouchi et al. 2010; Mortier et al. 2012). The RNS is restricted to plant species in the related orders Fabales, Fagales, Cucurbitales and Rosales, abbreviated FaFaCuRo clade (Soltis et al. 1995; Werner et al. 2014; Griesmann et al. 2018). The most recent (60 Mya) and the most studied of endosymbioses is that involving the plants of the Fabaceae family (previously known as Leguminosae), as well as the genus *Parasponia* (non-leguminous) of the family Cannabaceae, with certain bacteria of the Rhizobia family (Kistner and Parniske 2002; Lafay et al. 2006).

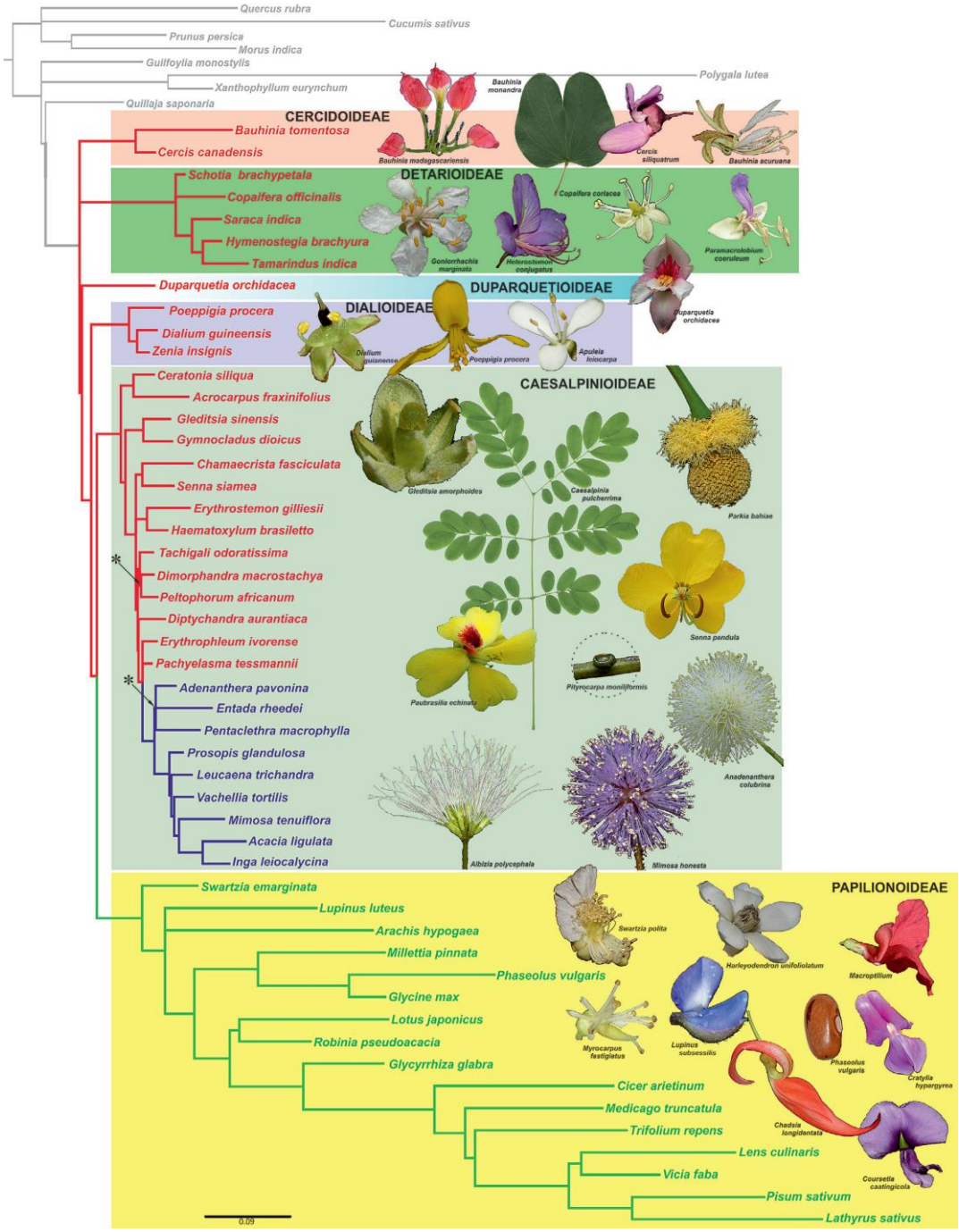


Figure 8 | Phylogeny and subfamily classification of the Leguminosae.
It is depicted on a 95% majority-rule Bayesian consensus tree based on analysis of 81 plastid peptide sequences. The six subfamilies are indicated by the coloured boxes. Coloured branches indicate the three traditionally recognised subfamilies: red the paraphyletic old-sense Caesalpinioideae, blue the Mimosoideae and green the Papilionoideae. From LPWG 2017.

Rhizobia	**Host plants**
Sinorhizobium meliloti	*Medicago spp.*
Rhizobium leguminosarum	
biovar viciae	*Pisum, Vicia*
biovar trifolii	*Trifolium*
biovar phaseoli	*Phaseolus*
Mesohizobium loti	*Lotus*
Sinorhizobium fredii	*Glycine*
Sinorhizobium fredii NGR234	*Broad scale* and *Parasponia* (non-legume)
Rhizobium tropicii	*Phaseolus, Leucaena*
Bradyrhizobium japonicum	*Glycine*
Azorhizobium caulinodans	*Sesbania*

Table 1 | Associations between *Rhizobia* and legumes.
From Dénarié et al. 1992; Sawada et al. 2003.

In addition to rhizobia, the soil contains filamentous actinobacteria of the genus *Frankia* that can infect the roots of so-called actinorhizal plants (*Casuarina*, *Alnus*, …) resulting in the formation of actinorhizal nodules (Huss-Danell 1997; Wall 2000). In the 1980s, Fessenden proposed the name "actinorhizal" for "actinorhizal nodules", by analogy with the term mycorrhizal (Prin and Duhoux 1996). Appeared about 80 Mya, actinorhizal symbioses are less studied than legume-rhizobia symbioses, but nevertheless have great ecological importance; they are seen as major contributors to overall N_2 fixation to sustain diverse forest and ecosystem settings. Moreover, this symbiotic relationship with *Frankia* shows significant differences with the rhizobial symbiosis, which fully justifies its study (Pawlowski and Bisseling 1996; Pawlowski and Sprent 2007; Franche et al. 2009).

1.1.3.1. Rhizobial symbiosis

1.1.3.1.1. The Legume plants

The legumes form a diverse and important family of Angiosperms, with more than 650 genera and 20 000 species. Legumes are the third largest family of higher plants and are second only to grasses in agricultural importance (Doyle 2001). They provide largest single source of vegetable protein in human diets and livestock feed. Legumes were traditionally divided into three sub-families: Mimosoideae, Caesalpinoideae and Papilionoideae however new phylogenomic study recognises six subfamilies in Leguminosae (Figure 8). Most cultivated legumes are found within the Papilionoideae, the sub-family with largest total number of genera. In legumes the root nodule is induced after infection with the soil bacterium *Rhizobium* or *Bradyrhizobium*. Of the three “traditionally” sub-families of legumes, over 90% of the Papilionoideae and Mimosoideae nodulate, whereas less than 30% nodulate in the paraphyletic old-sense Caesalpinioideae clade (Doyle 2001). Legumes are grown on approximately 250 Mha (Mega hectare) and they fix about 90 Tg (teragram) of N_2 per year (Kinzig and Socolow 1995). Legume productivity is theoretically independent of soil nitrogen status and they provide important grain and forage crops in both temperate and tropical zones (Cooper 2004).

Two legumes with favourable genetic attributes, namely *M. truncatula* and *Lotus japonicus*, have been selected as model species. These plants were selected to be model plants for their small genome size (between 450 and 500 Mbp) and the easiness of transformation by *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* (Boisson-Dernier et al. 2001; Chabaud et al. 2003). In the last decade the technical advances in molecular biology have allowed to construct many resources to identify the genes involved in nodulation and nitrogen fixation. For example in *M. truncatula*, the scientists constructed ESTs banks and studied the expression profile of many genes by using the microarray (Benedito et al. 2008; He et al. 2009). Moreover, different

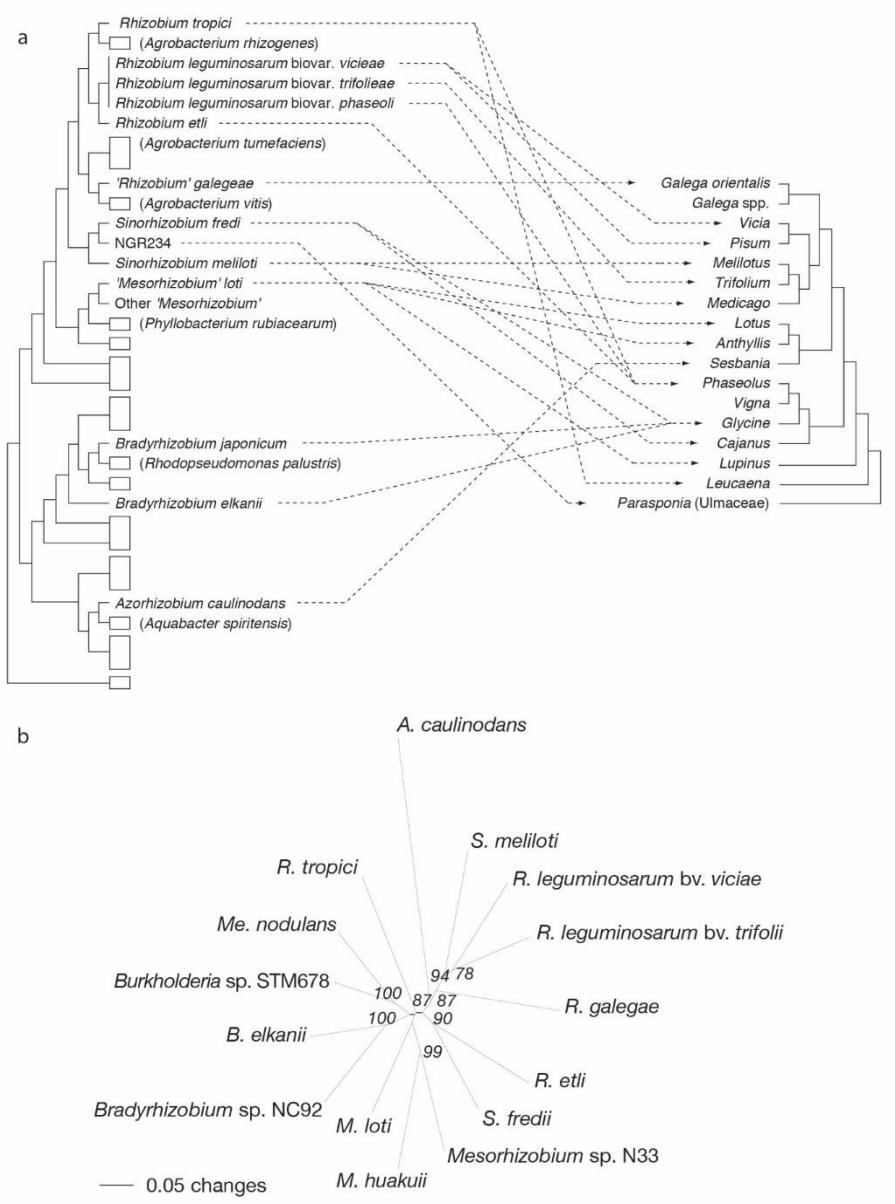


Figure 9 | Relationship between the phylogeny of *Rhizobia* and their symbiotic properties.
(a) Comparison of legume and bacterial molecular phylogenies. The bacterial phylogeny (left) is based on 16S rRNA gene sequences and shows three very distinct groups of bacteria involved in nitrogen-fixing symbiosis with legumes and *Parasponia* (Ulmaceae). Representative symbiotic bacteria are shown; lineages of non-symbiotic bacteria are shown by boxes, with a representative given in parentheses for lineages closely related to symbiotic groups. On the right, phylogenetic relationships are shown for selected legume genera nodulated by bacteria shown on the bacterial tree. Arrows connect bacterial symbionts with their plant hosts. The wide host-range of *Sinorhizobium fredii* NGR234 is shown only by indicating its ability to nodulate *Parasponia*. Although host-ranges of individual bacteria are likely to be wider than shown here, the lack of correlation between the 16S rRNA bacterial phylogeny and the rbcL phylogeny of Leguminosae is still apparent. (b) Unrooted phylogenetic showing the close phylogenetic relationship between the NodA of strain STM678 and those of a-rhizobia. The tree is based on full-length sequences and constructed by using the neighbour-joining method. Bootstrap values (% from 1,000 replications) are indicated. A, *Azorhizobium*, B., *Bradyrhizobium*. M, *Mesorhizobium*. Me; *Methylobacterium*. R, *Rhizobium*. S, *Sinorhizobium*. (a) From Doyle 1998. (b) From Moulin et al. 2001

population of mutants were generated using the chemical mutagenesis (Le Signor et al. 2009) or by using transposable elements (d'Erfurth et al. 2003; Tadege et al. 2008). In addition to all described above, the complete sequence of genome of *M. truncatula* (Tang et al. 2014) and of its symbiote (*Sinorhizobium meliloti*, (Barnett et al. 2001; Galibert et al. 2001)) gave the possibility to identify the genes implicated in symbiosis process via direct or reverse genetics tools.

1.1.3.1.2. Rhizobia

In the rhizobial symbioses, several bacterial groups induce nodules on the roots of legumes and one non-legume, *Parasponia* (Cannabaceae). For instance, among these bacteria, it could be quoted nine genera belonging to the Proteobacteria phylum and the α-sub-class (*Rhizobium*, *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Methylobacterium*, *Blastobacter*, *Devosia*) and two β-proteobacteria belonging to the genera *Burkholderia* and *Ralstonia* (Chen et al. 2001; Moulin et al. 2001; Rivas et al. 2002; Van Berkum and Eardly 2002; Vandamme et al. 2002; Young et al. 2001; Gyaneshwar et al. 2011) This symbiotic relationship between the plant host and rhizobia is specific (Table 1, Figure 9 a). For example, the legume *S. rostrata* can establish symbiosis with three groups of rhizobia but *Azorhizobium caulinodans* forms an effective symbiosis almost exclusively with *S. rostrata*. Moreover, another legume-like *Phaseolus vulgaris* is able to form symbiosis with several species of rhizobia like *Rhizobium tropicii*, *R. leguminosarum* bv. *phaseoli* and *R. etli*. At the other hand, bacteria like *R. leguminosarum* bv. *vicieae* is able to nodulate *Vicia sativa* and *Pisum sativum* (Doyle 1998). Although rhizobia have been studied for more than 100 years, symbionts have been identified for around 10% of the 720 genera of Fabaceae. It is therefore likely that new genera of rhizobia are found among the subclasses α and β of proteobacteria and perhaps even among other taxa.

Several strains are entirely sequenced, such as *Mesorhizobium loti* MAFF303099 (Kaneko et al. 2000), *Bradyrhizobium japonicum* USDA110 (Göttfert et al. 2001; Kaneko et al. 2002), *Sinorhizobium meliloti* 1021 (Barnett et al. 2001; Galibert et al. 2001), *Rhizobium leguminosarum* 3841 (Young et al. 2006), and *Bradyrhizobium* sp. BTAi1 and ORS278 (Giraud et al. 2007). For other rhizobia only "symbiotic islands" or symbiotic plasmids have been sequenced. This is the case, for example, with *Sinorhizobium fredii* NGR234 (536 kbp symbiotic plasmid (Freiberg et al. 1997)) or *Mesorhizobium loti* R7A ("symbiotic island" of 611 kbp (Sullivan et al. 2002)).

The phylogenetic analyses based on the 16S RNA made it possible to highlight a great evolutionary diversity of rhizobia. Rhizobia belong to some major groups of proteobacteria, but generally each group containing symbiotic bacteria is closer to a group without any symbiotic capacity (such as *Rhizobium* spp. close to *A. tumefaciens*) than to other groups of bacteria

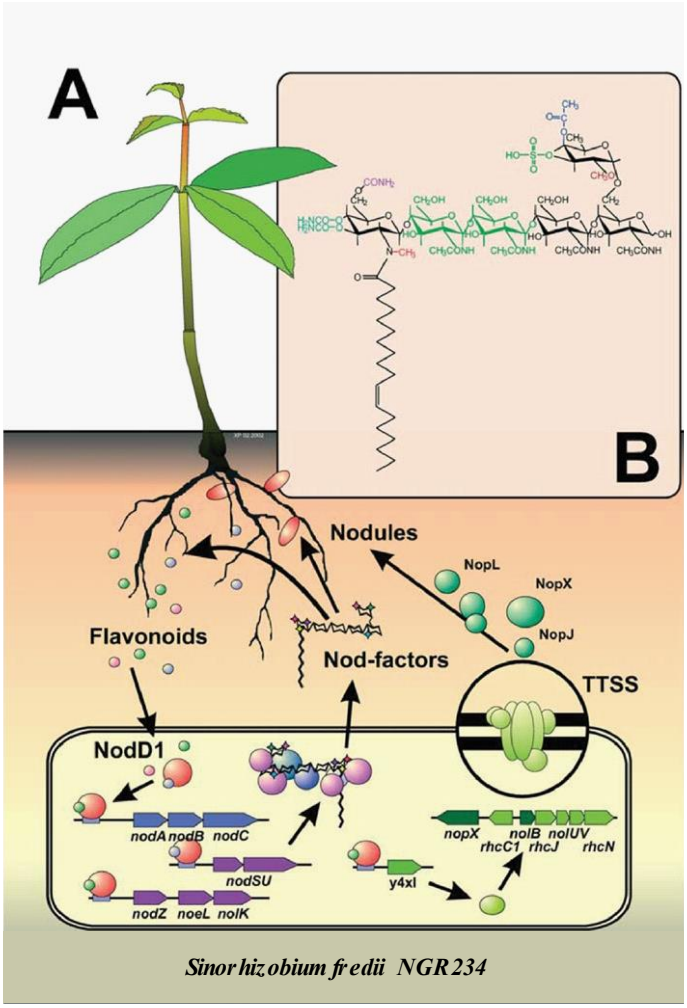


Figure 10 | Flavonoid-inducible determinants of nodulation in *Sinorhizobium fredii* NGR234.
(A) Flavonoids secreted from the roots trigger the expression of the rhizobial nodulation genes (*nod*, *nol* and *noe*) required for nodulation. Regulation of these genes is mediated by the transcriptional regulator NodD1. Most nodulation genes are involved in the synthesis of a family of nodulation signals called Nod-factors. In *S. fredii* NGR234, NodD1 also controls y4xI that regulates the expression of genes encoding components of a bacterial type III secretion system (TTSS). (B) Nod-factors are modified lipo-chito-oligosaccharides *i.e.*, *β*-1,4-linked oligomers of N-acetyl-D-glucosamine, with a fatty acid replacing the N-acetyl group on their non-reducing terminus. The Nod-factor core is synthesised by NodC (a N-acetyl-glucosaminyltransferase required for chain elongation), NodB (a deacetylase that removes the N-acetyl group at the non-reducing terminus), and NodA (an acyltransferase that links the acyl chain to the deacetylated oligosaccharide). Synthesis of *S. fredii* NGR234 Nod-factors requires a number of additional nodulation genes (e.g., *nodS* is involved in N-methylation, *nodU* in carbamoylation, and *nodZ* in fucosylation).
Modified from Broughton et al. 2003

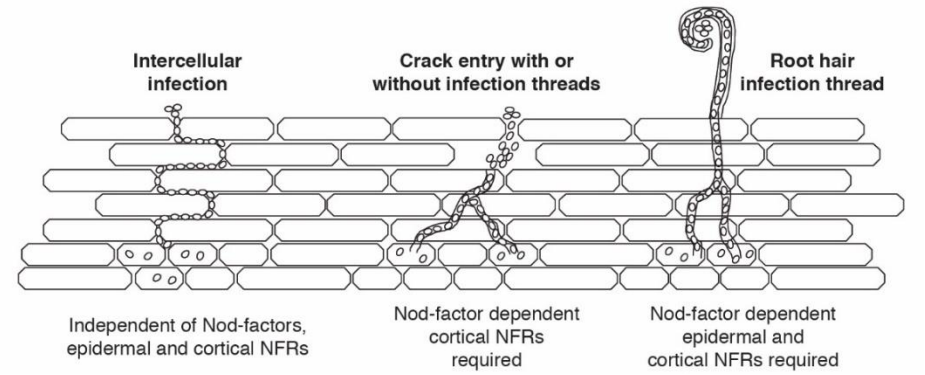


Figure 11 | Model of three kinds of infection mechanisms in legumes.
From Madsen et al. 2010

competent to nodulate (Figure 9 a). It is possible, however, that a phylogenetic analysis based on 16S RNA does not provide the right answers with respect to symbiotic origins. Indeed, many of the bacterial genes involved in nodulation are on plasmids and horizontal transfers may have played an important role in the evolution of the nodulation process. In addition, many bacterial nodulation genes are grouped into "symbiotic islands" (Doyle 1998). Thus, when the phylogenetic analyses are carried out from *nodA* gene (and no longer from 16S RNA), the distances between rhizobia are shorter (Figure 9 b), which again suggests a horizontal gene transfer (Moulin et al. 2001).

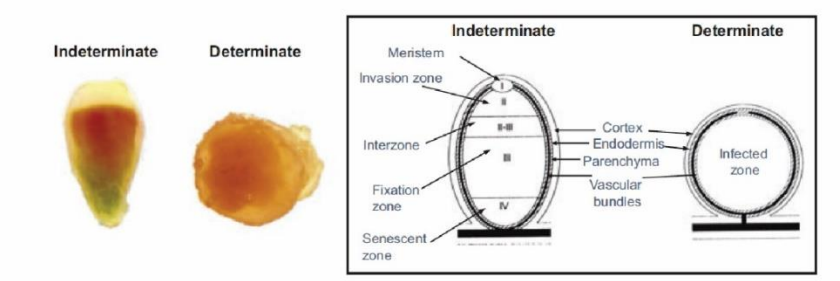
1.1.3.1.3. Nodule formation

1.1.3.1.3.1. Signal exchange

The establishment of the legume-rhizobia symbioses is initiated by the exudation of flavonoids compounds (*e.g.* genistein, naringenin, luteolin) from the host plant (Miklashevichs et al. 2001). These substances act as both chemoattractant to the rhizobia and inducers of the nod genes in the rhizobia (Figure 10). Thus, led to the bacterial synthesis of LCOs also named Nod factors (Figure 10 b). Perception of Nod factors by the host legume results in numerous responses involved in infection and nodule formation, including root hair (RH) deformation, development of preinfection threads, cortical cell divisions, and induction of nodule-specific genes (ENOD genes) expressed early in nodule development (Schultze and Kondorosi 1998; Miklashevichs et al. 2001).

1.1.3.1.3.2. Infection and nodule development

Three kinds of infection mechanisms allow symbiotic bacteria to infect their host plants (Figure 11) (Madsen et al. 2010). The best studied of these involves infection via RH and is shared by the two model legumes *M. truncatula* and *L. japonicus*, as well as most temperate legumes (Figure 11). RH infection begins with the attachment of rhizobia to growing RH and the formation of infection foci, created either by individual RH tip curling or by contact between adjacent RH. Transcellular apoplastic infection through the RH then takes place via the progressive formation of a host-derived inwardly growing tubular compartment known as the infection thread (IT) (Brewin 2004). Rhizobia enter and divide within the IT, which subsequently traverses the entire RH and outer cortex. At the same time cortical cell divisions are initiated in the inner cortex that subsequently leads to nodule formation. The second mode of infection known as crack entry is often observed in tropical legumes (Figure 11). Rhizobia penetrate through cracks or wounds, usually at the base of lateral roots and form an infection pocket resulting from localized cell death at the site of penetration. ITs are formed from these infection pockets and subsequently invade the cortex as for RH infection (Figure 11). Finally, in a limited number of cases, rhizobia penetrate



	Indeterminate	Determinate
Host plant	Alfalfa, pea, *Medicago truncatula*	Bean, soybean, *Lotus*
Geographic origin	Temperate	Tropical and subtropical
Nodule shape	Elongated	Spherical
Initial cell divisions	Inner cortex	Outer cortex
Nodule growth	Cell division. Persistent meristem	Cell expansion
Flavonoids inducing *nod* genes	Isoflavones	Flavones, flavonones
Export of assimilated nitrogen	Amides	Ureides (*Lotus* is an exception)

Figure 12 | Comparison of indeterminate and determinate nodules.
The figure shows the major structural and biochemical differences between the two types of nodules. In the photographs, note the red colour in the fixation (III) zone of the indeterminate nodule and in the infected zone of the determinate nodule, which is due to the high concentration of leghemoglobin (Lb). Also note the green colour in the senescent (IV) zone of the indeterminate nodule, indicative of Lb degradation to biliverdin-like pigments. From Matamoros et al. 2017 diagram adapted from Franssen et al. 1992

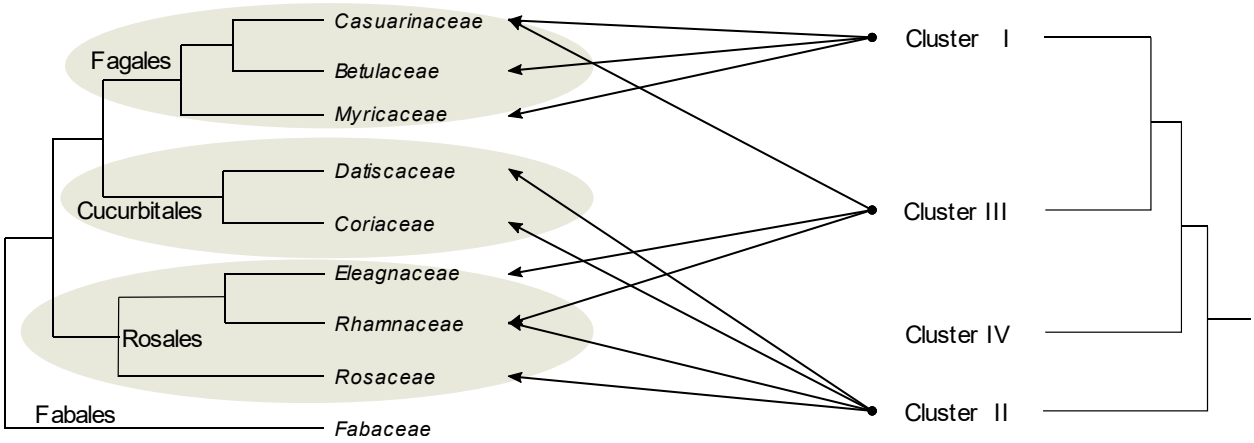


Figure 13 | Phylogenetic relationships between plant families containing actinorhizal species and the corresponding *Frankia* strains.
Strains of cluster IV are not able to induce root nodules
Adapted from Van Nguyen and Pawlowski 2017 for the phylogenomic tree of *Frankia* and Perrine-Walker et al. 2011 for the phylogenomic tree of actinorhizal plants and relationship between actinorhizal plants and groups of *Frankia* strains were based on Normand et al. 2007

through the intercellular spaces between epidermal cells and progress through the root cortex intercellularly, without forming ITs towards the nodule primordia where the bacteria are internalized (Figure 11).

1.1.3.1.3.3. Structure of legume nodules

Two types of legume nodules have been described, determinate and indeterminate (Pawlowski and Bisseling 1996) and this depends on the host plant (Trinick and Galbraith 1980). Indeterminate nodules have an apical meristem, the activity of which leads to the formation of a developmental gradient in the inner tissue. Close to the meristem, cells are infected by IT. Indeterminate nodules (Figure 12) can be unbranched (caesalpinoid type) or lobed (mucunoid and crotalarioid type (Corby 1988). In determinate nodules (Figure 12), the meristem stops its activity early in nodule development, and new infected cells mostly do not arise by infection, but by division of infected cells (Newcomb 1981b; Rolfe and Shine 1984). There are two types of determinate nodules, the desmodioid type, which occurs in the Phaseoleae and Loteae, and the aeschynomenoid type (Sprent 1995; Doyle 1998; Sprent 2001).

1.1.3.2. Actinorhizal symbiosis

1.1.3.2.1. The actinorhizal plants

With the exception of the *Datisca* genus which is a herbaceous perennial plant, actinorhizal plants are trees and shrubs distributed among three angiosperm orders (Fagales, Cucurbitales and Rosales), 8 families and 24 genera (Figure 13, Table 2), among which we find the Australian pine (*Casuarina equisetifolia*), the Russian olive (*Elaeagnus angustifolia*), the sweetgale (*Myrica scabies*) and the Alder (*Alnus* spp.) (Benson and Silvester 1993). They are capable of forming root nodules (Figure 14) as result of infection by a nitrogen fixing actinomycete called *Frankia*. In particular climatic conditions characterized by high humidity, cauline nodules can be observed at 130-150 cm above the ground in *Casuarina cunninghamiana* (Figure 14) (Prin et al. 1991).

Actinorhizal species play important roles in wild-land ecosystems and are used in land reclamation, range management, agroforestry, and horticulture. They are distributed on all continents except Antarctica. Silvester (1977) provided a map showing the geographical distribution of the different kinds of actinorhizal plants, some species of which have been introduced by man, who has introduced it for centuries into his cultural practices (horticulture and reforestation as *Casuarina* spp. and *Elaeagnus* spp.). As a result, their geo-ecological distribution is quite disparate; some species colonize temperate and tropical forests, others alpine, semi-arid (*Cercocarpus* and *Allocasuarina*) and lacustrine (*Alnus* spp. and *Myrica gale*) (Moiroud 1996; Dawson 2007; Bargali 2011).

Family	Genus	Number of species	Known nodulated	Native distribution
Betulaceae	*Alnus*	47	47	Europe, Asia, N. America, Andes Mtns.
Casuarinaceae	*Allocasuarina*	59	54	Australia
	Casuarina	18	18	Australia, Tropical Asia, S -W Pacific
	Ceuthostoma	2	2	Oceania
	Gymnostoma	18	18	Australia, New Caledonia, Sumatra
Coriariaceae	*Coriaria*	16	16	Mediterranean, Asia, New Zealand, N America
Datiscaceae	*Datisca*	2	2	Asia, N America, Europe
Elaeagnaceae	*Elaeagnus*	45	35	Europe, Asia, N America
	Hippophae	3	2	Europe, Asia
	Shepherdia	3	2	N America
Myricaceae	*Comptonia*	1	1	N America
	Myrica	60	28	All continents except Australia
Rhamnaceae	*Adolphia*	1	1	N America
	Ceanothus	55	31	N America
	Colletia	17	4	S America
	Discaria	10	5	S America, Australia, New Zealand
	Kentrothamnus	2	2	S America
	Talguenea	1	1	S America
	Trevoa	6	2	S America
Rosaceae	*Cercocarpus*	20	4	Mexico, S-W United States
	Chamaebatia	2	1	Sierra Nevada Mtns.
	Cowania	25	1	Mexico, S-W United States
	Dryas	3	1	Arctic
	Purshia	4	2	W-N America

Table 2 | Taxonomy and geographical distribution of actinorhizal plants.
From Dawson et al. 2008

Figure 14 | Examples of actinorhizal nodules.
(a) *Dryas drummondii* multilobed nodule (B. Billault-Penneteau) (b) *Discaria trinervis* multilobed nodule (From Chaia et al. 2010) (c) *Alnus rubra* nodule (R. Griffith) (d) *Casuarina equisetifolia* root nodules (e-f) Aeial nodule on trunk of *Casuarina cunninghamiana* (From Prin et al. 1991)

Pioneer species *par excellence* because of their role in the evolution of the parent rock to a true soil, actinorhizal plants grow in disturbed or low nitrogen soils: mining soils, sand dunes, arctic tundra, glacial moraines, and deposits volcanic (Moiroud 1996; Bargali 2011). Some actinorhizal plants species can even grow under a range of environmental stresses such as high salinity and heavy metal (Dawson 1990). This facility for adaptation has drawn great interest to actinorhizal plants, particularly to several species of Casuarinaceae such as *Casuarina glauca* (Figure 14), a tropical tree native of the east coast of Australia. It has been largely spread in several tropical and sub-tropical countries like China, India, Egypt and Senegal and can be used for costal area protection, agroforestry, and land reclamation in the tropics and subtropics.

Casuarina has appeared to be one of the most suitable actinorhizal plants for molecular analyses because in the past few years several molecular tools were developed including (i) genetic transformation procedures based on *A. tumefaciens* (Franche et al. 1997) and *A. rhizogenes* (Diouf et al. 1995) (ii) functional analysis of symbiotic genes based on RNA interference (Gherbi et al. 2008). Furthermore, various molecular resources are available (ESTs database, microarrays, BAC library and cDNA library (Hocher et al. 2006; Hocher et al. 2011)). Other actinorhizal plants have seen to be the object of projects that made them also model to studies *Frankia* symbiosis such as *Datisca glomerata* (Persson et al. 2011; Demina et al. 2013) and *Alnus* spp. (Périnet and Lalonde 1983; Swanson et al. 2015; Normand et al. 2018).

1.1.3.2.2. *Frankia*

The bacteria responsible for the formation of actinorhizae have been classified in the phylum actinobacteria, which represents one of the largest taxonomic units in the field of prokaryotes (Garrity et al. 2004). *Frankia* is an actinomycete genus of the family Frankiaceae, of the order Actinomycetales, suborder Frankineae, and subclass Actinobacterideae (Normand et al. 1996). It is a filamentous, branching, gram-positive actinomycete. In general, *Frankia* strains are much more promiscuous than rhizobia. So far no *Frankia* strain specific to a single host plant species has been described (Pawlowski and Sprent 2007; Perrine-Walker et al. 2011). However, host specificity is present at different levels and a broad correspondence can be defined between the phylogenies of *Frankia* strains and actinorhizal plants (Figure 13). *Frankia* has been characterized at the taxonomic level by a comparative analysis of the sequences of the *nifH* genes encoding the nitrogenase reductase, *glnII* which encodes glutamine synthetase II (Cournoyer and Lavire 1999; Hahn 2007; Nouioui et al. 2011) and the gene 16S ribosomal RNA (16S rRNA) (Normand et al. 1996). The results of these taxonomic studies have made it possible to distribute the different strains of *Frankia* in 4 different groups or "clusters" (Figure 13):

(i) Strains belonging to cluster I (*Alnus* cluster) were isolated from plants belonging to the Fagales clade and show a high level of host specificity, as they are only able to interact with plants belonging to this clade (Betulaceae, Myricaceae and Casuarinaceae). A subgroup within this cluster, the *Casuarina* strains (CcI3), appears to have evolved even higher levels of specificity as members of this subgroup are only able to nodulate two Casuarinaceae genera, *Casuarina* and *Allocasuarina* in natural conditions (Torrey and Racette 1989).

(ii) Strains belonging to cluster III (*Elaeagnus* cluster) have a broader host spectrum and can interact with plants belonging to two distant plant clades, the Rosales and the Fagales, more precisely with five plant families (Myriacaceae, Rhamnaceae, Elaeagnaceae, Gymnostoma and Casuarinaceae).

(iii) The third group of *Frankia* (cluster II or uncultured) has not yet been isolated in pure culture, but cross-inoculation experiments performed with crushed nodules also suggest a broad host range for members of this cluster that nodulate plants belonging to 4 families within the Rosales and Cucurbitales clades (Coriariaceae, Datiscaceae, Rosaceae and Rhamnaceae).

(iv) Cluster IV groups atypical *Frankia* strains (non-infective and/or non-nitrogen-fixing) isolated from the nodules of several actinorhizal plants such as *Coriaria*, *Datisca* and *Purshia*. Ramírez-Saad et al. (1998) demonstrated that root nodules of *Ceanothus caeruleus* contain, in addition to typical *Frankia*, actinobacteria that are phylogenetically linked but non-nitrogen-fixing and incapable of nodulation.

On the plant side, most actinorhizal species are nodulated by few *Frankia* strains belonging to the same cluster but a few genera like *Myrica* (Myricaceae), *Ceanothus* (Rhamnaceae) and *Gymnostoma* (Casuarinaceae) are highly promiscuous and accept a wide variety of *Frankia* strains from distinct clusters (Pawlowski and Sprent 2007).

Frankia is a heterotrophic, chemoorganotrophic, microaerophilic, mesophilic and neutrophilic microorganism (Lechevalier 1994). Unlike rhizobia (gram-negative bacteria), *Frankia* is a gram-positive, filamentous bacterium that is able to fix nitrogen freely within specialized vesicles, or in a symbiotic manner (Pawlowski and Bisseling 1996). *Frankia* isolates can be grown in a liquid medium, but very few strains are able to grow in solid media. They then form starfish-like colonies, or diffuse colonies with a loose network of hyphae, or compact colonies with hyphae growing profusely on the periphery. Due to their radial growth, bacteria of the genus *Frankia* have long been considered as fungi. *In vivo* as *in vitro*, actinomycete has three types of structures (Normand et al. 1996):

- The branched hyphae (or filaments) which constitute the vegetative form and form a mycelium.
- Diazovesicles, which are spherical cells in which the process of biological nitrogen fixation is carried out. They are differentiated in a nitrogen deficiency condition and observed in the terminal position of the hyphae. The presence of lipid layers of hopanoids around the vesicles ensures the protection of the nitrogenase complex against inactivation by oxygen (Tisa and Ensign 1987; Silvester et al. 2007).
- Multilocular sporangia are formed under conditions of depletion of the culture medium and in old nodules. They constitute a reproductive and resistance form of the microorganism (Schwintzer 1990).

The *Frankia* genome is characterized by a high content of Guanine + Cytosine (GC), more than 70% (Benson and Silvester 1993; Normand et al. 1996). Up to now, analysis of the four *Frankia* genomes sequenced have indicated a circular chromosome that ranges from 5.3 to 8.89 Mbp (Normand et al. 2007; Persson et al. 2011). A correlation between genome size, host range, and persistence in soils is suggested (Normand et al. 2007). The smaller genome comes from the cluster II *Datisca glomerata Candidatus Frankia Datiscae* (Persson et al. 2011). The *nif* genes are central to the symbiosis and their order is highly conserved among. Homologs of rhizobial *nod* genes are found only sporadically in *Frankia* spp. genomes and with low similarity to those found in rhizobia. *nodB* homologs are found in all strains and are generally annotated as polysaccharide deacetylases and present only around 35 and 45% of homology. Homologous sequences (with 25-42% of homology) were observed for *nodC* encoding N-acetylglucosamine transferase. These results suggest that *Frankia*'s signal molecules may be of a different nature from rhizobial Nod factors.

1.1.3.2.3. Nodule formation

1.1.3.2.3.1. Signal exchange

In actinorhizal plants, although the involvement of flavonoids in symbiosis is poorly understood, some evidence of chemo-attraction and proliferation of *Frankia* has also been reported in the rhizosphere of several species (Vessey et al. 2005).

As mentioned before, *Frankia* does not use a Nod-like signal molecules in early communication with their host plants. It has been previously shown that *Frankia alni* (ACN14a) produces RH deforming factor in culture supernatant that reacts with *Alnus glutinosa* RH inducing branching and curling of these cells (Cérémonie et al. 1999). This factor was shown to have a molecular weight below 3 000 Da, to be heat-stable (similar to rhizobial Nod factor) but also to be

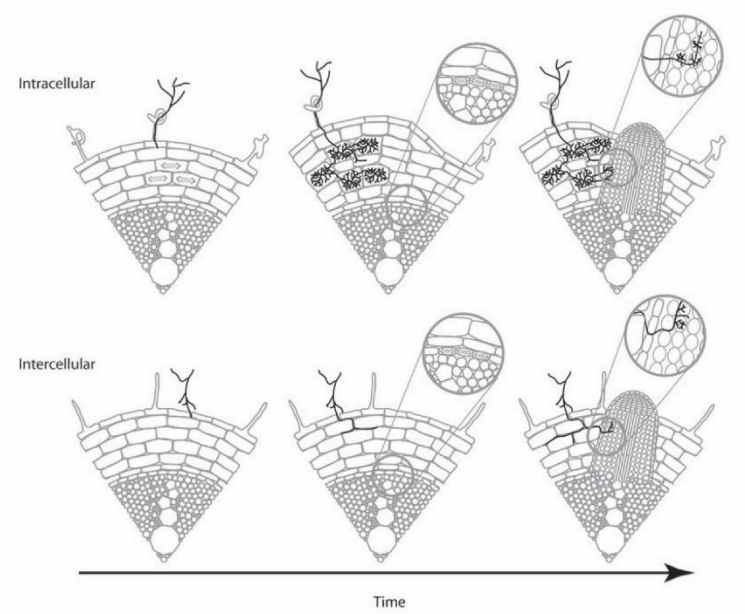


Figure 15 | Description of the two modes of infection of actinorhizal plants.
(a) During intracellular infection, root hairs become deformed in response to Frankia signals. Trapped Frankia hyphae penetrate and grow basipetally inside the root hair while being encapsulated by the host-derived membrane and a thin cell wall. Frankia remains intracellular while it progresses in the root cortex and invades first some prenodule and then nodule cells. (b) During intercellular infection, root hairs do not deform or branch, a prenodule is not formed and growth of Frankia in infected roots is through intercellular spaces. Frankia hyphae become intracellular when they invade the young nodule primordium.
From Perrine-Walker et al 2011

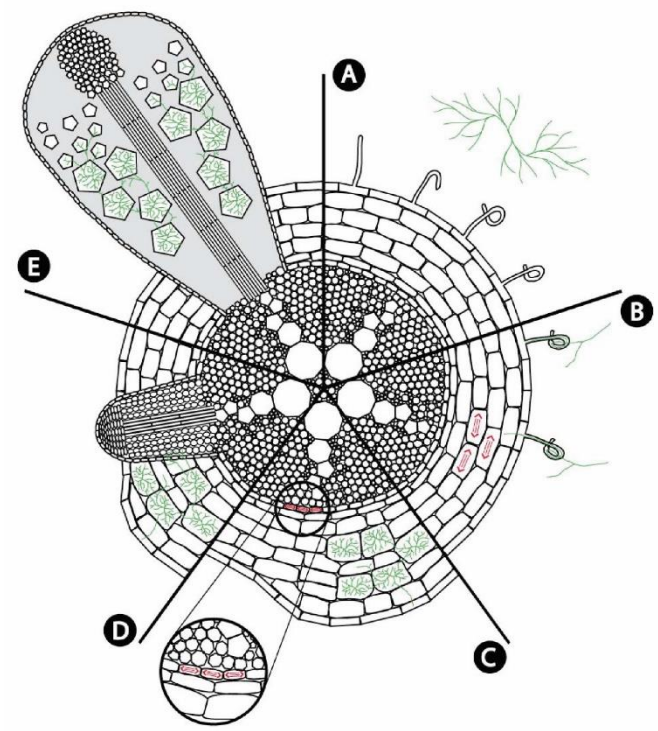


Figure 16 | *Casuarina glauca* nodule formation.
(A) Signal exchanges between the actinorhizal plant and Frankia lead to root hair infection. (B) Frankia penetrates a deformed root hair and triggers cortical cell divisions. (C) Dividing cortical cells are infected by Frankia hyphae and thus leading to the formation of a prenodule. At the same time, pericycle cell divisions occur in front of a xylem pole to form a nodule primordium. (D) Frankia hyphae coming from the prenodule invade the cortex of the nodule primordium. (E) mature nodules.
From Péret et al. 2007

hydrophilic and to resist to chitinases (contrary to rhizobial Nod factor). It has also been found that *Frankia alni* RH deforming factor was sensitive to pronase, an unpurified preparation from *Streptomyces griseus*. So far, the chemical structure of RH deforming factor is unknown.

1.1.3.2.3.2. Infection

Two types of *Franki*a infection can be found in actinorhizal plants by *Frankia*: intercellular and intracellular infection (Figure 15) and the type of infection depends on the host plant (Berry and A. Sunell 1990). The intracellular infection (Figure 15 a) (example of *Casuarina*) starts with RH curling induced by a *Frankia* signal after about 24 to 48 hours from inoculation (Figure 16). Depending on the species, all RH (*e.g. Casuarina* (Torrey 1976) or only some of them (*e.g. Comptonia* (Callaham et al. 1979) curl. *Frankia* penetrates the curled RH and infection proceeds intracellularly in the root cortex (Figure 16 b). At the same time, limited cells divisions occur in the cortex, leading to the formation of a small external protuberance called the prenodule (Figure 16 c). Most of prenodule cells are infected with *Frankia*. But actinorhizal prenodules do not evolve in nodules. Concomitant with prenodule development, mitotic activity occurs in pericycle cells opposite to a protoxylem pole, giving rise to an actinorhizal lobe primordium (Figure 16 c&d). The mature actinorhizal nodule consists of multiple lobes, each of which is a modified lateral root. A central vascular bundle is present in each lope, and *Frankia* is restricted to the cortical cells (Figure 16 e).

During intercellular infection (Figure 15 b), *Frankia* hyphae enter the root between epidermal cells, and colonize the root cortex intercellularly (Miller and Baker 1986; Racette and Torrey 1989). In contrast to rhizobia, *Frankia* does not depend on gaps in the root epidermis for entering the root. During cortex colonization , the root cortical cells secrete an electron-dense pectin and protein rich material into the intercellular spaces, and the formation of a nodule primordium is induced in the root pericycle (Liu and Berry 1991; Valverde and Wall 1999). Through an intense branching of hyphae, simultaneous with a continuous invagination of the plant plasma membrane, the primordium cells from the apoplast are infected by *Frankia* hyphae. Intercellular infection takes place in host plants of the Rhamnaceae, Elaeagnaceae and Rosaceae families. *Frankia* induce the formation of multiple lobed root nodules composed of modified lateral roots without root caps, a superficial periderm, a central vascular system (in contrast to the peripheral vasculature of legume nodules), and infected cells in the expanded cortex.

1.1.3.2.3.3. Nodule development

In both intracellular and intercellular modes of infection, nodule development starts with the induction of mitotic activity in pericycle cells as a fashion of lateral roots, opposite or at an angle of 45 degrees from the xylem pole (Torrey 1976). The number of root primordia initiated was shown to vary according to plant family: usually one or two in *Casuarina* (Torrey 1976), up to 14 in *Comptonia* (Callaham and Torrey 1977). In *C. cunninghamiana* (Torrey 1976), the root primordia traverse the cortical tissue in the midst of infected prenodule cells, whereas in *A. glutinosa* (Carmona 1974) and in *C. glauca* the root primordia develop outside the infected tissue. The meristematic cells of the primordia always remain free of *Frankia* by an unknown mechanism. While the nodule primordium develops, *Frankia* hyphae infect young cortical cells and start invading the nodule cortex acropetally.

1.1.3.2.3.4. Structure of actinorhizal nodules

In actinorhizal plants, the formation of the nodule primordia takes place in the root pericycle and the nodule consists of multiple lobes, each representing a modified lateral root without a root cap and with infected cells present in the cortex. Actinorhizal nodules conserve the structure of a lateral root with a central vascular bundle and peripheral infected cortical tissue (Bogusz et al. 1996; Pawlowski and Bisseling 1996). Four zones were characterized in actinorhizal nodules (Figure 17):

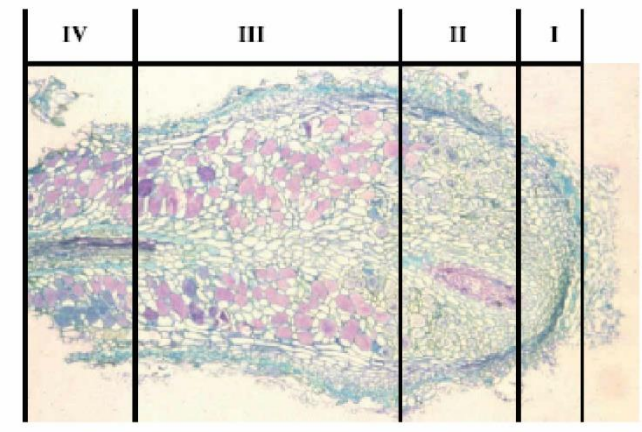


Figure 17 | *Casuarina glauca* nodule structure.
Nodule zones are indicated: I, meristem zone; II, infection zone; III, nitrogen fixation zone; IV, senescence zone
From Obertello et al. 2003

- **The meristem zone (I)** at the apex of nodule, this zone is responsible for indeterminate growth of nodule and always free from *Frankia*
- **The infection zone (II)** is adjacent to the apical meristem, the hyphae infect some of the new cells derived from meristem activity that subsequently enlarge.
- **The fixation zone (III)** is composed of infected and uninfected cells. Within this zone, infected host cells are hypertrophied. *Frankia* hyphae and vesicles are present, *Frankia nif* genes, coding for the nitrogenase complex, are expressed (Pawlowski et al. 1995), and the nitrogenase protein is detected (Huss-Danell and Bergman 1990). Therefore, in this zone, active nitrogen fixation takes place and the uninfected cells are smaller than infected cells.
- **The senescence zone (IV)** in older nodule lobes where host cytoplasm and endophyte degeneration is observed (Newcomb and Wood 1987), *nifH* expression is switched off, and nitrogenase activity is lost in legume and actinorhizal nodules (Vikman et al. 1990; Swaraj et al. 1993).

1.1.4. Brief overview of the endosymbiotic genetic

1.1.4.1. CSG: Common Symbioses Genes

Because root endosymbioses are energy-consuming processes, symbiotic establishment is tightly controlled by the plant. The development and functioning of endosymbioses (AM or RNS) rely on the orchestration of complex processes in both partners, such as induction of infection and organogenesis programmes, facilitation of the microsymbiont in planta survival in the presence of plant defence responses and increased capacity of nutrient flows. The underlying molecular mechanisms involve close coordination of the regulation of gene expression in both partners. To initiate a symbiosis, the two partners must first recognize each other. It is therefore an exchange of signals between the host plant and its future symbiont. Thus, flavonoids or strigolactones (SL) present in root exudates induce the expression of bacterial or fungal genes responsible for the synthesis and export of LCO compounds: the Nod or Myc factors respectively. These factors are then recognized by specific plant receptors. The symbiotic factor signal is then transmitted to the target tissues of the plant through a signalling pathway. This signal transmission leads to the activation of many genes allowing the establishment and control of symbiosis. These signals are perceived and integrated through an increasingly characterized signal path, which involves CSG shared between both types of root endosymbioses (Figure 6) and assumed to be co-opted from the ancestral AM by the evolutionary more recent RNS (Figure 18; (Singh and Parniske 2012; Genre and Russo 2016). The characterization of this pathway is largely based on work on the *L. japonicus* and *M. truncatula* legumes models. However, in the past few years, global

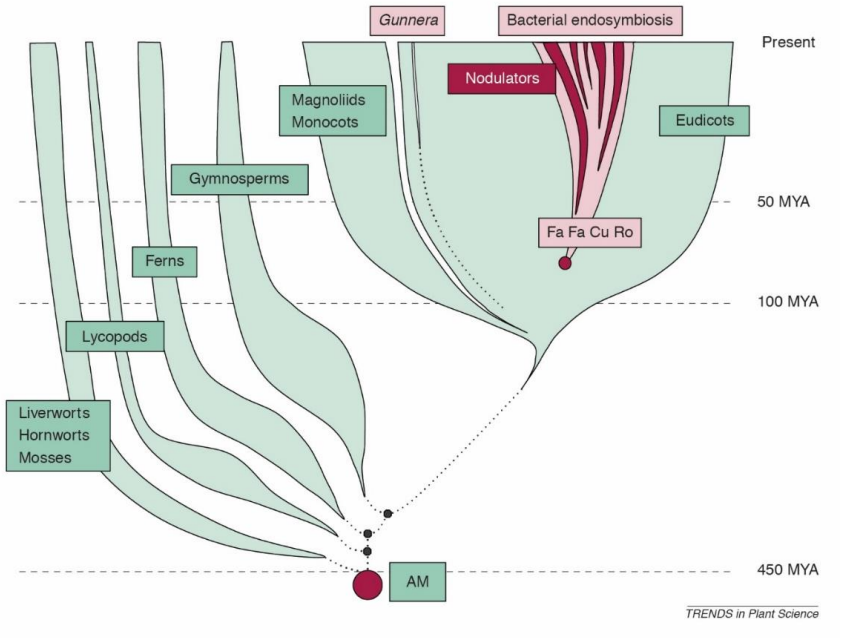


Figure 18 | Evolution of plant root endosymbioses.
Molecular clock data suggest the diversification of arbuscular mycorrhizal fungi occurred ~460 million years ago (MYA), concomitantly with the colonization of land by plants. The only known intracellular symbioses between plants and bacteria are the root nodule symbioses and the endosymbiosis formed between *Gunnera* spp. (Gunnerales) and strains of Nostoc (cyanobacteria). Root nodule symbioses are restricted to a clade within the Eurosid I, consisting of Fabales, Fagales, Cucurbitales and Rosales (Fa Fa Cu Ro). The oldest fossil nodule-like structures, possibly legume nodules, originate from the late Cretaceous (65 MYA). Nodulation has multiple origins within the Fa Fa Cu Ro clade. From Kistner and Parniske 2002

Legume symbioses			Actinorhizal symbioses	
Gene name *Lotus*/*Medicago*	Expression in nodule *vs* root	Mutant phenotype	Species	Expression in nodule *vs* root
NFR1/*LYK3*	root	nod-/inf- (for *lyk3*)	Ag, Cg*, Dt*, Dg	similar
NFR5/*NFP*	similar	nod-	Cg*, Dt*, Dg	similar
SymRK/*DMI2*	similar	nod-/myc-	Ag, Cg*, Dg*	similar
CASTOR/*POLLUX*/*DMI1*	nod	nod-/myc-	Cg/Dg	nod/nd
NUP133	root	nod-/myc-	Cg/Dg	similar/nd
CCamK/*DMI3*	nod	nod-/myc-	Ag, Cg*, Dt* / Dg	similar/nd
CYCLOPS/*IPD3*	nod	nod- (S) /myc-	Cg / Dg	similar/nd
CRE1/*HK1*	similar	nod-	Cg / Dg	root/nd
NSP1	nod	nod-/myc-	Ag / Dg	similar/nd
NSP2	nod	nod-/myc-	Dg	nd
ERN1	similar	inf-	Cg* / Dg	similar/nd
ERF1	root		Ag	similar
NIN	nod	nod- (IT, NP)-	Cg*, Dg	nod
NF-Y Complex	nod		Ag, Cg / Dg	similar/nd
SYMREM1	nod	inf-	Ag, Cg, Dg	nod
CERBERUS/*LIN*	nod	inf-/myc-	Cg / Dg	nod/similar
RIT/*NAP1*	similar	inf-	Dg	similar
PIR1	similar	inf-	Dg	nd
VAPYRIN	nod	inf-/myc-	Cg, Dg	nod
RPG	nod	inf-	Ag	nod
PUB1	nod		Cg, Dg	root
HMGR1	root		Ag, Cg / Dg	nod/nd
LATD/*NIP*	similar	nod-	Ag, Cg	similar

Table 3 | Actinorhizal putative orthologues of legume genes encoding proteins involved in Legume root nodule symbiosis.
–: defective; nod: nodule enhanced; myc–: defective in mycorrhiza; inf: infection; S: symbiosome; IT: infection thread; NP: nodule primordium; nd: not determined; Ag: *Alnus*; Cg: *Casuarina glauca*; Dg: *Datisca glomerata*; Dt: *Discaria trinervis*. *: functional characterisation available or in progress.
From Svistoonoff et al. 2014

approaches allowed the generation of huge amounts of data about the genes involved in actinorhizal symbioses and have highlighted common element shared between legume-rhizobia and actinorhizal symbioses (Table 3; (Svistoonoff et al. 2013; Hocher et al. 2019). The symbiotic signalling pathway transmits the symbiotic signal from the plasma membrane to the nucleus (Figure 6), leading to nuclear calcium oscillations, which are decoded into downstream symbiosis-associated gene expression (Singh and Parniske 2012). To trigger this pathway, Myc-LCOs released by AM fungi are thought to be perceived by specific Lysin motif domain-containing receptor-like kinases (LysM-RLKs, also named LYK), like the Nod factors which are perceived by *Mt*NFP/*Lj*NFR5 (Nod Factor Perception / Receptor) and *Mt*LYK3/*Lj*NFR1 (Amor et al. 2003; Radutoiu et al. 2007; Smit et al. 2007; Genre et al. 2013; Zhang et al. 2015). Interestingly Zhang et al. (2015) found in *M. truncatula*, *L. japonicum* and *O. sativa* that *Mt*LYK3/*Lj*NFR1/*Os*CERK1 (Chitin Elicitor Receptor Kinase) were required for AM colonization and perhaps encode proteins necessary for Myc-LCO perception. Downstream of these receptors, several other transduction pathway proteins have been characterized: *Mt*DMI2/*Lj*SYMRK (Does not Make Infection / Symbiosis Receptor Kinase) are other plasma membrane LRR (leucine-rich-repeat) receptor kinase (Endre et al. 2002; Stracke et al. 2002), *Mt*DMI1/*Lj*CASTOR/*Lj*POLLUX are components of a cationic channel located at the nuclear envelope (Ané et al. 2004; Imaizumi-Anraku et al. 2005; Peiter et al. 2007; Riely et al. 2007), NUP85, NUP133 and NENA, are three nucleoporins characterized in *L. japonicus* (Kanamori et al. 2006; Saito et al. 2007; Groth et al. 2010). All these proteins contribute to the production of a calcium signal in the form of characteristic oscillations: the calcium spiking in root cells (mainly epidermal), a typical feature of both endosymbiosis signalling pathways (Oldroyd and Downie 2006; Maillet et al. 2011; Sun et al. 2015). In addition this calcium oscillation is generated by a two-component calcium transport, one responsible for calcium import into the nucleus (*Mt*MCA8) (Capoen et al. 2011), and the other one for the calcium release comprising CNGC15 a,b&c (Cyclic Nucleotide Gated Channel; (Charpentier et al. 2016). Then come proteins that decode and participate in the transduction of this calcium signal: *Mt*DMI3/*Lj*CCaMK, a nuclear-localized calcium-calmodulin-dependent kinase (Lévy et al. 2004; Mitra et al. 2004; Tirichine et al. 2006) and the transcription factor *Mt*IPD3/*Lj*CYCLOPS that interact together in the nucleus (Messinese et al. 2007; Chen et al. 2008; Yano et al. 2008; Horváth et al. 2011). Finally, two transcription factors of the GRAS family, NSP1 and NSP2 (Nodulation Signalling Pathway), occur downstream of this pathway (Kaló et al. 2005; Smit et al. 2005). Most mutants corresponding to these genes have a strong mycorrhizal and RNS phenotype, which inhibits both root penetration and subsequent stages of intraracinaric development, including arbuscular and/or nodular formation.

1.1.4.2. Transcriptional regulation during root symbioses

The endosymbiosis pathways trigger the activation of several transcription factors which then switch on the specific genetic program leading the establishment of the symbiosis. In the past decades, via forward and reverse genetic studies as also transcriptomic analysis, important key genes were identified. Downstream of these symbiotic pathways, the calcium oscillation decoding switches on specific genes for AM or RNS symbiosis. The precise mechanisms that lead to specific responses to AM fungi and rhizobia are still under studies. This might be explained in several ways: (i) as yet unknown patterns (e.g. phosphorylation pattern of CYCLOPS) are generated within the shared common parts of both symbiosis pathways (Singh and Parniske 2012; Limpens and Bisseling 2014) and/or (ii) independent supplementary pathways provide additional specificity modules that circumvent the shared pathways or involve unknown partners (Bonfante and Requena 2011; Genre and Russo 2016; Pimprikar et al. 2016).

It was found that CCaMK and CYCLOPS induce the expression of several symbiosis related transcriptional regulators belonging to the RWP-RK domain, GRAS domain, CAAT-box (*i.e.* Nuclear Factor-Y, NF-Y) and ethylene response factor (ERF) family (Schauser et al. 1999; Kaló et al. 2005; Smit et al. 2005; Combier et al. 2006; Andriankaja et al. 2007; Marsh et al. 2007; Middleton et al. 2007; Vernié et al. 2008; Gobbato et al. 2012; Schaarschmidt et al. 2013; Soyano et al. 2013; Cerri et al. 2016; Pimprikar et al. 2016; Cerri et al. 2017). While *NIN* (*nodule inception*), encoding an RWP-RK domain containing transcription factor, is considered nodulation specific, GRAS proteins, ERFs and NF-Y act in both symbioses.

The GRAS domain proteins NSP1 and NSP2 were initially placed on the nodulation specific pathway, but refined analysis also implicates a role in AM and, under asymbiotic conditions, in the SL biosynthesis pathway (Smit et al. 2005; Kaló et al. 2005; Maillet et al. 2011; Liu et al. 2011; Lauressergues et al. 2012; Delaux et al. 2013b). Downstream of CCaMK, *nsp1* and *nsp2* mutants in addition to reduced RH deformation, are impaired in rhizobial infection and root nodule formation (Mitra et al. 2004; Kaló et al. 2005; Smit et al. 2005; Heckmann et al. 2006). NSP1 and NSP2 form together a heterocomplex which has been shown to bind promoters of Nod factor-inducible genes, such as *ENOD11*, *ERN1* and *NIN* (Hirsch et al. 2009; Cerri et al. 2012). Further, *nsp1* and *nsp2* mutants show reduced AM fungal colonization (Delaux et al. 2013b; Maillet et al. 2011). These two GRAS transcription factor have been shown to be involved in activation of SL biosynthesis genes which may explain the delayed AM colonization phenotype (Liu et al. 2011).

DELLA, another GRAS domain protein was identified as important regulator during root nodule and AM symbiotic development (Floss et al. 2013; Yu et al. 2014; Fonouni-Farde et al. 2016; Jin et al. 2016; Pimprikar et al. 2016). *della* mutants were reduced in rhizobial colonization

and *ERN1* and *ENOD11* expression (Jin et al. 2016) and severely impaired in arbuscule formation (Floss et al. 2013). DELLA proteins inhibit gibberellic acid (GA) signalling which upon treatment of plant roots represses AM development, nodulation and rhizobial infection (Floss et al. 2013; Takeda et al. 2015; Jin et al. 2016; Pimprikar et al. 2016). Arbuscule development in presence of GA and spontaneous induction of *ENOD11* expression are observed whit ectopic expression of a dominant GA insensitive version of DELLA (Floss et al. 2013; Fonouni-Farde et al. 2016).

Additionally, two mycorrhiza specific GRAS proteins involved in AM signalling are RAM1 (Reduced Arbuscular Mycorrhization) and DIP1 (DELLA Interacting Protein). Both *ram1* and *dip1* mutants are impaired in arbuscules formation or branching (Floss et al. 2013; Yu et al. 2014; Fonouni-Farde et al. 2016; Pimprikar et al. 2016). RAM1 overexpression is sufficient to activate genes critical for arbuscule development in absence of symbiont. RAM1 activates genes involved in fatty acid biosynthesis such as *STR* and *RAM2*. *STR* encodes an ABC transporter that most probably is involved in the transfer of fatty acids to the fungus. RAM2, a glycerol-3-phosphate acyltransferase, is involved in lipid metabolism (Keymer et al. 2017) and required for hyphopodia formation during the pre-infection stage (Gobbato et al. 2013; Wang et al. 2012). Furthermore, RAM1 can induce the AM-specific phosphate transporter PT4 required for phosphate uptake at the arbuscules (Harrison et al. 2002; Breuillin-Sessoms et al. 2015; Volpe et al. 2016).

NIN, a nodulation specific RWP-RK domain containing transcription factor, is rapidly upregulated in response to Nod factor in a dependent manner of CYCLOPS (Yano et al. 2008; Horváth et al. 2011). NIN plays an important role for both nodule organogenesis and rhizobial infection (Schauser et al. 1999; Fournier et al. 2015). Expression of many infection-related genes are dependent on NIN (Haney and Long 2010; Qiu et al. 2015). NIN has been shown to bind the promoter of some *NF-Y* subunit (Soyano et al. 2013) (presented below), *EPR3* (ExoPolysaccharide Receptor; (Kawaharada et al. 2017) and *NPL1* (Nodulation Pectate Lyase (Xie et al. 2012)). NPL activity is required for the localized degradation of PCW pectin during IT formation in RH. Beside its role as positive regulator of RNS, NIN creates a negative long-distance feedback loop though direct targeting of *CLE-RS1* and *CLE-RS2*, which induces expression of small root derived peptides that are perceived in the shoot to activate the autoregulation of nodulation pathway and restrict the number of nodules (Soyano et al. 2014).

The subunits of the heteromeric CAAT-box binding protein complex, NF-YA1 and NF-YB1 control different steps of nodulation, including rhizobial infection in the epidermis and the persistence of nodule meristems (Mantovani 1999; Combier et al. 2006; Laloum et al. 2013; Soyano et al. 2013; Laporte et al. 2013). They play roles during the Nod factor signalling cascade, acting downstream of NIN and upstream of ERN1 (Laloum et al. 2014).The *nf-ya1-1* mutant

displays abnormal bulbous IT causing their frequent early arrest and formation and functioning of the nodule meristem is blocked (Laporte et al. 2013). Several transcriptome analyses have revealed NF-Y subunit-encoding genes that are upregulated during mycorrhization in different plant species (Gomez et al. 2009; Czaja et al. 2012; Gaude et al. 2012). For a more complete overview of the NF-Y see Zanetti et al. (2017).

ERN1 promoter is directly targeted by NSP1, NSP2 and NF-YA1 (Hirsch et al. 2009; Laloum et al. 2014) and rhizobia-induced expression of *ERN1* in the epidermis is dependent on CYCLOPS (Cerri et al. 2017). ERN1 has a nodulation specific function and is predominantly associated with infection initiation and progression. It activates the *ENOD11* promoter via the Nod factor-box in a separate region than NSP1/NSP2 (Cerri et al. 2012; Cerri et al. 2016) and directly binds to and transactivates the *EPR3* promoter during epidermal infection (Kawaharada et al. 2017). *ern1* mutants are impaired in nodule organogenesis, while its closest homolog *ern2* mutants develop infected, but less colonized and prematurely senescent nodules (Cerri et al. 2016). In contrast, the double mutant is completely blocked in rhizobial infection and nodule organogenesis, demonstrating functional redundancy between both transcriptional regulators (Cerri et al. 2016). Interestingly, *ERN2* expression is upregulated in AM infected cells (Cerri et al. 2012).

1.1.4.3. Few words on genome-wide studies between host and nonhost species

Genes involved in AM or RNS symbiosis have been first identified by forward genetics through mutant screens. Then reverse genetics done on genes based on their expression pattern have allowed the identification of others important symbiotic genes. However, these approaches do not allow the identification of genes which could be members of functionally redundant gene families or genes that have vital function. Regarding these points and also in order to investigate more broad symbiotic evolution aspects among land plant, several phylogenomic studies were performed. Delaux et al. (2014), Favre et al. (2014) and Bravo et al. (2016) have done genome-wide comparisons on several plant species containing AM host and non-host plant (from monocot to dicot species). By postulating that non-host plants have lost orthologs of putative symbiotic genes, these genome-wide comparison studies identified numerous candidate genes (known and yet unknown) with potential roles in AM symbiosis. Only 14 genes overlap in these three lists, however the rest of the lists remains interesting data in order to identified new important AM genes. Phylogenomic analysis resulted in 166 genes from Delaux et al. (2014), 1 632 from Favre et al. (2014) and 138 from Bravo et al. (2016). Similar approaches were performed in order to investigate on the evolutionary hypothesis about a predisposition event and/or about the different gain or loss of RNS (Griesmann et al. 2018; van Velzen et al. 2018). These two studies have highlighted multiple independent loss of symbiosis and the importance of *NIN* and *RPG*

(Rhizobium-directed Polar Growth) genes in RNS evolution. These genome-wide comparisons methods are opening a new avenue for research with the potential to unravel the genetic constraints that drive the evolution of symbioses and the discovery of uncharacterized molecular mechanisms important for root symbioses.

Even through the genome-wide studies presented above, symbiotic pathways are well study in AM on model plants like *Zea mays*, *O. sativa*, *Petunia* x *hybrida*., *M. truncatula* and *L. japonicus*. While RNS studies are mainly focus on rhizobia symbiosis. Actinorhizal plants, that include plants from Fagales, Cucurbitales and Rosales, are less studied due to the fact that they are mainly woody plants with long life cycle and lack genomic data and/or established method for transformation (stable or not) for example. The actinorhizal model are more gathered in the plant family which interact with *Frankia* cluster I and III which could be cultured, in opposition of the cluster II that seems to be obligate symbionts. In this last group of plants, *Datisca* is the only herbaceous actinorhizal plant so used as model. However, the Rosaceae performing RNS are poorly studied. Among which there is the genus *Dryas* which was the genus used during this thesis and will be presented in the next sections.

1.2. *Dryas*: basal actinorhizal genus of Rosaceae

1.2.1. The Rosaceae

Among the Rosales, Rosaceae is a fairly large angiosperm family in the order Rosales, with about 3 000 species, 3 subfamilies, 16 tribes, and 88–100 genera (Figure 19) (Hummer and Janick 2009; Phipps 2014). The family has a global distribution, in particular in Northern Hemisphere temperate forests. The Rosaceae encompasse many well-known and economically important species, such as edible fruits from temperate zones (Janick 2005) as well as ornamental plants, but also some forestry and medicinal or neutriceutical cultures. Indeed, it has many species of fruit trees and shrubs such as apple (*Malus domestica*), pear (*Pyrus communis*), or raspberry (*Rubus idaeus*), as well as perennial fruit species, strawberry (*Fragaria* spp.), ornamental species such as rose (*Rosa* spp.), photinia (*Photinia* spp.), Maule's quince (*Chaenomeles japonica*), or cinquefoil (*Potentilla* spp.) or forest species, cherry (*Prunus avium*) (Figure 19). Based on FAO statistics, the total world production of the edible rosaceous fruits in 2005 was about 113 million tonnes, with an estimation of $45 billion. Rosaceous plants would have been worth at least $180 billion in 2005 when the global value of almonds, cut roses, rose plants, and other products is added. In 2012, 48% of all the fruit and nut crops produced in the USA were rosaceous plants and their production value exceeded $12.56 billion (Jung et al. 2013).

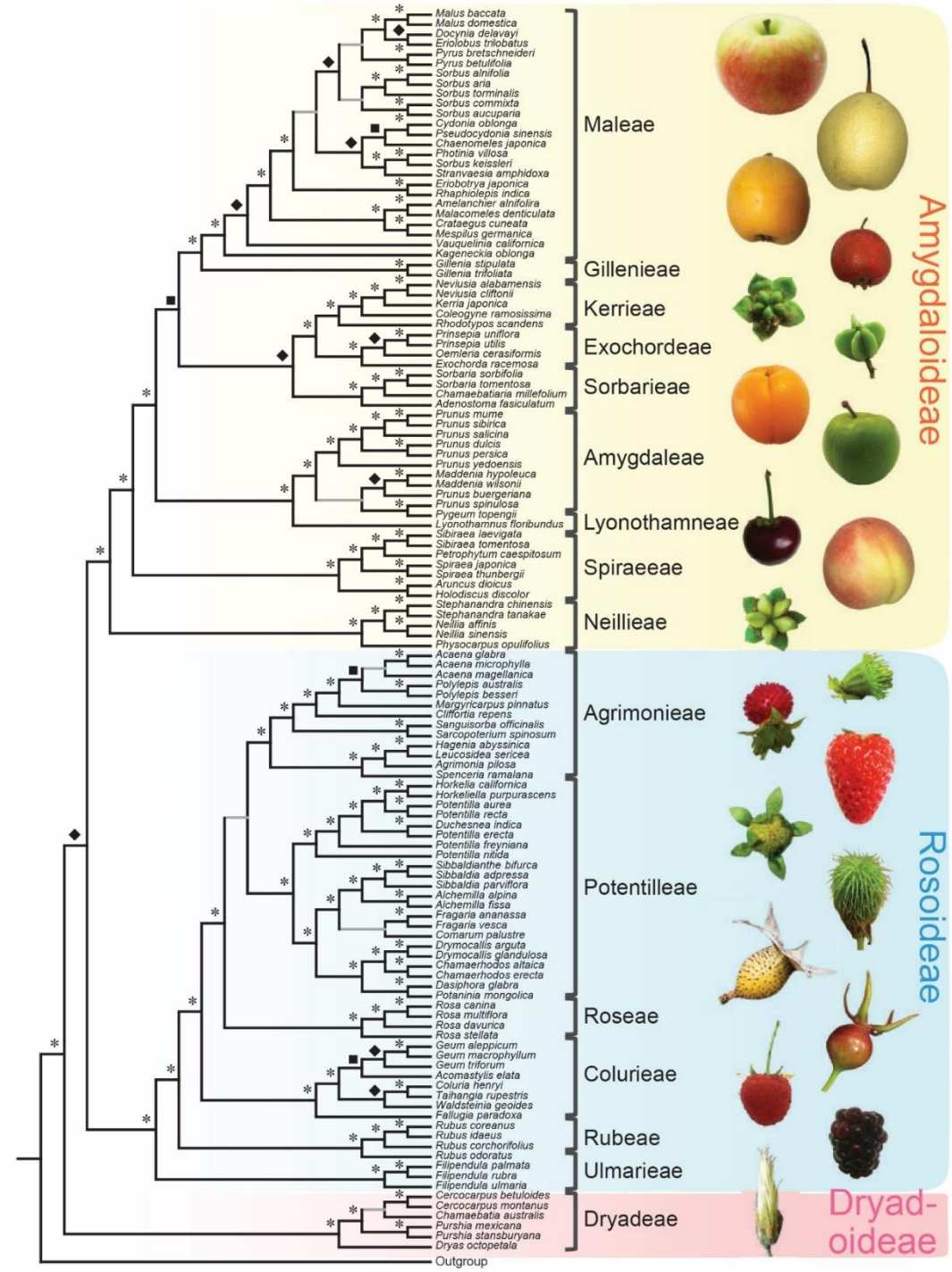


Figure 19 | A summary of Rosaceae phylogeny and Rosaceae fruit morphologies.
On the left is a summary tree with results from five coalescence analyses of 882, 571, 444, 256, and 113 gene sets, respectively, and a concatenation analysis using the 113-gene supermatrix. Topologies consistent in all six trees are drawn in black lines. Grey lines show uncertain relationships, with some trees support the topology. Asterisks (*) indicate 100% supports in all six trees. Diamonds indicate more than 90% supports in at least five trees and more than 85% supports in all six trees. Squares indicate more than 80% supports in at least three trees and more than 40% supports in all six trees. Plant photographs on the right show the diversity of Rosaceae fruits. The left row (from the top) includes *Malus pumila* (apple), *Eriobotrya japonica* (loquat), *Kerria japonica*, *Prunus armeniaca* (almond), *Prunus* sp. (cherry), *Spiraea thunbergii*, *Duchesnea indica*, *Potentilla supina*, *Rosa laevigata*, *Rubus* sp. (raspberry), and *Dryas octopetala*. The right row (from the top) includes *Pyrus bretschneideri* (pear), *Crataegus pinnatifida*, *Exochorda racemosa*, *Prunus salicina* (plum), *Prunus persica* (peach), *Agrimonia pilosa*, *Fragaria* x *ananassa* (strawberry), *Geum aleppicum*, *Rosa* sp., and *Rubus fruticosus* (blackberry).
From Xiang et al. 2016

Rosaceae are characterized by alternate leaves, which vary from simple to trifoliate, palmate, or pinnate. As secondary recognition criterion for Rosaceae, their whole leaves or smaller leaflets have often a more or less oval aspect with serrated edges. Flowers are typically composed of 5 (rarely 3 to 10) separate sepals and a comparable amount of petals. Stamens are at least five, but often much more, usually in multiples of five. Many Rosaceae flowers, especially those of the Rosoideae subfamily, have several to many simple pistils, or a single one composed with numerous styles resulting from the union of the pistils at their base, with the separate styles. Either way, the result is a distinctive, fuzzy-looking centre surrounded by lots of stamens. Flowers are almost always perfect with radial symmetry. Many very distinctive kinds of fruit are produced by Rosaceae species (Figure 19) (Potter et al. 2007; Phipps 2014). Indeed, they can be fleshy fruits (*e.g.* apple or pear which have a relatively soft core and several seeds), drupes (with a single seed in a hard central shell: peach, plum, cherry), and dry achenes (with a single seed surrounded by a thin wall: *Dryas* Figure 23 a, *Cercocarpus*). Furthermore, some species produce aggregate fruits such as drupetum (a group of tiny drupelets loosely attached to a central structure, as in raspberry), achenetum (multiple achenes from a single flower), sometimes with a fleshy enlarged receptacle (strawberry) or an enveloping hypanthium (fused lower portions of the sepals, petals and stamens, as in rose), and follicetum (several pod-like structures each with one or more seeds, from a single flower).

Previously, largely according to fruit and other morphological characteristics, four main subfamilies in the Rosaceae family used to be considered: Rosoideae *s.l.* (with aggregate fruits, rose and strawberry), Maloideae (with over 30 genera including apple and pear), Prunoideae (or Amygdaloideae *s.s.*, with drupes: cherry, plum, peach), and Spiraeaoideae (Potter et al. 2002). However, recent molecular studies have suggested three subfamilies in this family, with two large ones, Rosoideae *s.s.* and Amygdaloideae *s.l.*, having 2 000 and 1 000 species, respectively, and a small one, Dryadoideae, with fewer than 30 species (Morgan et al. 1994; Potter et al. 2007; Chin et al. 2014; Phipps 2014; Xiang et al. 2016). The last evolutionary trees trace the ancestor of Rosaceae separated from other families as far back as 120 Mya. Around the boundary between the Early and Late Cretaceous, almost 20 My after the beginning of the Rosaceae family, the Dryadoideae shrub subfamily split from the other rosaceous plants, immediately followed by a divergence of the two largest subfamilies (Xiang et al. 2016). The number of chromosomes is variable: $x = 7$ for the Rosoideae subfamily, 9 for the Dryadoideae subfamily, and for the Spiraeoideae subfamily, it is variable according to the genus (8 for the genus *Prunus*, 9 for the genera *Kerria* and *Spiraea*, 17 for the genera *Malus* and *Pyrus* (Xiang et al. 2016). Importantly, the species richness of Rosaceae could be partly related to polyploidization and species radiation in the family history, with evidence for polyploidy events in the two larger subfamilies (Talent

and Dickinson 2005; Dickinson et al. 2007; Lo et al. 2010; Considine et al. 2012; Fougère-Danezan et al. 2014).

In recent years there has been a general interest to sequence different genomes of the botanical family Rosaceae. On the Genome Database of Rosaceae website, actually there are 14 species genomes sequenced representing 7 genera. Among those genomes we can quote apple (Velasco et al. 2010), strawberry (Shulaev et al. 2011) and peach (Verde et al. 2013). Each of these species is a specific genome model for the subfamilies Malaideae, Rosoideae and Amygdeloideae, respectively, which allows more precise understanding of the biochemical and physiological processes which occur in each of these taxa (Shulaev et al. 2008) and provide an excellent system to conduct comparative and evolutionary studies of fruits and Rosaceae family. Unfortunately, there is no reference genomes for the most basal clade of the Rosaceae, indeed no Dryadoideae species were sequenced until the beginning of this thesis.

1.2.2. *Dryas drummondii* and *Dryas octopetala* description

Dryas were shown to be the most basal genus of the Rosaceae (Potter et al. 2007; Xiang et al. 2016) (Figure 19), with a separation of the Dryadoideae clade to the two other Rosaceae subfamily estimated about 101.6 Mya (Xiang et al. 2016). In terms of biomass, the genus *Dryas* is among the most important of all artic plant genera, together with *Carex* (sedges) and *Salix* (willows) (Grau et al. 2014). It is also an important constituent of the alpine heats of calcareous temperate mountains in Europe, in Asia south to Japan and in western North America (Hultén and Fries 1986).

During glaciations, continuous distribution areas were fragmented into isolated subunits in which plants potentially evolved apart. After glaciation, formerly isolated populations dispersed and often met again (McGraw 1995). By this secondary contact, hybridization often occurred followed by polyploidization or introgression (Stebbins 1984). These processes have been important for the development of the arctic flora (Murray 1997). the *Dryas octopetala–integrifolia* complex (Elkington 1965) is one of the known example of hybridization at the diploid level without polyploidization in Greenland. Due to the isolation during glaciations with the potential hybridization of the closely related species, *Dryas* taxonomy is controversial, with estimates of species/subspecies numbers ranging from 3 to 23 (Hultén 1959; Elkington 1965; Hultén and Fries 1986; Yurtsev 1997; Elven et al. 2003). The three main recognized species are: *D. drummondii*, *D. integrifolia* and *D. octopetala*.

In this thesis, we focused on *D. drummondii* and *D. octopetala* for their polymorphic RNS trait, and omitted *D. integrifolia* because of its high similarity with *D. octopetala* (Skrede et al. 2006), the latter being more accessible and better researched.

1.2.2.1. *Dryas* natural habitat

Dryas are pioneer plants, found all over the Eurasian and American arctic tundra, north to the border of the 'Polar Desert' and it is also an important constituent of the alpine heaths of temperate mountains in Europe, in Asia south to Japan, Altai and Caucasus, and in western North America south to southern Rocky Mountains. *D. octopetala* has nearly circumpolar arctic and alpine range, it predominantly grows in Eurasia but also extends across Beringia and into the Rocky Mountains (Hultén and Fries 1986) (Figure 20 b). The species is absent from the Canadian Arctic and western Greenland. Whereas, the range of *D. drummondii* is limited to gravelly riverbeds at scattered sites below the tree line in North America (Figure 20 a).

Artic and alpine tundra are characterized by extreme conditions which are challenging for plant growth due to factors such as low moisture, low mean temperature, UV stress, extreme variation in radiation and seasonality, during the growing season. These conditions help to create an ecosystem with low nutrient supply, low average biomass productivity and impose strong selection pressures on plants. *Dryas* follow the retreat of the glaciers. They stay predominant in the landscape until the development of bigger shrubs or trees. Whether in Alpine or in arctic biome, this genus present a predilection for calcareous soil with few organic matter (less than 10 cm of depth (Jorgenson et al. 2009) and for alkaline soil. *Dryas* are present in soil having a pH range from 5.5 to 8 (Jorgenson et al. 2009), however they are growing most luxuriantly within a pH range of 7.8-8.5 (Lawrence et al. 1967). Chemical changes under *Dryas* vegetation are more marked than physical changes. These include increasing soil acidity, organic carbon, nitrogen and calcium carbonate. For example, at Glacier Bay in Southeast Alaska, *D. drummondii* grow on lands which are proportionately quite high in limestones and marbles, giving a pH at 8.0-8.4, for the fine soil fraction. The degree of acidification is slight, reaching 7.7 after 20 years, under a *Dryas* mat. Acidification is accompanied by a moderate leaching of calcium carbonate ($CaCO_3$). Indeed under *Dryas*, the proportion of $CaCO_3$ in the five first soil centimetres is around 3.5% compared to the 8% on area bare of ice for 20 years (Lawrence et al. 1967). And based on data of Crocker and Major (1955), there is in 25 years an increase of 1 030 $kg.ha^{-1}$ of organic carbon in the top five centimetres of soil, compared to initially, under *Dryas* (and so without taking count of the *Dryas* mat itself).

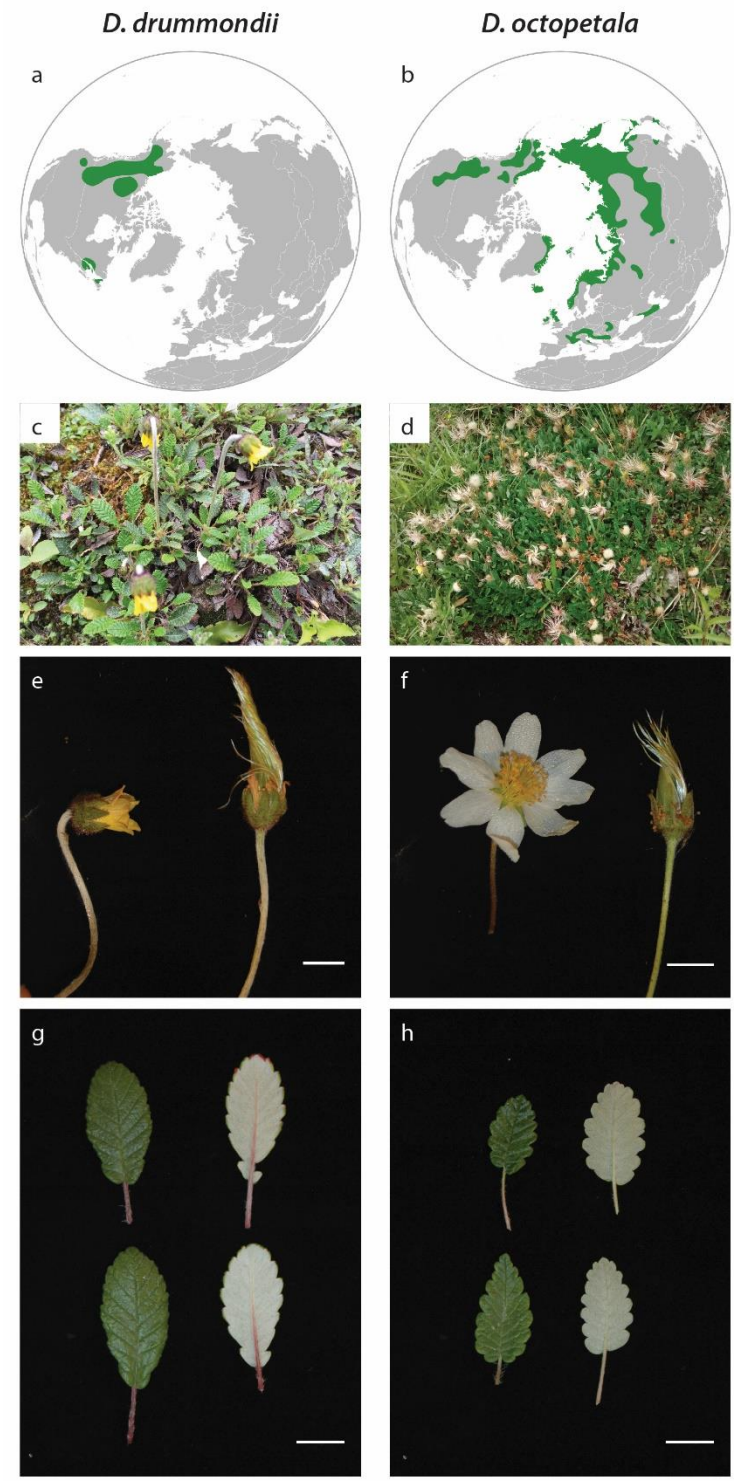


Figure 20 | Characteristics of *Dryas drummondii* and *Dryas octopetala*.
(a&b) Geographical repartition of *D. drummondii* (a) and *D. octopetala* (b) based on Central Yukon Species Inventory Project and Aarnes et al. 2012. (c&d) *Dryas* spp. growing under natural conditions. (e&f) Flower in full bloom and in fructification of *D. drummondii* (e) and *D. octopetala* (f). (g&h) Petiolated leaf morphology in *Dryas* spp. (g) obovate with dentate margins *D. drummondii* leaves; (h) ovate with dentate to sinuate margins *D. octopetala* leaves. Scale bars = 1cm.
Pictures B. Billault-Penneteau; (e&f) From Billault-Penneteau et al. 2019

1.2.2.2. *Dryas* spp. morphology and reproduction

Dryas consists of long-lived shrubs and it is one of the hardiest of all woody plant groups and forms the vegetation carpet in dry and damp gravelly sites all over the arctic tundra (Figure 20 c&d).

The petiolated *Dryas* leaves are leathery, tardily deciduous or evergreen, with stipules rising from leaf axils, linear-lanceolate in shape, tomentose (Figure 20 g&h). However, *D. octopetala* leaves (Figure 20 h) are narrower with generally ovate or elliptic shape with dentate or sinuate margins, while *D. drummondii* leaves (Figure 20 g) are larger and longer with dark green colour, rugose adaxial surface, densely tomentose abaxial surface, oblong shape and with dentate but less marked margins.

The entire genus is diploid (2n=18) (Potter et al. 2007), and the plants are mainly outcrossers (McGraw and Antonovics 1983) without any special adaptation to vegetative reproduction, even though clonal proliferation occurs (Wookey et al. 1995). It is a perennial plant, which in some studies was described to attain its reproductive maturity in the fifth year (Lawrence et al. 1967), suggesting that a long time is needed to complete a life cycle from seeds to seeds. Clonal growth in *Dryas* enables individuals to persist and grow in extreme environments where sexual proliferation is often unsuccessful (Wookey et al. 1995). Individual clones of *D. octopetala* can live more than 100 years (Kihlman 1890; Crawford 1989).

In the wild, *Dryas* flower primordia are formed during the summer. This precedes far in advance the flowering, which occurs shortly after snowmelt with most individuals flowering within a month. *Dryas* flowers have a positive heliotropism movement to maximize the amount of sunlight reflected on the petals and on the mass of pistils at the centre of the flower (Kevan 1975; Kjellberg et al. 1982; Krannitz 1996). It was shown that flowers that track the sun are warmer, have pistils that develop faster and produce heavier seeds(Kevan 1975). Pollination in *Dryas* is assumed to be insect-dependent, with only a low level of wind pollination (Kevan 1975; Roslin et al. 2013; Tiusanen et al. 2016). The flowers (Figure 20 e&f) are perfect (hermaphrodites), with regular symmetry, solitary, and scapose (on naked stalks borne above a dense rosette of leaves). *Dryas* flowers do not fit to the general Rosaceae floral formula (✶K5C5An*5G∞), as they present height sepals and height petals with numerous stamens and pistils; and give the following floral formula: ✶K8C8A∞G∞. The most obvious differences between the two species are in the shape and colour of the flower in full bloom. Indeed, *D. octopetala* flowers (Figure 20 f) are characterized by a relatively large size, around 2 cm, fully open with white petals and a yellow centre, whereas *D. drummondii* flowers (Figure 20 e) are nodded, never fully open and completely yellow.

The fruit of *Dryas* (Figure 23 a) is an achene, bearing long, silky-feathery white hygroscopic hairs which contract and twist upon wetting, but expand and straighten in drying (Lawrence et al. 1967) forming a plumed fruit. These hairs are modified from the persistent styles and help spread the attached seed via light winds. Observations in the wild suggest that the mortality rate is high in the wild in the first few years and relatively few of the delicate seedlings survive (McGraw and Antonovics 1983).

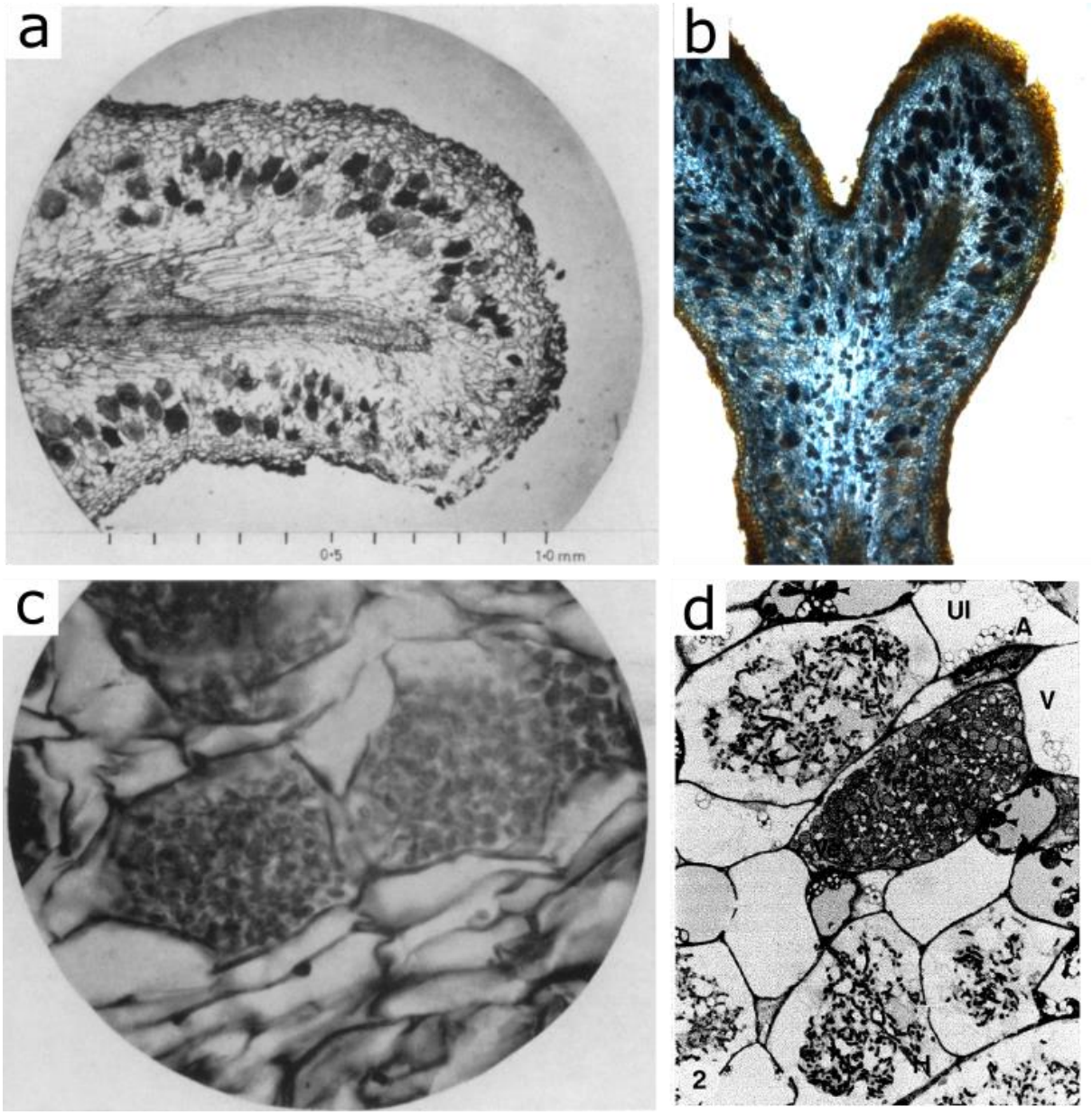


Figure 21 | *Dryas drummondii* nodule structures.
(a&b) Longitudinal section through a root nodule lobe of *D. drummondii* from Lawrence et al. 1967 (a) and from a nodule found in the botanical garden of Munich stained with trypan blue (unpublished, B. Billault-Penneteau) (c) Cortex cells filled with the endophyte showing vesicles and hyphae. x 1300. (d) Light microscopy showing infected and uninfected (UI) cells. The uninfected cells contain a large central vacuole (V), numerous amyloplasts (A) with large round starch granules, and phenolic inclusions (arrows). Both types of infected cells are shown: cells containing only hyphae (H) and one cell containing both endophytic hyphae and vesicles (Ve). x 600.
From Newcomb 1981

1.2.3. *Dryas* spp. and root nodule symbioses

1.2.3.1. *D. drummondii* and *Frankia* symbiosis

Among almost 100 Rosaceae genera only four of them are described as nodulating plants: *Cercocarpus*, *Chamaebatia*, *Dryas*, and *Purshia*. They are all gathered and forming the Dryadoideae subfamily. In 1952, Lawrence discovered that *D. drummondii* at Glacier Bay, Alaska, possessed root nodules (Lawrence et al. 1967). In 1963, Allen et al. (1964) found brownish aggregation of nodules on the roots of *D. drummondii* from another region of Alaska. Nodulated plants have been found on the flood plain gravels of the St Lawrence River by Schoenike and on shingle flats at Mount Robson, Canada, by Tisdale et al. (1966), so their occurrence is not restricted to Alaska. These nodules consisting of typical metamorphosed roots which, on the basis of light microscopic observations, were similar to *Alnus* spp. nodules both in nodule and endophyte morphology. The nodules are red orange in colour and have a coralloid appearance due to the presence of many nodule lobes (Figure 14 a, Figure 21 b). The nodules ranged between 1 and 3 cm in diameter. The main differences between the situations in *Alnus* spp. and *Dryas* sp. seemed to be the difference in depth at which the nodules were located. Instead of being superficial, often immediately below the surface or even partly above the surface as in alder, they were usually found 15 cm or more beneath the surface. At Glacier Bay they were found on young plants at or near the ends of tiny feeder rootlets, 30-50 cm away from the base of the stem. Some nodules could have a flattened appearance because they grow pressed close against the rocks. In Lawrence et al. (1967), it is suggested that perhaps the rocks provide some required mineral nutrient, or perhaps the scarifying effect of rock is needed to allow entrance of the nodulating microorganism. Numerous attempts of other workers to confirm the presence of nodules on *D. drummondii* have failed. One of the explanations is the point that nodules were easily torn away from the roots. Indeed, they are found at or near the ends of tiny feeder rootlets growing between and around rocks. Moreover, *Frankia* strain interacting with *Dryas* sp. is obligate symbiont. Several efforts were made to induce nodulation of *D. drummondii* however only one publication has achieved this goal, Kohls et al. (1994). In this publication *D. drummondii* nodules are in the same number that on other Rosaceae but less than in other actinorhizal plants. Kohls et al. (1994) alluded to the fact that rosaceous plants are slow to nodulate.

Studies of soil under *D. drummondii* mat show an increase in N (Crocker and Major 1955; Viereck 1966; Lawrence et al. 1967). For example Lawrence et al. (1967) have shown that in 25 y under *Dryas* sp. the top 5 cm of soil there was an increasing of around 60 $kg.ha^{-1}$. They have also demonstrated with ^{15}N test that *D. drummondii* nodules were capable of fixing atmospheric N. Fresh sections of *D. drummondii* nodules reveal a cream-coloured interior. Light microscopic studies demonstrated an organization similar to that of other actinorhizal root nodules. Each nodule

lobe is surrounded by a brownish layer of rather small phelloderm-like cells. They consist of a thick cylinder of cortex surrounding a central well-developed vascular bundle. A poorly defined meristem is situated at the distal end of the vascular cylinder. The infected cells are restricted to the outer half of the cortical cylinder but are not present in the outermost cortical cell layers. The infected ones are immediately conspicuous because of their darkly stained contents and by their shape. Indeed, they have a larger diameter (up to 40 µm in width, up to 80 µm in length) than the neighbouring uninfected cortical cells. The inner cortical cells which remain uninfected tend to be elongated. They usually contain large amyloplasts with several starch granules. The uninfected cells often have phenolic deposits in the large central vacuole (Figure 21)

1.2.3.2. Doubts on the nodulation of the other *Dryas* spp.

It is currently assumed that within *Dryas* genus only *D. drummondii* is nodulating and the other species are considered as non-host. In some publications it was assumed that *D. octopetala* or *D. integrifolia* can be nodulated, but these affirmations were contested. The assertion that *D. integrifolia* or *D. octopetala* are symbiotic nitrogen fixers has worked its way into the literature. This was resulting from a "domino effect". Indeed as Markham (2009) explained, the first time that these two species were reported as nodulating, was in Lawrence et al. (1967). They reported that observations by Sprague in 1952 revealed that the roots of *D. octopetala* and *D. integrifolia* also have root structures similar to nitrogen fixing nodules. However, the authors did not report any microscopic examination of nodules or measurement of nitrogen fixation in this species. Then in summarizing the results of International Biological Program surveys, Bond (1976, 1983) listed *D. integrifolia* and *D. octopetala* as actinorhizal species based on the work of Lawrence et al. (1967), even though additional searches for nodules were reported as unsuccessful. Afterwards others referring directly to the Lawrence et al. (1967) paper (Svoboda and Henry 1987); Bond's 1976 paper (Torrey 1978; Lechevalier 1983); citing papers that cite Lawrence et al. (1967) (Okitsu et al. 2004), citing papers that cite Bond's 1976 paper (Melville et al. 1987); using no citation at all (Sondheim and Standish 1983); or citing Sondheim and Standish (1983) (Kohls et al. 1994). But no evidence was found or added. Moreover, in studies at both Glacier Bay (Kohls et al. 2003) and the Rocky Mountains of Alberta (Kohls et al. 1994), analysis of stable isotopes suggested that *D. integrifolia* does not fix nitrogen in the field, nor were any nodules observed when they were searched for at this site. Also, Deslippe and Egger (2006) were not able to find Frankia-related genes in roots or soil from around eight *D. integrifolia* plants from Ellesmere Island.

1.3. Thesis objectives

The research team of Martin Parniske has focused for several years on studying the molecular processes that lead to the cellular reprogramming and redifferentiation associated with interactions between plants and symbiotic microorganisms. Likewise, it is explored natural diversity and ecological approaches to identify allelic variation governing symbiotic important traits. One group of closely related plants evolved to become independent of nitrogen from the soil by engaging in root nodule symbiosis. Considering either the *multiple gains* hypothesis or the *single gain* hypothesis, nodulation evolved exclusively in four related orders, the Fabales, Fagales, Cucurbitales and Rosales (FaFaCuRo). The European Research Council (ERC) funded project "Molecular inventions underlying the evolution of the nitrogen-fixing root nodule symbiosis (Evolving Nodules)", in which my thesis was integrated, builds on the underlying idea that the root nodule symbiosis evolved by co-opting AM pre-existing developmental program. Using a combination of phylogenomic, transcriptomic and genetic approaches, the team would like to systematically investigate and compare the prewired connections between signalling pathways and developmental modules present in non-host and host relative plants, to identify components acquired by nodulating plant species. To investigate evolutionary hypotheses related to nodulation, Rosaceae is a particularly appealing family of plants. Most genera of the Rosaceae including economically valuable targets such as apple and strawberry are non-nodulating. Four of the hundred Rosaceae genera form ancestral, lateral root-related actinorhiza nodules with Frankia actinobacteria. These nodulating Rosaceae are gathered in the basal subfamily Dryadoideae. Thus, a major step towards independent cultivation of nitrogen fertilizers for sustainable agriculture could be achieved by retracing the evolutionary steps within this plant family..

However, there are no established reference species within the Dryadoideae. Nevertheless, *Dryas* genus stands out due to the presence of two closely related species with a divergent ability to nodulate: *D. drummondii* and *D. octopetala*. My thesis project focused mainly on the study of these genera and therefore proposed to investigate their genetic divergences related to root symbiosis. With this aim in mind, various approaches and collaborations have been developed, grouped into three main parts: (i) the establishment of *Dryas* as a model genus, (ii) the study and establishment of *Dryas* spp. root endosymbioses and finally (iii) a genetic comparison of both *Dryas* spp. in order to study the genetic evolution that leads to the endosymbiosis polymorphism within this plant genus.

1.4. Structure of thesis results and allocations of collaborators' results

All my works along with the one from my collaborators are presented in the following sections of the Results chapter: section 2.1 gathers the different methods and protocols developed to introduce *Dryas* spp. in a laboratory context and to allow genetic and molecular analyses while sections 2.2 and 2.3 are they devoted to comparative studies between both *Dryas* spp. at the root symbiosis (2.2) and genetic (2.3) levels.

The results presented in section 2.1 have been published in Frontiers in Plant Science (Billault-Penneteau et al. 2019) and the *D. drummondii* genome (section 2.1.7) was used in Griesmann et al. (2018) published in Science. The results presented in sections 2.2 and 2.3 will be part of a second future publication.

In section 2.1.2, the growth tests presented in Figure 24 were performed together by me and in collaboration with Leon Van Ess during his Bachelor trainee under my supervision. The Figure 27, in section 2.1.4, comes from Billault-Penneteau et al. (2019) and was carried out by Aline Sandré. All genome sequencing and annotation were done by the Dr. Shifeng Cheng's team from the BGI. *D. drummondii* genome quality analysis (Figure 31, Table 5 and 6, Supplemental S2 in section 2.1.7) are issue from Maximilian Griesmann's work (Griesmann et al. 2018). In section 2.2.3, the ectomycorrhizal visualization on *D. octopetala* roots (Figure 33) and the PCR done on *D. octopetala* roots from Switzerland Alps (Figure 34) were done by Prof. Didier Reinhardt. The genomic comparisons presented in section 2.3 use as a first step the OrthoFinder files generated by Maximilian Griesmann. These data files, based on the first version of the annotated *Dryas* spp. genomes, search for the orthologous genes of *M. truncatula* in each *Dryas* spp. independently. The Figure 36 d from section 2.3.2 is issue from a collaboration with Aline Sandré. Indeed she cloned the *Dryas* spp. *RAM1* genes and performed the *L. japonicus* root transformation and I carried out all the AM inoculations and observations.

2. Results

2.1. Adaptation of *Dryas* spp. in a laboratory context

2.1.1. Seed sterilization et germination

Fungal contamination of *Dryas* spp. seeds was often observed, whether they were collected in the field or obtained from professional seed producers. Indeed, white fungal hyphae growing out from the seeds on agar plates (independently of the agar source) always led to seedling death at an early stage. *Dryas* spp. seeds were quite sensitive to different surface sterilization procedures (Figure 22): any traces of ethanol would completely inhibit germination, while the size and thinness of the seed coat rendered the use of sulphuric acid risky. Furthermore, contaminating fungi were more resistant to bleaching than other sterilizing agents tested. (Figure 22) However, after stratification of *Dryas* spp. seeds at 4˚C, most efficient sterilization and highest germination rates were observed by using concentrated hydrogen peroxide. Indeed approximately 100% of *D. octopetala* seeds and between 95 and 100% of *D. drummondii* seeds were free of contamination after the procedure. For *D. drummondii*, the maximal germination level (85%) was obtained 8 days post sterilization (Figure 23 c), whereas the maximum germination rate of *D. octopetala*, *ca.* 40%, was only reached 12 days post sterilization (Figure 23 c).

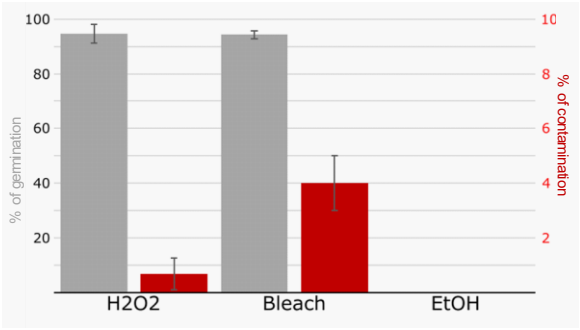


Figure 22 | Influence of sterilization solutions on *Dryas drummondii* seeds.
Percentage of germination (in grey) and of contamination (in red) of *D. drummondii* seeds after a treatment with H_2O_2 (30% hydrogen peroxide), or with Bleach (1.2% of NaClO in 0.1 % of SDS) or with EtOH (0.05% of Tween80 in 70% Ethanol). Displayed are means (n = 3 biological replicates with at least 100 seeds per replicate) and standard errors.
(data unpublished)

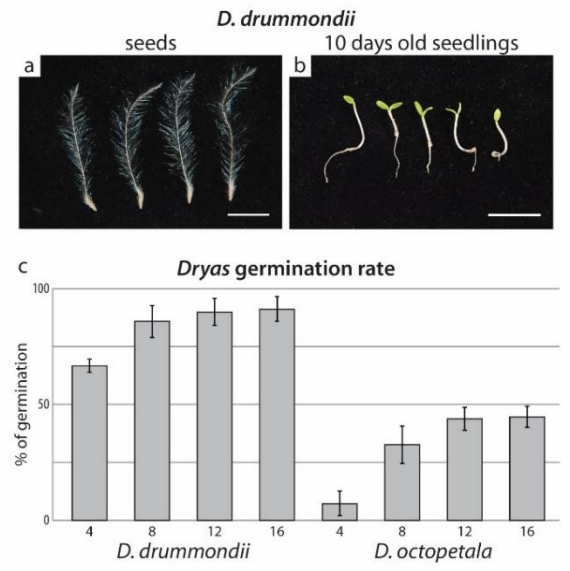


Figure 23 | *Dryas* spp. from seeds to seedlings.
(a) Isolated anemochorous silky-feathery achenes from *Dryas drummondii*. (b) 10-day-old seedlings of *D. drummondii*. Scale bars denote 1cm. (c) Time course of *Dryas* spp. germination after seed surface sterilization. Displayed are means (n = 4 biological replicates with at least 100 seeds per replicate) and standard errors.
From Billault-Penneteau et al. 2019

2.1.2. Growth systems

Once germinated and grown on agar plates with classical plant medium like B5 or MS (Gamborg et al. 1968; Murashige and Skoog 1962) (Duchefa, Haarlem, The Netherlands) or Fåhraeus medium (Fåhraeus 1957), *Dryas* spp. seedlings turned reddish, likely due to anthocyanin production, a response typically interpreted as stress- or defence-related. This anthocyanin production was less pronounced when the seedlings were grown on 0.4% Gelrite agar with ¼ strength Hoagland medium (Figure 23 b). Moreover, after two weeks on plates, *Dryas* spp. plantlets grown on ¼ strength Hoagland medium showed darker green cotyledons and further developed root systems than on B5 medium. In addition, compared to plants grown on "FAB" medium (medium developed especially for Fabaceae plants and used as routine in our lab for *Lotus japonicus*) both *Dryas* spp. present longer principal root length on ¼ Hoagland and *D. drummondii* present also more lateral roots (Figure 24).

Upon germination on plates, *Dryas* spp. plantlets were transferred to a hydroponic system (Figure 25 a) with ¼ strength Hoagland solution. In this system *Dryas* spp. plants grew and developed healthily (Figure 25 b); they formed well-developed primary and lateral roots (Figure 25 b). Altogether, *Dryas* spp. plants adapted very well to the hydroponic system tested. The absence of gel and soil substrates makes this system particularly well suited for root system analyses as it offers the opportunity to perform non-invasive observations of the roots.

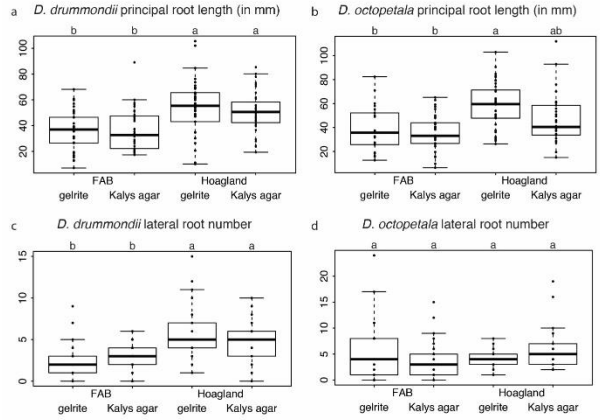


Figure 24 | *Dryas* spp. root growth on different agar sources and medium.
D. drummondii (left) and *D. octopetala* (right) root length in mm (a&b) and lateral root number (c&d) after 6 weeks of growth on plate containing either 0.4% gelrite or 1% Kalys agar with FAB or ¼ Hoagland 1mM KNO_3. (4 replicates with at least 20 plantlets per replicate).
In collaboration with Leon Van Ess during his Bachelor trainee under my supervision. (data unpublished)

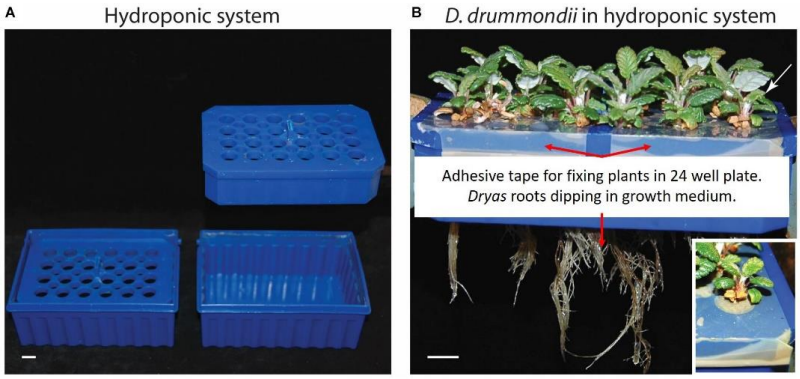


Figure 25 | Hydroponic system for *Dryas* spp.
(a) Overview of the hydroponic system: assembled (left) and split up (right). (b) Hydroponic culture of *Dryas drummondii* after 7 weeks in the hydroponic system; the white rectangle shows a close-up view of the plant labelled with a white arrow. Scale bars denote 1cm.
From Billault-Penneteau et al. 2019

2.1.3. Propagation

For clonal propagation by cuttings, 2-3 cm of *Dryas* spp. stems containing one node were transferred into moist soil (Figure 26 b&c) in a small growth container with a transparent plastic lid for conservation of high humidity levels. Once the shoots had successfully rooted, the plants were transferred into single pots and grew normally in standard greenhouse conditions. In a first trial, three series of *ca.* 20 cuttings per species were grown in a greenhouse at different times of the year. Under these conditions, 65% to 95% of the *Dryas* spp. cuttings developed roots within three weeks in the absence of hormonal treatments (Figure 26 c). A second test was performed with classical horticultural propagating substrate and with peat pellet (Jiffy®, Denmark), and for 32 explants per *Dryas* spp. (*D. drummondii*, *D. octopetala* and the hybrid *D.* x *suendermannii*) and per substrate. During this assay all *Dryas* spp. presented 100% of growing cuttings in propagating substrate contrary to the ones in peat pellet (81,25% for *D.* x *suendermannii*, 75% for *D. drummondii* and 56.25% for *D. octopetala*; Table 4).

In this study, flower and seed production did not occur when plants were grown at a distance of 2 m from standard high-pressure mercury vapour lamps (providing 90 µmol.m^{-2}.s^{-1} at the plant level) used in initial trials. However, when the plants were placed at a distance of 2 m under high-pressure sodium vapour lamps (150 µmol.m^{-2}.s^{-1}) for 16 h per day, flowering was induced (Figure 26 a) and seeds were produced (Figure 23 a) within less than a year after germination, whether the plants originated from cuttings or from sexual propagation. This is shorter than the five years required for *D. drummondii* according to Lawrence et al. (1967).

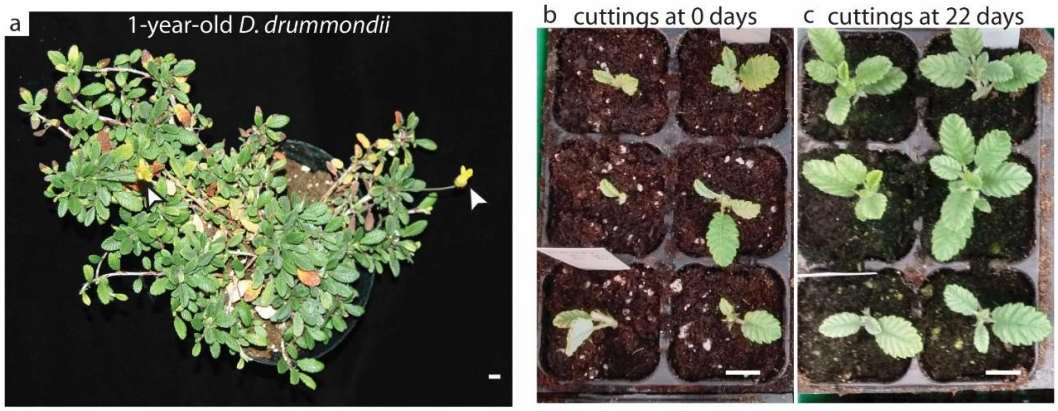


Figure 26 | Sexual and vegetative propagation of *Dryas* spp.
(a) 1-year-old *D. drummondii* plant flowering (white arrows indicate flowers). (b&c) Cuttings of *D. drummondii* at day 0 (e) and after 22 days of growth (f). Scale bars denote 1 cm.
From Billault-Penneteau et al. 2019

	starting explants	Rooting after 3 weeks on propagating substrate		peat pellet	
		number	percentage	number	percentage
D. drummondii	32	32	100	24	75
D. octopetala	32	32	100	18	56,25
D. x *suendermannii*	32	32	100	26	81,25

Table 4 | Percentage of rooting of *Dryas* spp. cuttings on different substrates.
(data unpublished)

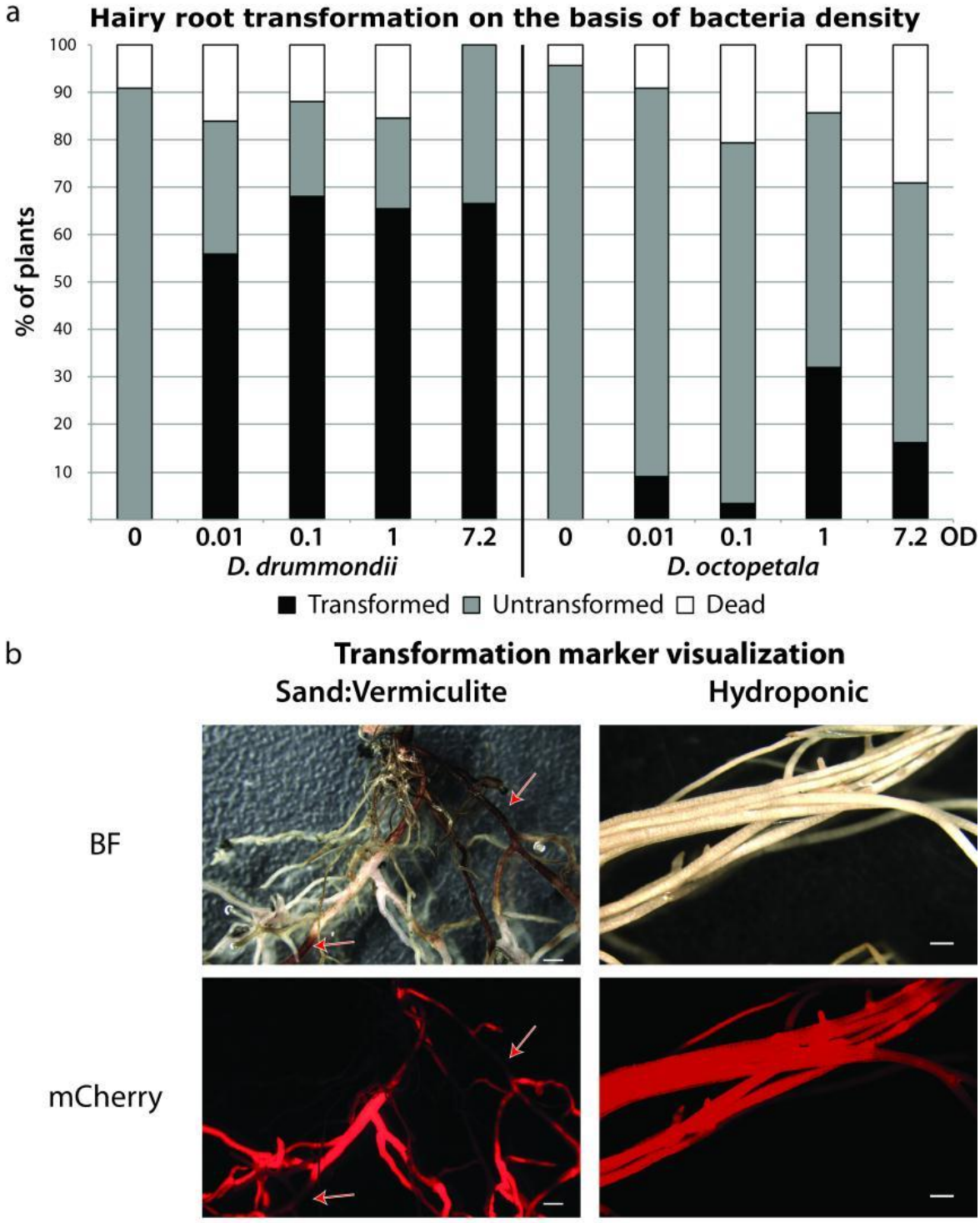


Figure 27 | *Agrobacterium rhizogenes*-mediated transformation of *Dryas* spp.
(a) Success rates of hairy root transformation of *D. drummondii* and *D. octopetala* depended on the bacteria density. Percentages of dead (white boxes), surviving untransformed (grey boxes) and transformed (black boxes) plants were determined five weeks after transformation on plants grown in Petri dishes. Transformation was determined based on mCherry fluorescence. (b) Visualization of the mCherry transformation marker of *D. drummondii* hairy roots after seven weeks of growth in sand:vermiculite (left panel) vs. growth in the hydroponic system (right panel). Red arrows point at lignified part of the roots. BF = bright field; mCherry = mCherry fluorescence. Scale bars denote 1cm.
From Billault-Penneteau et al. 2019

2.1.4. *Agrobacterium rhizogenes* transformation

We established a protocol for hairy root transformation in *Dryas* spp. by combining and adapting *Medicago truncatula* and *L. japonicus* protocols. The *Agrobacterium rhizogenes* strain AR1193 (Stougaard et al. 1987) was used because it was shown, for some species such as pea, to be more efficient than other strains (Clemow et al. 2011) and for practical reasons (being the strain routinely used in our lab for *L. japonicus* transformation). For protocol optimization, we transformed *Dryas* spp. using *A. rhizogenes* suspensions at different cell densities. Five weeks after transformation, the composite plants on plate were evaluated. For *D. drummondii*, a transformation efficiency of 55 to 70% was obtained under all conditions tested, while *D. octopetala* plants died more frequently in response to infection with *A. rhizogenes*. The use of higher bacterial densities had a negative effect on plant survival, while lower bacterial densities reduced transformation efficiency. Here, the best compromise between low mortality and transformation rate for *D. octopetala* was observed when the *A. rhizogenes* suspension was adjusted to an optical density (OD_{600}) of 1. However, the transformation efficiency was still low with only 30% (Figure 27 a). The experiment was repeated three times using an *A. rhizogenes* suspension adjusted to an OD_{600} of 1 on *ca.* 70 seedlings per species. In all cases, the results were the same: 55 to 70% transformation for *D. drummondii*, maximally 30% transformation for *D. octopetala*.

Up to seven weeks after transfer to pots or to the hydroponic system, transgenic roots showed healthy growth and expressed the transformation marker mCherry driven by the ubiquitin promoter (Figure 27 b). However, roots growing in solid substrates (such as sand:vermiculite) developed sections with increased lignification, hence more autofluorescence and opacity. This led to the quenching of the mCherry signal as highlighted by the red arrows in Figure 27 b. In contrast, when plants were grown in the hydroponic system, lignification was less pronounced. Thus, the hydroponic system is well suitable for the observation of fluorescent proteins in *Dryas* spp. hairy roots.

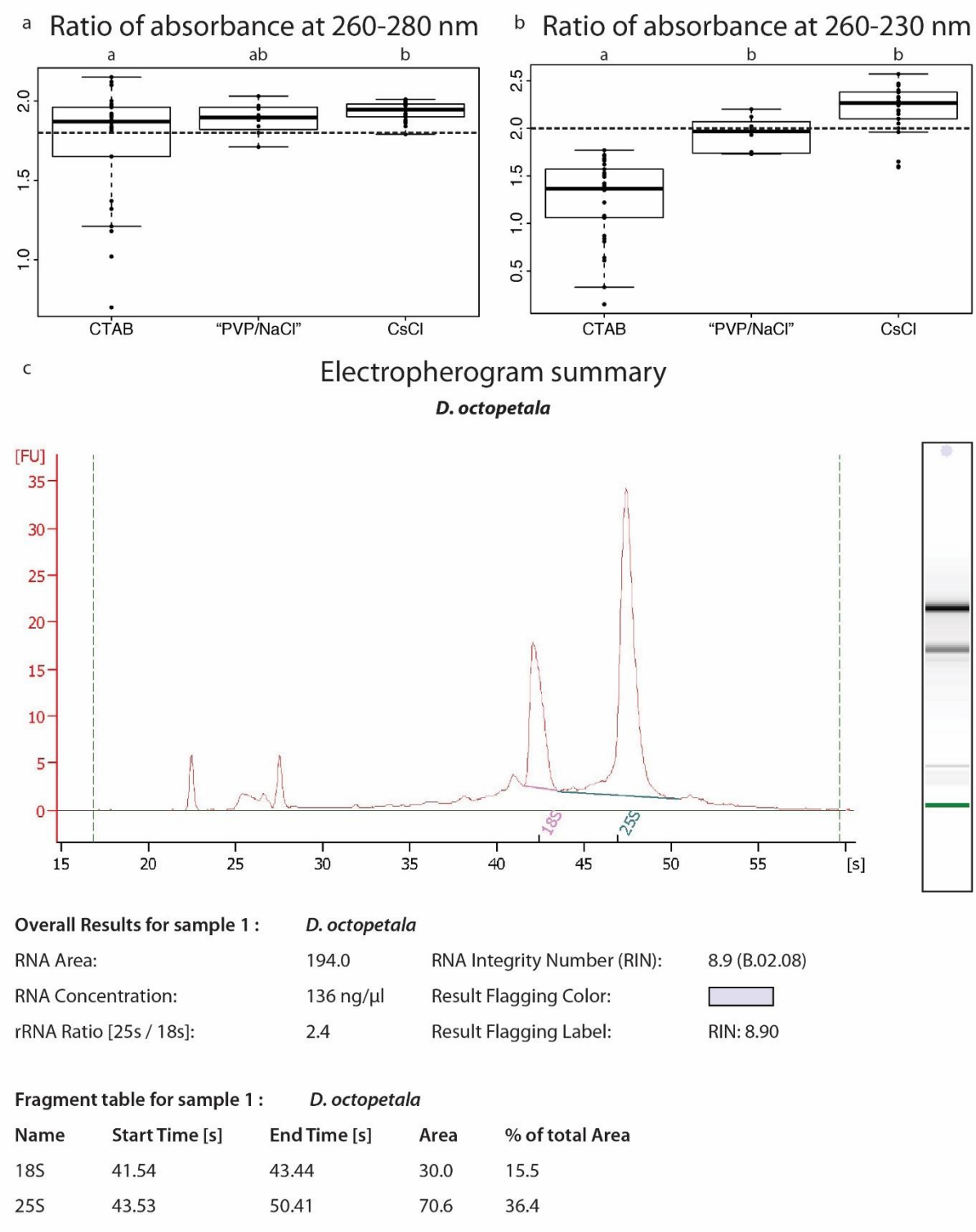


Figure 28 | Nucleic acid extractions from *Dryas* spp.
(a,b) Ratios of UV absorbance at 260 nm vs. 280 or 230 nm, from DNA samples isolated from *Dryas drummondii* and *Dryas octopetala* using three different methods: the classical CTAB method = "CTAB"; an adapted CTAB method for difficult plants = "PVP/NaCl" and a method involving a Caesium chloride gradient centrifugation = "CsCl". Theoretically, a 260/280 ratio (a) for nucleic acids vs. protein of at least 1.8 (dashed line) is generally accepted as denoting "pure DNA". 260/230 (b) values for nucleic acids vs. polysaccharides should be higher than 2.0 (dashed line). All DNA isolations were performed on 30 biological replicates per method. (c) Agilent Bioanalyzer electropherogram analysis of RNA isolated from *D. octopetala*, showing RNA integrity as determined by an RNA Integrity Number (RIN) of 8.9
From Billault-Penneteau et al. 2019

2.1.5. Nucleic acid extraction

For molecular biological studies, DNA and RNA have to be isolated with high purity, integrity and yield to be used for sequencing or reverse transcription, respectively. This was particularly challenging since the woody nature of *Dryas* and the composition of the leaves adapted to harsh environmental conditions lead to the presence of contaminants interfering with nucleic acid extraction protocols.

We tested different DNA extraction protocols on *D. drummondii* and *D. octopetala*, performing at least 30 extractions per method. DNA isolated from *Dryas* spp. with classical CTAB extraction protocols had an UV absorbance ratio at 260/280 of *ca.* 1.8, but the 260/230 ratio was always below 1.8, indicating polysaccharide contamination (Figure 28 a&b). Several established DNA extraction methods were tested, but none of them led to a yield and purity sufficient for robust PCRs and *de novo* whole genome sequencing. However, a CTAB protocol adapted for recalcitrant plant material (Khanuja et al. 1999) ("PVP/NaCl") by addition of polyvinylpyrrolidone (PVP), followed by a high salt lysis buffer and extraction with chloroform:isoamyl alcohol (24:1, v/v), resulted in good quality DNA suitable for PCRs with reproducible results (Figure 28 b). Yet, the DNA yield and quality required for genome sequencing was so far only achieved using a modified Dellaporta et al. (1983) lysis protocol followed by a caesium chloride (CsCl) gradient centrifugation as described by Ribeiro et al. (1995). The DNA extracted using this last method was used for *de novo* whole genome sequencing performed as a part of an international consortium (EVONOD) in collaboration with the BGI (Beijing Genomics Institute, China). The first version of the *D. drummondii* genome was used in a phylogenomic comparison study by Griesmann et al. (2018). The first version of *D. octopetala* genome was also obtained but still not published.

The Spectrum^{TM} Plant Total RNA Kit (Sigma-Aldrich) was used in order to extract RNA from different organs of *Dryas* spp. When Polyclar AT was added during the grinding step for recalcitrant samples (*e.g.* mature leaves and lignified roots), this method resulted in RNA of suitable integrity and purity for the performance of reverse transcription-quantitative PCR as indicated by the RNA integrity number (Figure 28 c).

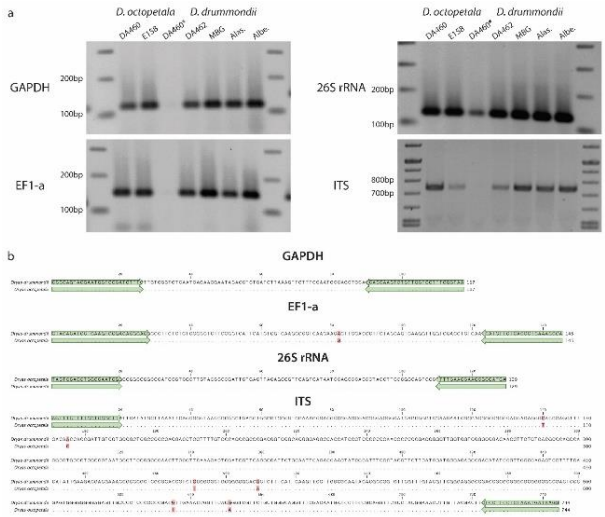


Figure 29 | PCR of housekeeping genes in *Dryas* spp.

(a) PCR products were amplified from different marker genes (GADPH, EF1-a, 26S rRNA and ITS) using gDNA from *Dryas octopetala* ecotypes ("DA460" and "E548") and *Dryas drummondii* ecotypes ("DA462", "MBG", "Alas." and "Albe.") extracted with the PVP/NaCl method (DA460* represents gDNA of *D. octopetala* ecotype DA460 extracted with the CTAB method). (b) Nucleotide alignment of the fragments from *D. drummondii* ecotype DA462 and *D. octopetala* ecotype DA460. Matching residues are marked as dots and differences are highlighted in red. Green arrows highlight the primers used.

From Billault-Penneteau et al. 2019

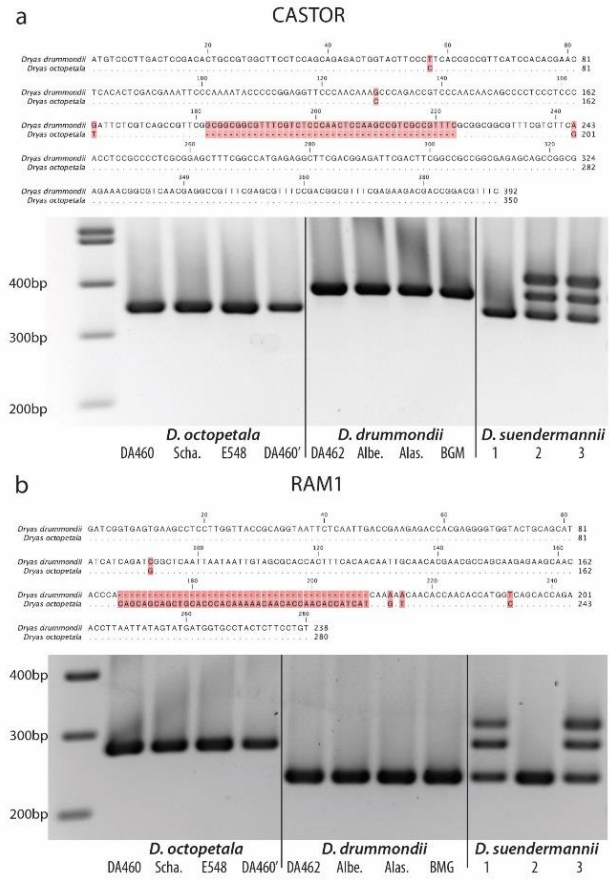


Figure 30 | Species specific deletion / insertion polymorphisms in two symbiotic genes [*CASTOR* (a) and *RAM1* (b)].

Upper panel: alignment of the gene region between *D. drummondii* and *D. octopetala*; matching residues marked as dots and differences highlighted in coloured background. Lower panel: PCR of the DIPs region in 4 different genotypes of *D. octopetala* and *D. drummondii* and 3 different *D. x suendermannii* hybrids. *Dryas octopetala* genotypes used: ecotype "DA460" from the seed producer Jelitto©, "Scha." from German Alps in Schachen, and "E548" from Italian Alps. *Dryas drummondii* ecotypes used: "DA462" from the seed producer Jelitto©, "Able." from Alberta, "Alas" from Alaska (both obtained in the Millennium seed bank of the KEW) and "BMG" from the botanical garden of Munich.

(unpublished data)

2.1.6. Robust PCR amplification

PCRs and subsequent sequencing of the amplified products was performed to test the quality of the isolated DNA samples. Primers were designed based on the first version of the *D. drummondii* genome. First, the targets were regions in the internal transcribed spacer (ITS) of nuclear ribosomal DNA, 26S ribosomal RNA (26S rRNA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the elongation factor 1-alpha (EF1-a). Using *D. octopetala* gDNA as template, fragments were amplified and sequenced as well. The high sequence conservation in these regions highlights the similarity between *D. drummondii* and *D. octopetala*. Indeed, the size of amplicons of both species were similar and their sequences presented few single nucleotide polymorphisms (Figure 29).

Second, primers were designed based on both genomes, in order to target coding regions of genes encoding proteins involved in root symbioses. Sequencing of the amplified products enabled us to confirm or improve some gene annotations (see part 2.3). PCR fragment sequences revealed deletion/insertion polymorphisms (DIPs) between the two species in the *CASTOR* and *RAM1* genes. Compared to *D. drummondii* in *D. octopetala*, the *CASTOR* gene has a 42 bp deletion at the beginning of the coding region (Figure 30 a). The *RAM1* gene contains a 42 bp insertion in the region coding for the variable part of the protein (Figure 30 b and 38). These patterns were confirmed to be species-specific by testing several different ecotypes of both *Dryas* spp. All *D. drummondii* and *D. octopetala* genotypes analysed contained consistent homozygous allelic versions of *RAM1* and *CASTOR* (Figure 30). PCR performed with these primers and on hybrids of *D.* x *suendermannii* showed different levels of hybridization as some were heterozygous for these regions and others were homozygous. For both genes, the third bands observed in heterozygous hybrids have been confirmed as the result of PCR artefacts. Indeed, by mixing *D. drummondii* and *D. octopetala* gDNA these third bands also appeared (data not presented).

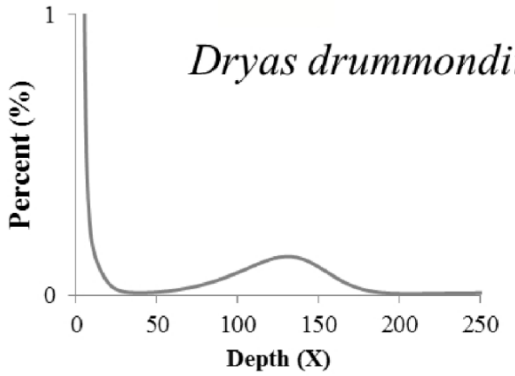


Figure 31 | 17-kmer frequency distribution of *Dryas drummondii* sequenced genome.
From Griesmann et al. 2018

Statistics of genome assembly

# of DNA libraries	Predicted size	Main Assembler
8	253 Mb	Soapdenovo
Gapfilled scaffold		
Total length (bp)	N50 (bp)	Assembly Percentage
233Mb	979 416	92,11%
Gapfilled scafitg		
Total length (bp)	N50 (bp)	Assembly Percentage
229Mb	35 876	90,55%

Evaluation of genome assembly completeness using BUSCO

Complete	Single	Duplicate	Fragment	Missing
90,80%	86,40%	4,40%	3,90%	5,30%

Table 5 | Statistics and evaluation of *Dryas drummondii* genome.
From Griesmann et al. 2018

Statistic of annotated genes.

Total	Gene number	25 030
	exon number	130 647
	intron number	105 617
	intron length (bp)	46 502 539
Average	mRNA length (bp)	3 208,54
	cds length (bp)	1 350,66
	exon number	5,22
	exon length (bp)	258,77
	intron length (bp)	440,29

Evaluation of gene models using BUSCO.

Complete	Single	Duplicate	Fragment	Missing
90,30%	87,90%	2,40%	1,90%	7,80%

Table 6 | Statistics and evaluation of *Dryas drummondii* annotated genes.
From Griesmann et al. 2018

2.1.7. First *Dryas* sp. genome version (*D. drummondii* in Griesmann et al. 2018)

Eight DNA libraries for *de novo* whole genome sequencing were constructed with *Dryas* sp (one of 170bp, 250bp, 350bp, 500bp, 800bp, 2kb, 6kb, and 10kb). DNA extracted by CsCl gradient method. RNA extracted from leaves and seedlings were used by the BGI in order to assist gene prediction. The transcripts were mapped to the protein-coding gene models, identified using the MAKER-P pipeline (version 2.31) (Campbell et al. 2014), in order to obtain gene characteristics (size and number of exons/introns per gene, distribution of genes, features of splicing sites, *etc.*).

The first *D. drummondii* genome version was presented and used in Griesmann et al. (2018). The genome size was estimated at 253 Mbp. A total length of 233 Mbp were sequenced. It is estimated to be assembled at 92.11% (Table 5). This genome was been assembled at the scaffold level, with 62 N50 scaffolds at 979 kbp of length (Table 5). The graphic representing the 17-kmer frequency distribution (Figure 31) of *D. drummondii* genome presents only one clear peak without intermediate peak or shoulder. This indicates a low heterozygosity rate.

25 030 genes models were predicted and 86.64% of them are annotated (Table 6 and Supplemental S2). Those predicted genes have an average of 5.22 exons with an average length of 258.77 b. The mean of the intron length is estimated at 440.29 b (Table 6). Transposable elements (TEs) represent around 29% of the genome, with 13.33% of the genome which are retro-TEs and 16.18% which are DNA-TEs (Supplemental S2).

The same analysis for *D. octope*tala genome could not be presented in this thesis (see section 3.1.3) but it is on going.

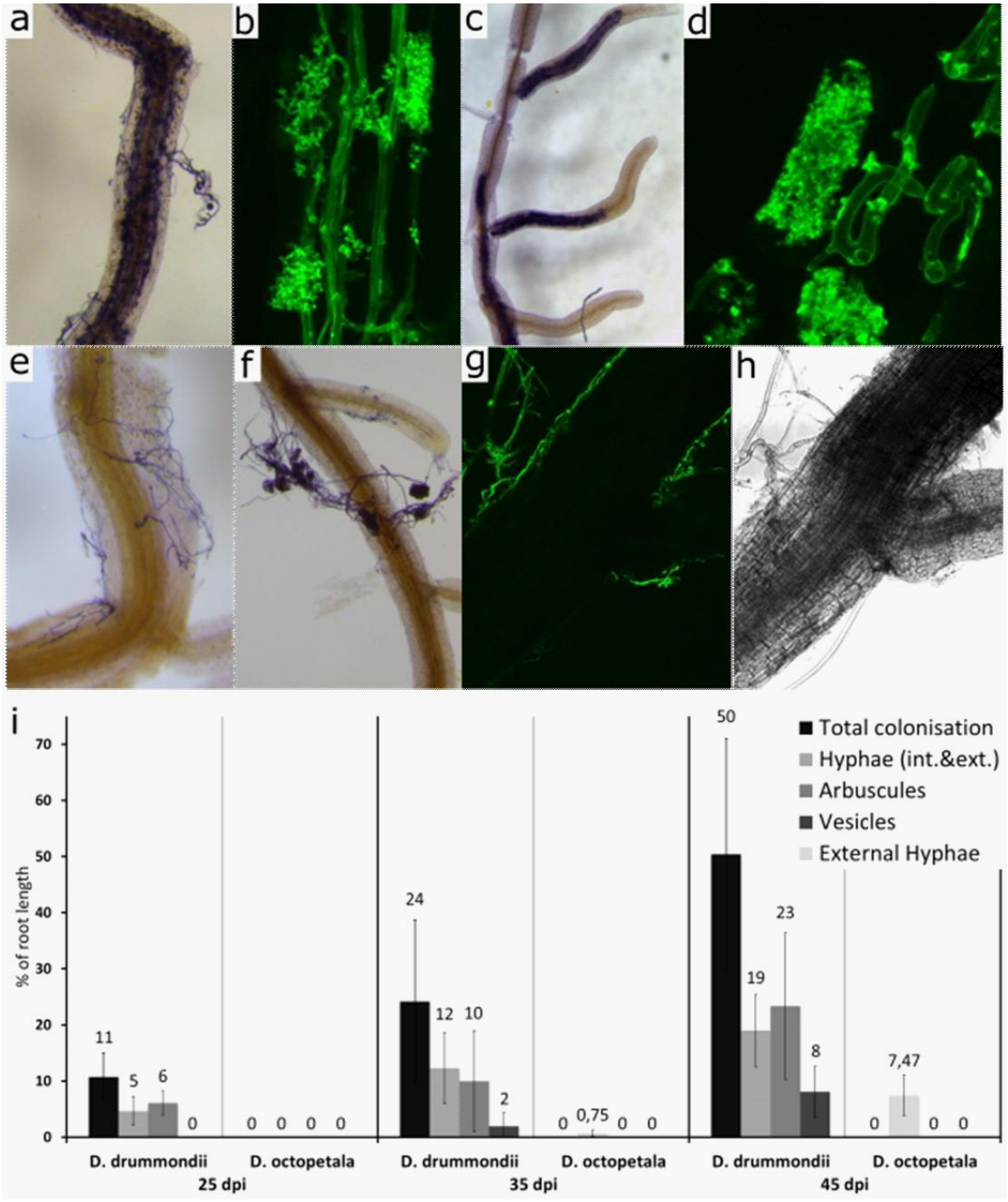


Figure 32 | Detection of Arbuscular mycorrhizal fungi within *Dryas drummondii* and *Dryas octopetala.*
(a-h) Microscopy analysis of *D. drummondii* and *D. octopetala* AM inoculation. Roots of *D. drummondii* (a to d) and *D. octopetala* (e to h) 45 days post inoculation either with *Rhizophagus irregularis* (a, b & e) or *Gigaspora rosea* (c, d, f-h). AM structures are visualized either with black ink (a, c, e & f) or with WGA-Alexa- Fluor488 (b, d, g & h) staining. Note that all *D. drummondii* roots present internalization of fungi and Arum-type arbuscules fully filling the host cells (b&d) whereas *D. octopetala* only carries external hyphae, as it is highlighted by the WGA-Alexa- Fluor488 fluorescence picture (g) with the bright-field (h). (i) Time-course of *Dryas* spp. AM quantification. Percentage of root length colonisation was carried out at 25-, 35- and 45-days post-inoculation. The hyphae, the arbuscules and the vesicles were counted in *D. drummondii* and the external hyphae for *D. octopetala*.
(data unpublished)

2.2. Endosymbioses within *Dryas* spp.

2.2.1. Attempt of *D. drummondii* nodulation

A first assay to nodulate *D. drummondii* plants was conducted on 12 weeks-old plants growing in pot containing soil sample from Alaska. This soil was collected close to wild *D. drummondii* and was containing few dry nodule sections. A second test was done by using one *D. drummondii* crushed nodule, found in the botanical garden of Munich. Both inoculum starting material have allowed each the inoculation of only pot. Two *D. drummondii* plants were present per pot. The root systems were carefully observed under a binocular microscope, 24 weeks post-inoculation. Both attempts to obtained *D. drummondii* nodulated plants have failed.

2.2.2. Arbuscular mycorrhiza symbiosis formation within *Dryas* spp.

Even though *D. octopetala* does not perform root nodule symbiosis (RNS), formation of ectomycorrhizal (ECT) symbiosis was reported with more than 50 fungi species. However, interaction with arbuscular mycorrhizal (AM) fungi has never been described. So in order to complete the overview of root symbioses in *Dryas* genus, both *Dryas* spp. were grown in chive (*Allium schoenoprasum*) nurse pots containing either *Rhizophagus irregularis* or *Gigaspora rosea* fungi. Under these conditions of high inoculation pressure, all *D. drummondii* root systems contained intra-radical fungi structures. Roots inoculated with either of the two fungi, stained with black ink after 45-days post inoculation show all characteristics AM-symbiosis structures, such as internal hyphae, vesicles and arbuscules (Figure 32 a-d). Confocal microscopy pictures of colonized roots stained with fluorescent wheat germ agglutinin (WGA-Alexa- Fluor488) allow to visualize arbuscules that completely fill the plant cells. In all colonized roots of *D. drummondii*, the arbuscules were of the Arum-type, regardless of which of the two fungal species used. (Figure 32 b&d) On the other hand, no fungal colonization was observed on the roots of *D. octopetala*, even though external hyphae surrounding the roots were present. (Figure 32 e-h)

In a second experiment, a time course analysis of colonization was conducted. *Dryas* spp. plants grown in sterile substrate were inoculated with 300 spores of *R. irregularis* per plant, which corresponds to an intermediate inoculation pressure. Root length colonization (determined by presence/absence of fungal hyphae, arbuscules and vesicles) was measured at 25-, 35- and 45-days post inoculation.

At the last timepoint analysed, 50% of *D. drummondii* root length was colonised, whereas in *D. octopetala*, only extraradical hyphae could have been observed on 7% of the root length (Figure 32 i). In all inoculation configurations that was from spores or from nurse plants, with or without cocultivation with *D. drummondii*, internalisation of fungi inside *D. octopetala* roots was never observed.

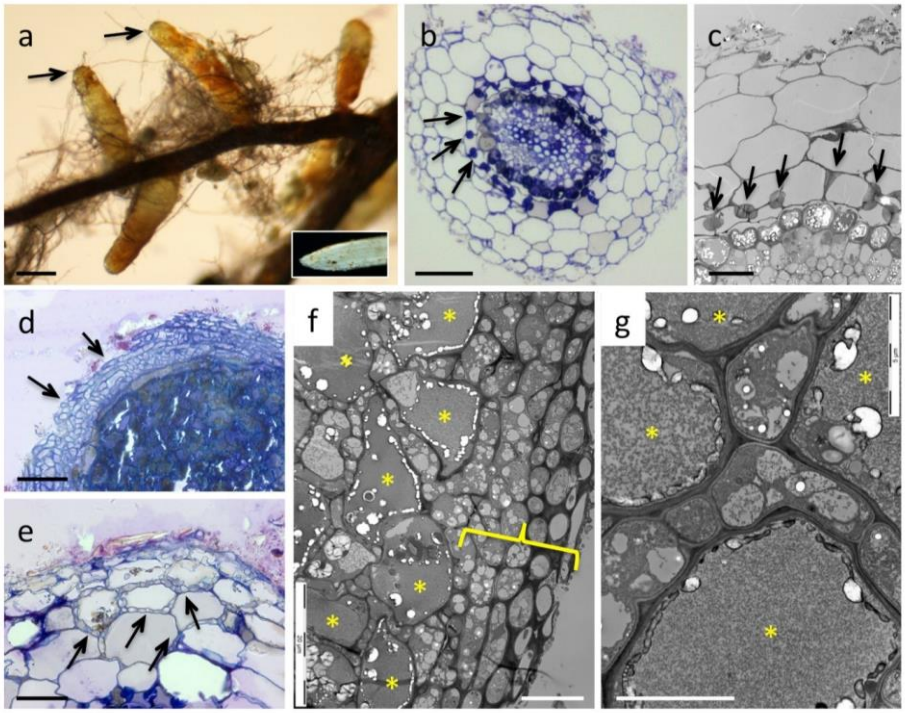


Figure 33 | Microscopic analysis of mycorrhizal *Dryas octopetala* roots.
(a) *D. octopetala* root system exhibiting extraradical mycelium, and the typical ectomycorrhizal rounded root tips (arrows). Inset: A non-mycorrhizal root. (b) Semithin cross-section of a non-colonized root; Note the cell wall thickenings of the endodermal cells (arrows). (c) Electron micrograph of a section as in (b). (d) Semithin section of a colonized root tip with extraradical colonization forming a mantle around the root (arrows). Note the dense highly cytoplasmic root cells. (e) Semithin section of a colonized root behind the root tip. Fungal hyphae proliferate between highly vacuolated cells, forming a Hartig net (arrows). (f) Electron micrograph with a close-up of a colonized root as in (e). Note the hyphal mantle (yellow bracket). Host cells (marked with asterisks) are surrounded by the Hartig net. (g) Close up of the Hartig net. Size bars: 100µm in (a), 50µm in (b), 20µm in (c)-(e), 10µm in (f), and 5µm in (g).
From Prof. Didier Reinhardt (data unpublished)

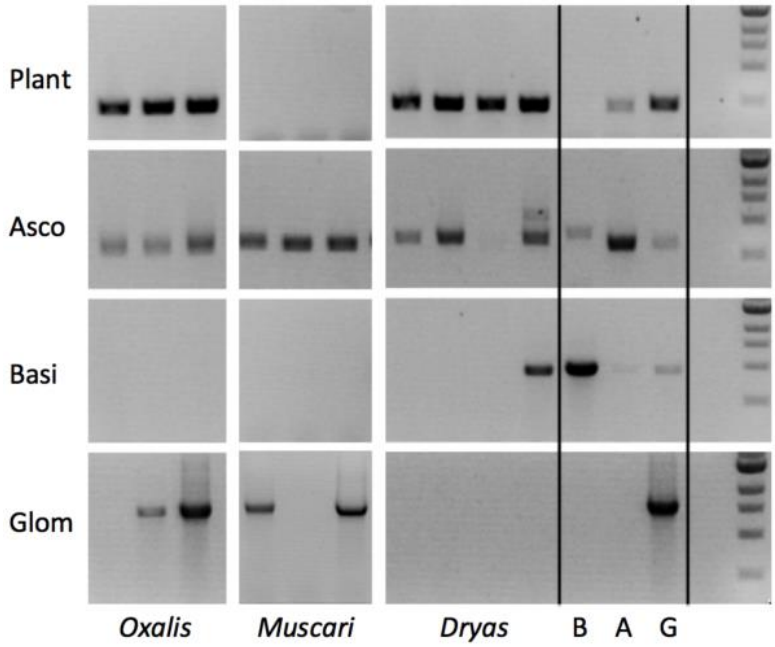


Figure 34 | Identification of fungal identities in *Dryas octopetala* roots.
Genomic DNA of colonized roots of *D. octopetala* were analysed by PCR with primers designed to preferentially amplify ribosomal genes of plant (Plant), ascomycetes (Asco), basidiomycetes (Basi), or Glomeromycetes (Glom). B, A, and G represent genomic DNA from a basidio- asco-, and glomeromycete, respectively, as positive controls (*Agaricus bisporus*, *Penicillium roqueforti*, *Rhizophagus irregularis*). Several individuals from *D. octopetala*, *Oxalis corniculatus*, and *Muscari armeniacum* were sampled from the same location.
From Prof. Didier Reinhardt (data unpublished)

2.2.3. Absence of Glomeromycota in the *D. octopetala* roots fungi community (From Prof. Didier Reinhardt).

In order to eliminate the hypothesis that the lack of AM symbiosis with *D. octopetala* roots in sterile substrate could be due to these gnotobiotic system or to the fact that only two AM fungi species were tested, in situ observations were carried out by Prof. Didier Reinhardt.

Root systems of *D. octopetala*, collected in the Switzerland Alps, were characterized by the presence of an abundant extra-radical mycelium and rounded root tips, which are typical structures of ECT roots (Figure 33). Cross-sections of the roots highlighted the extensive extra-radical mycelium in the colonized root tips forming a dense hyphae mantle, and also the fungi hyphae growing between the host cortical cells forming the Hartig net (Figure 33). In order to complete the overview of the fungi community found to be associated with *D. octopetala* roots, together with those of the neighbouring plants, presence of fungi from three different phyla was investigated (Figure 34). To do so, PCRs with primers specific of Ascomycota, Basidiomycota or Glomeromycota ribosomal region were performed on DNA extracted from roots. As expected, Ascomycota and Basidiomycota fungi DNA could be amplified from *D. octopetala* roots, but no Glomeromycota fungi DNA was found in those samples. However, fungi belonging to this last phylum were present in the soil close to *Dryas* sp., as amplicons were obtained in total DNA extracted from neighbouring growing mono and dicotyledonous plants (*Muscari armeniacum* and *Oxalis corniculata*).

By these results, we confirm the observation of Fitter and Parsons (1987) that *D. drummondii* interacts with AM fungi. Furthermore, we describe for the first time an unexpected rare case of AM-symbiosis polymorphic trait within the same plant genus.

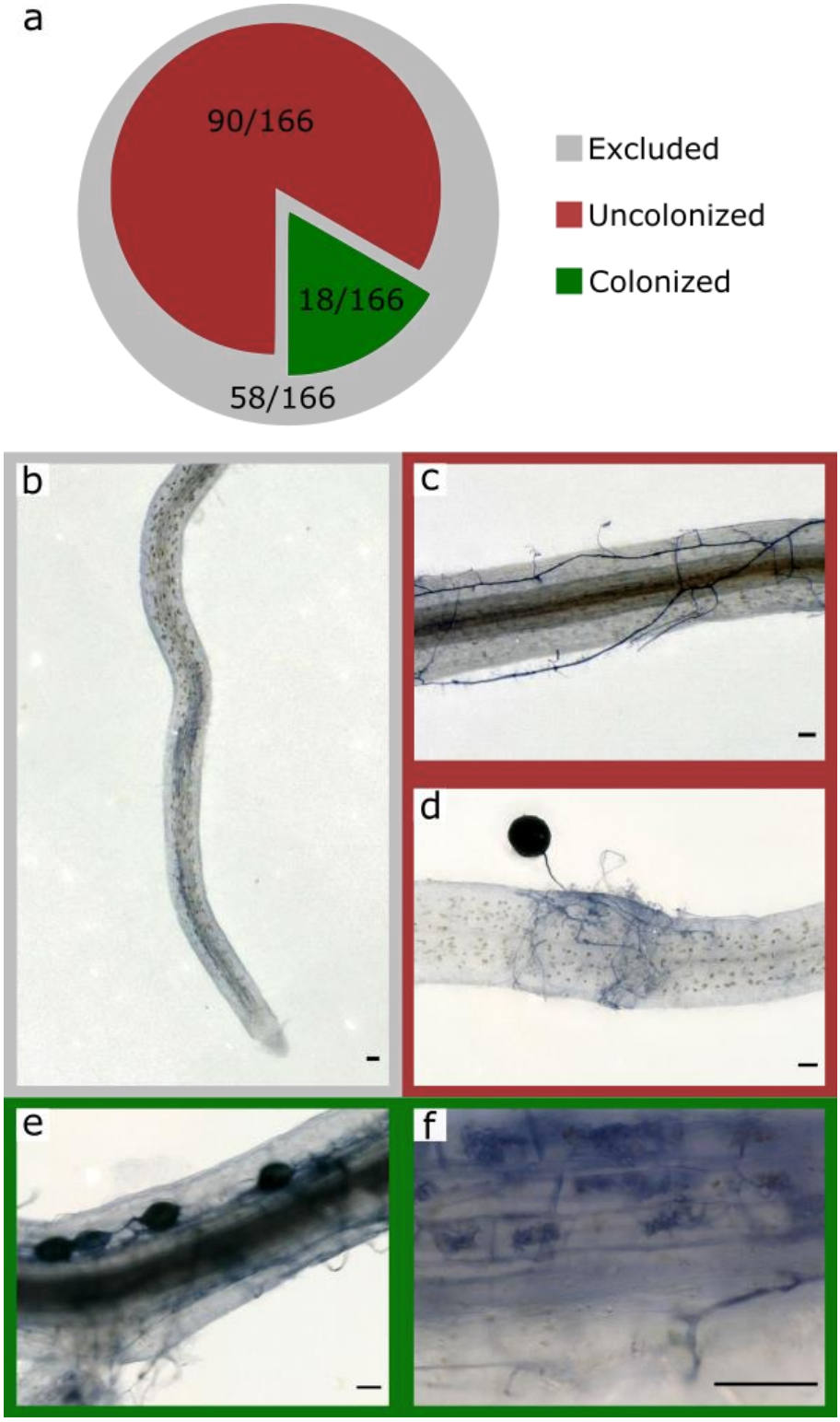


Figure 35 | Screening of AM symbiosis in hybrid *D.* x *suendermannii*.
(a) Diagram of the *D.* x *suendermannii* root systems analysed for AM symbiosis. (b to f) Pictures of representative hybrid roots after black ink staining. 166 hybrids plants were analysed 45 days post inoculation. In grey, all roots without any external hyphae (b) were excluded to avoid false-negative non-symbiotic plants. (c&d) Root systems exhibiting external hyphae but without intraradicular fungi structures are considering as symbiosis incompetent and represent 83% (in red). Note in c the external hyphae that progress along the root but never penetrate it like those progressing from a germinating spore in d. (e&f) 17% of the *D.* x *suendermannii* roots systems with external hyphae are colonized (in green). Note in e the presence of vesicles inside the root and arbuscules in f. Scale bars denote 50µm. (data unpublished)

2.2.4. Screening of endomycorrhizal symbiosis in hybrid *Dryas* x *suendermannii*

Seeds of *D.* x *suendermannii*, hybrid species between *D. drummondii* and *D. octopetala*, were obtained from the botanical garden of Vienna. AM inoculation of these hybrid plants were carried out, in order to study a genetically dominant or recessive trait of the symbiosis. This should help in the near future to narrow down and/or to determine which loci are important for the endosymbiosis polymorphic trait between *Dryas* spp.

A first roots screening was done on 166 *D.* x *suendermannii* plants. These plants were cultivated in chive nurse pots or with a minimum of 500 spores per plants to assure a high inoculation pressure. Each pot contained at least three plants. Based on the AM quantification time course done previously, the observations were carried out after 45 days post inoculation. In order to limit the risk of false negative regarding non-AM-symbiosis competent plants, the analysis only considered plants with at least the presence of external hyphae associated to the roots. After this filter a total of 108 plants were kept. Among these plants, 83% of the hybrids were considered as non-AM competent. The other root systems (17%) had internal structures characteristic of the AM symbiosis, as shown on Figure 35.

For this first screening only a sample of all root systems were observed in order to keep the plants alive for future analysis. Indeed, these plants are currently the object of vegetative propagation for a second AM screening and DNA extractions. In the Discussion section (part 3.2.3) the reasons why it is difficult to conclude on the dominant or recessive nature of the AM symbiosis in *Dryas* species are presented.

2.3. Genomic investigation on symbiotic genes

In order to identify the causative factors of the endosymbiotic polymorphic trait, preliminary genomic studies were performed. During these genetic comparisons, among the different reference genomes of model plants in the endosymbiosis field, *M. truncatula* was chosen as starting point. This choice is due to its genome quality in term of sequencing, annotation and compilation. On the basis of the *M. truncatula* genome, investigations were carried out on the presence or absence of orthologous genes in both *Dryas* spp.

	Gene name	Mt ID in genome v4.0	Dd ID in genome v1.0	Do ID in genome v1.0	CDS alignment gap	CDS alignment %identity
1	CASTOR	Medtr7g117580	Drydr411S21715*	Dryoc57S26677*	48	97,7
2	DMI1 / POLLUX	Medtr2g005870	Drydr34S20655	Dryoc124360S19988	9	98,8
3	CNGC15a	Medtr1g064240	Drydr51S22868	Dryoc370S24899	0	99,6
4	CNGC15b	Medtr4g058730				
5	CNGC15c	Medtr2g094860				
6	RAM1	Medtr7g027190	Drydr690S13573	Dryoc1788S22993	42	96,9
7	RAD1	Medtr4g104020	Drydr295S10168	Dryoc3491S13853	0	98,9
8	SEC13	Medtr3g054090	Drydr344S03618	Dryoc1259S11032	0	99,1
9	ELYS	Medtr5g012940	Drydr16S16592	Dryoc217S23390	0	99,1
10	NENA / SEH1	Medtr6g072020	scaffold86_380101_383274***	scaffold1123_8809_12224***	0	99,6
11	NUP133	Medtr5g097260	Drydr987S14816	Dryoc124459S10279	0	98,7
12	NUP85	Medtr1g006690	scaffold146_2300723_2306964***	scaffold124437_332063_338305***	0	99,4
13	IPD3 / CYCLOPS	Medtr5g026850	Drydr202S08474	Dryoc138S22273	30	98,1
14	DMI3 / CCaMK	Medtr8g043970	Drydr32S20242	Dryoc124299S01333	0	99,5
15	NSP1	Medtr8g020840	Drydr490S04636	scaffold4202_cov310_586076_587730***	0	98,8
16	NSP2	Medtr3g072710	Drydr40S04007	scaffold124289_483573_485114***	27	96,1
17	RAM2	Medtr1g040500	Drydr94S24667	Dryoc9385S07187	0	98,6
18	STR1	Medtr8g107450	Drydr192S07836	Dryoc138S22210	0	99,0
19	STR2	Medtr5g030910	Drydr87S24490	Dryoc756S06838	3	98,9
20	PT4	Medtr1g028600	Drydr2S08009	Dryoc124371S02116	15	97,8
21	VAPYRIN	Medtr6g027840	Drydr46S04331	Dryoc745S06808	6	97,8
22	CCD7	Medtr7g045370	Drydr41S21629	Dryoc1414S22435	0	99,0
23	CCD8	Medtr3g109610	Drydr146S16037	Dryoc124437S10120	0	98,7
24	MAX2	Medtr4g080020	Drydr229S02232	Dryoc1462S03615	21	97,6
25	DELLA1	Medtr3g065980	Drydr153S07143	Dryoc1731S03822	21	97,9
26	D14	Medtr1g018320	Drydr146S15906	Dryoc1272S21620	0	99,6
27	D14L	Medtr5g016150	Drydr284S02892	Dryoc3458S24773	0	98,5
28	NIN	Medtr5g099060.1	Drydr380S11191	Dryoc1938S12434	69	96,8
29	RPG	Medtr1g090807	Drydr103S00110	Dryoc317S13600	201	93,4
30	PUB1	Medtr5g083030	Drydr2S08284	scaffold124369_cov327_1986538_1988622***	15	98,0
31	LIN / CERBERUS	Medtr1g090320	Drydr276S09581	Dryoc122805S17489*	15	98,5
32	NUP96	Medtr5g097890	Drydr219S18017*	Dryoc1653S03770*	0	99,7
33	NUP107	Medtr8g022010	Drydr490S22714	Dryoc1269S21585	0	99,7
34	NUP160	Medtr1g063180	Drydr338S20595*	Dryoc570S26469*	0	99,4
36	NFP / NFR5	Medtr5g019040	Drydr368S03767	Dryoc124277S01099/Dryoc124277S01098**	0	98,6
37	DMI2 / SYMRK	Medtr5g030920	Drydr303S03268	Scaffold46413_cov288_532480_542645***	0	98,7

* -> *Dryas* gene annotation redone with FGENESH+
** -> 2 *Dryas* gene annotations corresponding to one gene in the other *Dryas*
*** -> *Dryas* gene annotation done with FGENESH+

Table 7 | Comparison of important endosymbiosis genes orthologous between both *Dryas* spp.
Determination of the orthologous genes in *Dryas* spp. of the *Medicago truncatula* ones. Pairwise comparison of the predicted *Dryas* spp. CDS was done based on MUSCLE alignment.
(data unpublished)

2.3.1. Presence / absence of genes

As a first step, based on the symbiotic toolkit described by Delaux et al. (2013a) and in combination with the symbiotic genes used in routine by our team, a list of the 37 important known genes for root endosymbioses (AM and also RNS) in *M. truncatula* was established (Table 7). This list covers the genes of the common parts within the symbiosis signalling pathways and some specific ones for nodulation or AM symbiosis. In collaboration with Griesmann and based on the first *Dryas* spp. genomes version, orthologous genes of this list were obtained with OrthoFinder then confirmed by synteny for both *Dryas* spp. In some cases, the *Dryas* spp. gene annotations needed to be redefined. Indeed, some *Dryas* spp. gene predictions were presenting supernumerary introns between both *Dryas* spp. or compared to *M. truncatula* or *L. japonicus* orthologous ones. Sometimes two predicted genes in *Dryas* sp. were corresponding to two exons in the other *Dryas* sp. or in *M. truncatula*. So in these suspicious situations, the nucleotide sequence was submitted to a bioinformatic gene prediction tool: FGENESH+ (Solovyev 2007). This was done in order to confirm or invalidate the original gene prediction. Sometimes, the orthologous gene has only been found in one of the *Dryas* spp. or even not at all. In these particular cases more careful investigations were done by blasting the *M. truncatula* gene and/or protein on the full *Dryas* spp. genomes. And in parallel the *M. truncatula* gene was used as query in order to find syntelogous region in *Dryas* spp. And when one *Dryas* sp. gene was annotated it was also used as query in order to compare and confirm the syntelogous gene found previously. When best blast hit and syntelogous region were corresponding, the *Dryas* sp. genome regions were submitted to FGENESH+ in order to predict a gene annotation. (See the example of *NSP1* gene which was unannotated in *D. octopetala* genome on Supplemental S3&4). After confirmation to have the *Dryas* spp. orthologous genes, alignment of them side by side between *D. drummondii* and *D. octopetala* was performed.

The table 7 shows the gene identification of the *M. truncatula* gene in correlation with the orthologous ones in *Dryas* spp. As it could be seen in this table, none of the genes in this list are missing or fragmented in *Dryas* spp. genomes. Moreover, looking closer to the predicted coding sequence (CDS) sequences only few SNPs (single nucleotide polymorphism) are found between both *Dryas* spp. Some genes are showing gaps, called DIPs such as *RAM1*, *CASTOR*, *CYCLOPS* or *PT4*. However, all these DIPs in the coding parts are a multiple of 3, this doesn't bring frame shift. Investigations were done on one of these genes with a DIP: *RAM1* (presented below in part 2.3.2).

a

	Mt gene name	Mt ID in genome v4.0	Lj gene name	Lj ID in genome v3.0	Dd ID in genome v1.0	Do ID in genome v1.0	CDS alignment gap	CDS alignment %identity
1	MtLYR10	Medtr7g029650	LjLYS16	Lj1g3v3834250	Drydr361S03721*	Dryoc1436S22612*	18	97,7
2	MtLYR8	Medtr5g042440	-	Lj0g3v0102179	DrydrC1210588S14895	scaffold2870_cov282_247003_250754***	0	99,7
3			-	Lj0g3v0124999				
4	LYK11	Medtr8g014500	LjLYS5	Lj3g3v2318170	Drydr344S20828	Dryoc1259S21404	0	99,5
5			LjLYS4	Lj3g3v0290100				
6	LYK10	Medtr5g033490	LjLYS3/EPR3	Lj2g3v1415410	scaffold13_1394870_1398848***	Dryoc1254S21264*	3	98,4
7	-	Medtr1g094730	-	Lj5g3v1811460	scaffold105_258915_260153***	Dryoc679S15237	0	99,2
8			-	Lj0g3v0106309				
9	-	Medtr2g095180	-	Lj3g3v1101700	Drydr51S04719*	Dryoc370S05289	12	98,1
10					Drydr51S12899	Dryoc370S05288	0	98,2
11	MtLYR3	Medtr5g019050	LjLYS12	Lj2g3v1828320	Drydr368S03773	Dryoc3475S05200	6	98,1
12	MtLYR2	Medtr1g021845	-	**Lj0g3v0145339**	Drydr368S03769	Dryoc3475S24779	**premature STOP codon in *D. octopetala***	
13	MtLYR4	Medtr5g085790	LjLYS13	Lj2g3v2899910	Drydr60S04994	Dryoc3780S05443	48	97,4
14	MtLYR7	Medtr3g080170	LjLYS14	Lj2g3v2899900				
15	LYK8	Medtr2g024290	LjLYS7	Lj6g3v1812110	Drydr64S23432	scaffold3035_cov254_60194_55004***	**N sequence in the middle of *D. octopetala***	
16			-	Lj6g3v1812100				
17	LYK9	Medtr3g080050	LjLYS6	Lj6g3v1055580	-	-	-	-
18	LYK3	Medtr5g086130	LjNFR1	Lj2g3v2904690	scaffold316_408621_413861***	Dryoc2271S23481*	0	99,4
19	LYK2	Medtr5g086330						
20	LYK7	Medtr5g086030	LjLYS1	Lj2g3v2904610				
21	LYK6	Medtr5g086040						
22	-	Medtr5g086080	-	-				
23	LYK5	Medtr5g086090	LjLYS2	Lj2g3v2904640				
24	LYK4	Medtr5g086120						
25	LYK1	Medtr5g086540						
26	MtNFP	Medtr5g019040	LjNFR5	Lj2g3v1828350	Drydr368S03767	Dryoc124277S01099/Dryoc124277S01098**	0	98,6
27	MtLYR1	Medtr8g078300	LjLYS11	Lj4g3v0912440				

b

	OthoFinder tree n° (S3 part. 4)	Mt ID in genome v4.0	Lj ID in genome v3.0	Dd ID in genome v1.0	Do ID in genome v1.0	CDS alignment gap	CDS alignment % identity	Remarks
1	1	Medtr1g009270	Lj0g3v0133349	Drydr146S16082	Dryoc124516S20838	30	97,2	**gap in the kinase domain of *D. octopetala* (confirmed by PCR-sequencing)**
2		Medtr8g087420	**Lj1g3v1991630**					
3		Medtr8g101260	/	Drydr232S08845	Dryoc124437S10104	0	98,4	
4		Medtr6g093050	Lj5g3v0196811					
5		Medtr2g105900	Lj0g3v0094389	Drydr490S04685	scaffolf124556_cov322_468519_465940***	30	97,1	
6	2	Medtr5g075630	/	Drydr285S09810	Dryoc136S21976	21	97,2	gap in *D. drummondii*
7		Medtr3g062500	Lj0g3v0115709					
8		Medtr7g073710	Lj1g3v2536060	Drydr291S19673	Dryoc124346S19689	0	99,6	
9		Medtr5g068210	Lj2g3v2173020	Drydr2S08214	scaffold124369_cov327_1413875_1415942***	0	99,4	
10		Medtr3g093710	Lj1g3v1113880					
11	3	Medtr2g030380	Lj0g3v0361919	Drydr328S03463*	Dryoc1123S00266	0	99,4	
12		Medtr5g087780	Lj2g3v2984880	Drydr241S02441	Dryoc124348S01977	0	98,9	
13		Medtr3g094710	Lj1g3v1207870	Drydr22S18037	Dryoc124390S20279	0	98,9	
14	4	Medtr6g470960	Lj0g3v0019189	Drydr192S07840	Dryoc138S22207	6	99,0	gap in *D. drummondii*
15			Lj0g3v0019209					
16		Medtr8g107470	Lj0g3v0328989					
17		Medtr5g078080	Lj0g3v0161519	Drydr27S09379	Dryoc1938S12468	0	99,0	
18	5	Medtr5g009660	Lj2g3v2087830	scaffold615_122873_119733***	Dryoc124325S19332*	0	99,0	
19		Medtr8g099195	Lj4g3v3002540					
20		Medtr3g090660	Lj1g3v0934380	Drydr2S08026	Dryoc124371S02112	0	98,9	
21		Medtr3g090665						
22		Medtr1g028890	Lj0g3v0308309					
23	6	Medtr4g014070	Lj0g3v0356809	Drydr238S18604	Dryoc11743S07757	0	99,6	
24		Medtr5g083480	Lj0g3v0055039	Drydr2S08262	scaffold124369_cov327_1816345_1818990***	0	99,1	
25			Lj2g3v2843040					
26	7	Medtr8g021350	/	Drydr490S04627	Dryoc2378S04362	24	98,1	gap in *D. drummondii*
27		Medtr2g042710	/	Drydr45S12376*	Dryoc2953S13472*	0	98,6	
28		Medtr4g124990	Lj5g3v1749280					
29	8	Medtr4g113100	Lj0g3v0346099	Drydr443S12157	Dryoc4986S26064	0	98,7	
30	9	Medtr1g107460	Lj5g3v2113420	Drydr459S12667	Dryoc939S27999	0	98,8	
31		Medtr4g094958	/	Drydr284S19423	Dryoc124303S18811	0	98,9	
32	10	Medtr4g094885	Lj6g3v2158590	Drydr284S19431	Dryoc124303S18798*	15	98,4	gap in *D. drummondii*
33		Medtr3g102180	Lj1g3v1686670	Drydr146S15940	scaffold842_cov316_609846_612519***	0	98,8	
34	11	Medtr3g078250	Lj1g3v0415090	Drydr295S10075	Dryoc1290S11294	0	99,5	
35		/	Lj4g3v1535150					
36		Medtr2g023150	Lj0g3v0043799	Drydr226S18342	Dryoc1228S17759#	39	97,1	**no gap (sequencing error found by PCR-sequencing)**
37	12	/	/	Drydr146S00962	Dryoc124399S09791	9	98,0	gap in *D. octopetala*
38		Medtr5g055470	/	Drydr94S24708	Dryoc124421S20489	0	99,5	
39		Medtr1g110280	Lj5g3v2182520					
40	13	/	/	Drydr94S06024	Dryoc124421S20488	0	99,2	
41		Medtr1g109580	Lj5g3v2179880	Drydr458S22222	Dryoc3488S13827	0	99,3	
42			Lj5g3v2179770					
43		/	Lj0g3v0291629					
44	14	Medtr7g070200	Lj1g3v2377960	Drydr13S15552	Dryoc1047S16865	0	99,0	
45		Medtr4g014350	Lj3g3v1381520					
46	15	Medtr7g103440	Lj1g3v4693100	Drydr270S09399	Dryoc5949S26711	0	99,0	
47		Medtr1g061590	Lj0g3v0154969					
48			Lj5g3v0705400					
49	16	Medtr8g046290	Lj0g3v0073209	Drydr2S01726*	scaffold124369_cov327_1081750_1070041***	0	99,5	
50			Lj0g3v0073219					
51	17	Medtr4g074080	Lj3g3v3500180	Drydr96S14706	Dryoc124252S18122	18	98,3	gap in *D. drummondii*
52	18	Medtr1g052425	Lj0g3v0328559	Drydr49S22603	Dryoc5307S14592	0	99,5	
53	19	Medtr7g059225	Lj1g3v3464950	Drydr380S11325	Dryoc7545S15634	0	99,6	
54	20	Medtr6g069030	Lj2g3v0561100	Drydr192S07906	Dryoc124309S18845*	0	99,7	
55	21	Medtr2g008740	/	Drydr265S09332	Dryoc124300S18768/Dryoc124300S01356**	66	94,6	**Fragmented gene**
56	22	Medtr5g094380	Lj2g3v3234610	Drydr64S23384	Dryoc124371S09594*	3	98,6	gap in *D. drummondii*

* -> *Dryas* gene annotation redone with FGENESH+
** -> 2 *Dryas* gene annotations corresponding to one gene in the other *Dryas*
*** -> *Dryas* gene annotation done with FGENESH+

Table 8 | Comparison of LYMS-RLK and LRRIII-RLK genes orthologous between both *Dryas* spp.
Determination of the orthologous genes in *Lotus japonicus* and *Dryas* spp. of the *Medicago truncatula* LYSM-RLK (a) or LRRIII-RLK (b). List from the iTAK database. Pairwise comparison of the predicted *Dryas* spp. In the column "Remarks" when it is notified "gap in *D. drummondii*" it means that there is a gap in *D. drummondii* sequence compared to *D. octopetala* and the *D. octopetala* one is similar to the other Rosaceae. The *L. japonicus* genes affected in a LORE 1 insertion line used in part 2.3.3 are highlighted in red. (data unpublished)

With the fact that AM fungi never enter inside the *D. octopetala* roots, a second set of genes investigations were performed in the upstream part of both symbiosis signalling pathways. Indeed, genetic comparison was carried out focusing on three families of receptor-like kinase (RLK) which contained important symbiosis genes. These families are the LYSM-RLK (Lysin motif domain-containing RLK; including the nod-factor receptor or perception genes: *NFP* and *NFR*), and two LRR-RLK (leucine-rich-repeat RLK): the subfamilies LRR-III (*LjALB1*; aberrant localization of bacteria inside nodule1) and LRR-I-1 (also named ShRKs because contained all the SYMRK homolog receptor kinases). As previously, the same process was carried out on the 22 LYSM, 34 LRR-I-1 and 47 LRR-III of *M. truncatula*, extracted from the iTAK database (Zheng et al. 2016).

The LRR-I-1 is a huge gene family which is derived from several series of gene duplications and translocated on the same chromosome. Indeed in *M. truncatula* there are 65% of these genes on the chromosome 8. This made the fine determination of each orthologous gene quite difficult (Supplemental S5 part 5). However, orthologs of all *D. drummondii* LRR-I-1 were found in *D. octopetala* (data not shown).

For the two other investigated RLK families, we could easily find and distinguish all genes. There is no absence of orthologous genes between both *Dryas* spp. In some cases, for one gene in *M. truncatula* several orthologous ones could be found in both *Dryas* spp. and *vice versa*. For example, in the first list, there are three versions of *CNGC15* in *M. truncatula* and only one in both *Dryas* spp. (Table 7 lines 3-5); just like, it is found one gene in both *Dryas* spp. for seven homologous ones in *M. truncatula* (from *LYK1* to *LYK7*) or three in *L. japonicus* (*NFR1*, *LYS1* and *LYS2*); Table 7 lines 18-25). And in another instance, *MtLYR8* has two homologous genes in *Dryas* spp. (Table 8 a lines 15,16). This highlights some specific duplications during the evolution of the green lineage that separates the Fabaceae from the Rosaceae. As for the first list of genes when nucleotide sequences were aligned few SNPs were found. Also, several gene predictions were submitted to FGENESH+ in order to confirm or redefine the annotations; afterwards some sequence regions were amplified by PCR and resequenced. Some rare SNPs were invalided, this is the case for example for the *Dryoc1228S17759* (Table 8 b line 11).

Globally the orthologous investigation and comparison on the 37 genes of the first list (Table 7) and on these three RLK families (Table 8 a&b) have not highlighted an absence of gene between both *Dryas* spp. Focusing on the LYSM and LRR-III families, only few of them (one LYSM and two LRR-III) are presenting polymorphism that could be resulting in a difference of phenotype or protein functionality. One LRRIII present a gap in the kinase domain of *D. octopetala* (Table 8 b line 1) and another one is fragmented (due to 375bp insertion in *D. octopetala*, Table 8 b line 55). Whereas one LYSM present a frame shift bringing a premature

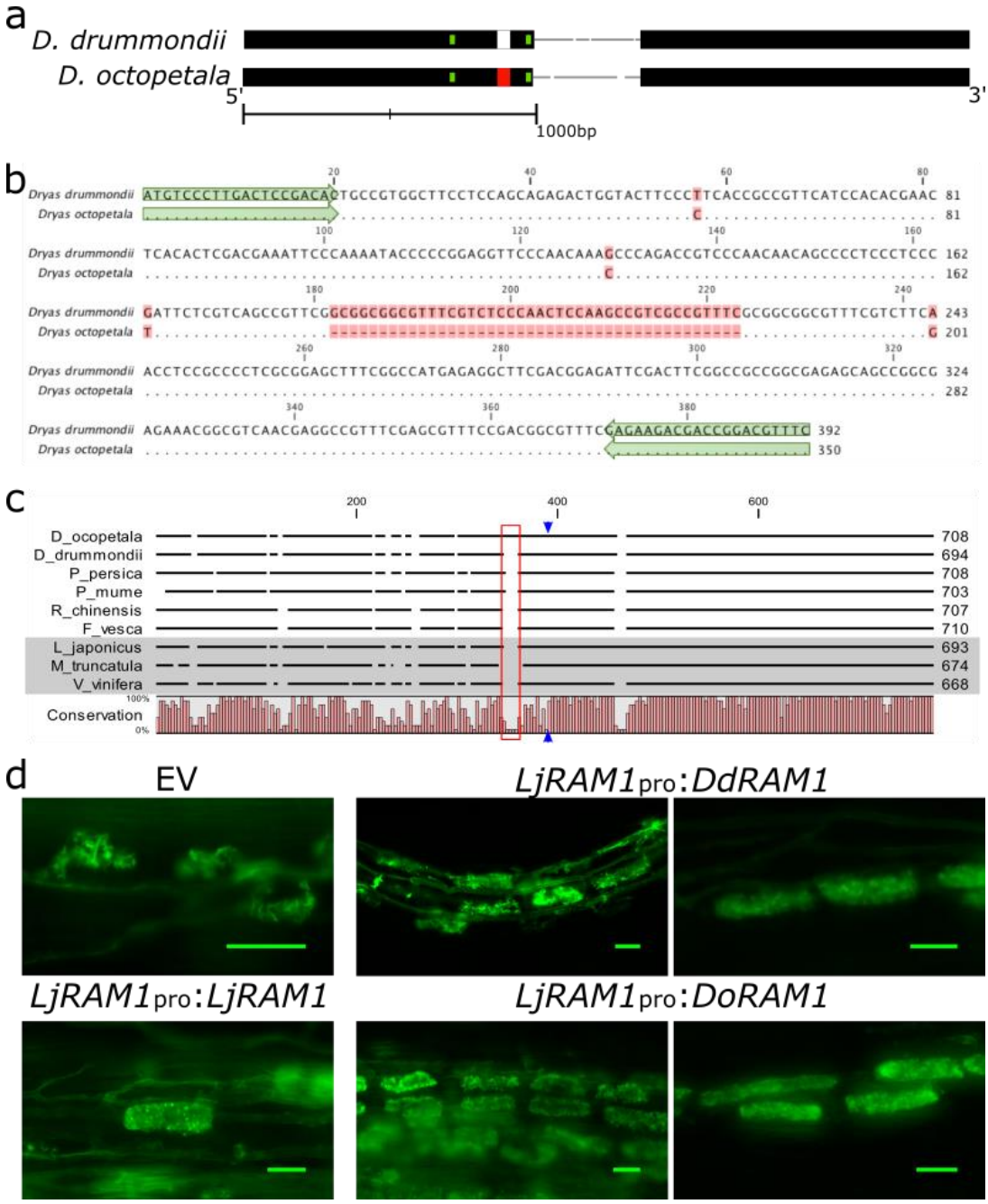


Figure 36 | Analysis of the deletion/insertion polymorphism in *Dryas* spp. *RAM1*.
(a) Alignment of the gene structure of *Dryas* spp. *RAM1* with locations of the *D. octopetala* insertion (red box) and of the primers used bellow (green boxes). Black boxes indicate exons separated by introns (grey lines) and gaps are represented in blank. (b) Nucleotide alignment of the fragments from *D. drummondii* ecotype DA462 and *D. octopetala* ecotype DA460. Matching residues are marked as dots and differences are highlighted in red. Green arrows highlight the primers used. (c) Alignment of the full RAM1 protein from different Rosaceae (*D. octopetala*, *D. drummondii*, *Prunus persica*, *Prunus mume*, *Rosa chinensis* and *Fragaria vesca*) and from *Lotus japonicus*, *Medicago truncatula* and *Vitis vinifera* (non Rosaceae plants are with grey background). *D. octopetala* specific insertion is highlighted with the red box. Bleu arrows show the separation between both exons. (d) Laser scanning confocal images of *L. japonicus* hairy roots colonized by *Rhizophagus irregularis*. *ram1-3* mutant transformed with an empty vector control (EV), *ram1-3* transformed with a genomic fragment containing the *L. japonicus*, *D. drummondii* or *D. octopetala* *RAM1* gene, and a 1,861 bp *LjRAM1* promoter fragment upstream of the transcriptional start site at 5 weeks post-inoculation are shown. Scale bars denote 50µm. The fungus is stained with WGA-Alexa- Fluor488.
In collaboration with Aline Sandré who cloned the *Dryas RAM1* genes and performed the root transformation (data unpublished)

STOP codon in the middle of the gene (Table 8 a line 12) and a second one could not be defined due to the presence of an ambiguous residues sequence (Table 8 a lines 15-16). Preliminary investigations on two of these putative candidates are presented in a next part (part 2.3.3).

2.3.2. *RAM1*: a deletion/insertion polymorphism case study

As presented during the first targeted genetic comparison performed, all symbiotic genes were present in *D. octopetala,* but some important ones have striking DIPs. This is the case for the *RAM1* gene which has an insertion of 42bp in *D. octopetala*. This insertion is located at the end of the first exon (Figure 30 and 37 a&b), which is considered as the variable part of this gene family. This DIP was consistently confirmed by PCR done on several ecotypes and genotypes of both *Dryas* spp. Moreover, those PCR have highlighted the homozygous status of each *Dryas* spp. independently of the genotype or ecotype. In addition, the alignment of RAM1 from diverse AM plants (*M. truncatula*, *L. japonicus*, *Vitis vinifera* and Rosaceae, such as *Prunus persica*, *Prunus mume*, *Rosa chinensis* and *Fragaria vesca*) highlights that this insertion is specific to *D. octopetala* (Figure 36 c).

This insertion of a multiple of three does not bring a frame shift but adds 14 amino acids to the protein. In order to verify if this insertion leads to a functional protein or to a loss of function, interspecies complementation tests were done. *Dryas* spp. *RAM1* genes from both species have been cloned (from the START codon to the STOP codon including the intron) then introduced in the *L. japonicus ram1-3* mutant. A mutation on *LjRAM1* gene does not lead to a non-AM phenotype. However, this mutant is characterized by the formation of aborted arbuscules (Pimprikar et al. 2016); Figure 36 d). After hairy root transformation of *ram1-3* mutants with either native *L. japonicus* or *Dryas* spp. *RAM1* under control of *LjRAM1* promoter, plants were inoculated with *R. irregularis* prior laser confocal observations of the arbuscules. A transformation with a dummy sequence at the place of the gene of interest (empty vector, EV) was used as negative control (Figure 36 d). As presented in Pimprikar et al. (2016) the complementation of this mutant with the native *LjRAM1* allows to restore the wild type arbuscules phenotype (Figure 36 d). *L. japonicus ram1-3* mutants transformed with *D. drummondii RAM1* exhibit wild type arbuscules, filling totally the host cells. This shows that the *D. drummondii* RAM1 protein is functional and that the interspecies cross complementation works (Figure 36 d). Surprisingly this cross complementation is also working with the *D. octopetala* gene version. Indeed, arbuscules in the *ram1-3* mutant transformed with the *RAM1* gene containing the 42 nucleotides of the *D. octopetala* insertion, are filling fully the host cells, as the wild type (Figure 36 d).

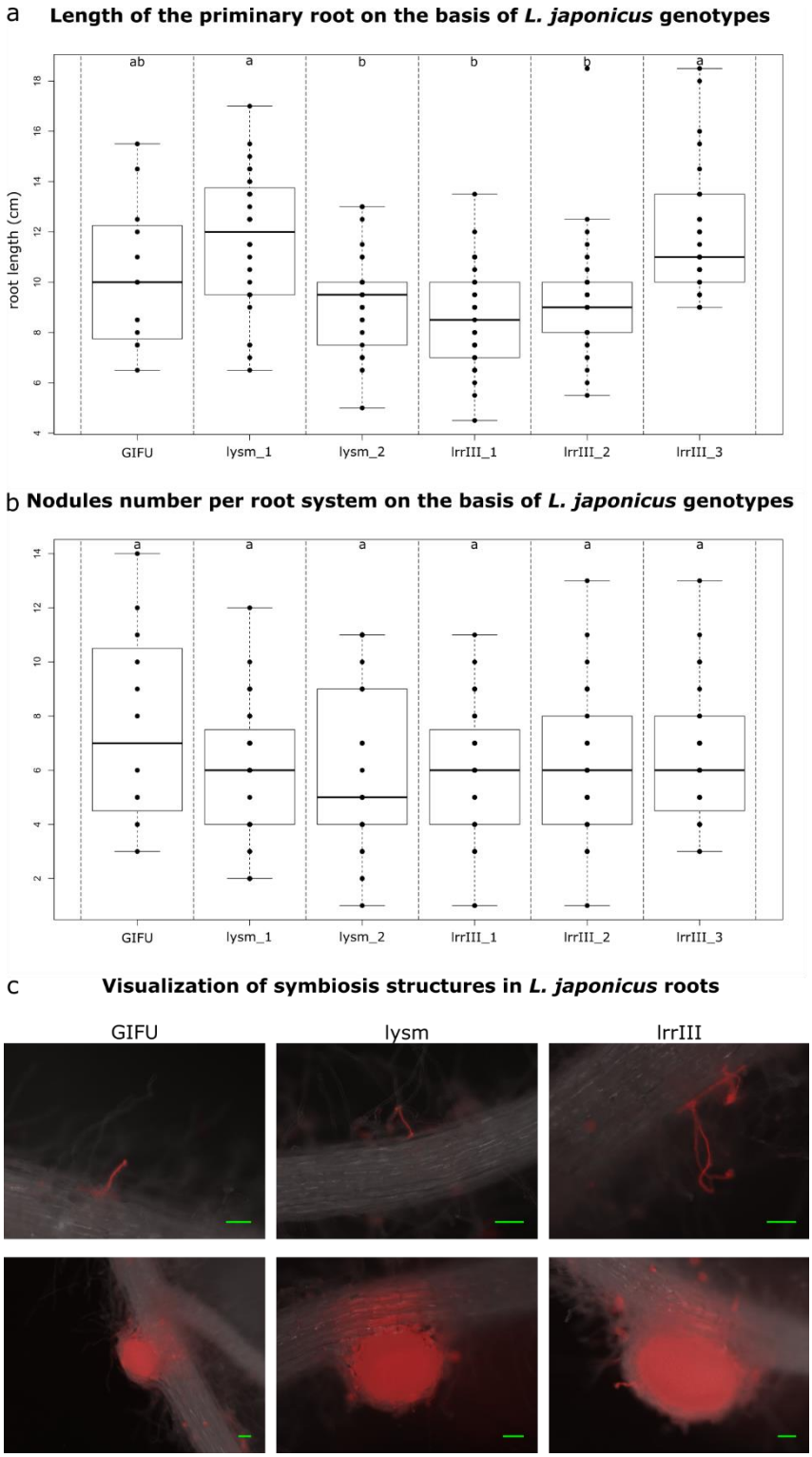


Figure 37 | Phenotyping of *Lotus japonicus* LORE 1 insertion lines.
(a&b) Determination of the primary root length (in cm; a) and of the nodule number per root system (b) in wild type *L. japonicus* (GIFU), in two LORE 1 insertion lines affecting a LYSM gene (Lj0g3v0145339; lysm_1 and lysm_2) and in three LORE 1 insertion line affecting a LRRIII gene (Lj1g3v1991630; lrrIII_1, lrrIII_2 and lrrIII_3). (c) Visualisation of representative symbiotic structures in the different lines used in a. *Mesorhizobium loti* expressing DsRED highlight the infection threads shape (upper panel) and the colonization of nodules (lower panel). Roots were analysed 5 weeks after inoculation. 2 replicates with an average of 14 plantlets per genotypes for each replicate. Lines reference and seed bags number are listed in Supplemental S6. Scale bars denote 50µm
(data unpublished)

2.3.3. LORE1 analyses of the LYSM and LRR-III candidates

From the second genes comparison done on the receptor kinase families (Table 8 a&b) some genes were presenting striking differences, that could lead to distinct phenotypes. Indeed, as mentioned in part 2.3.1, one LYSM and two LRR-III present between both *Dryas* spp. premature STOP codon and/or frame shift. These genes were either not described in *L. japonicus* or *M. truncatula* or not investigated for symbiosis. In front of the lack of available mutants in *Dryas* spp., the mutant version of the orthologous genes in *L. japonicus* were obtained in the LORE 1 *Lotus* mutant bank (Małolepszy et al. 2016). However, on these three *Dryas* spp. genes, one of the LRR-III does not have ortholog in *L. japonicus* (Table 8 b line 55 and Supplemental S6). In order to limit the risk of misleading observations due to background mutations, several lines with exonic insertion into the gene of interest and as few other insertions as possible were selected (Supplemental S6). Two and three mutant lines were respectively ordered for the remaining LYSM and LRR-III candidates. When homozygote status was confirmed for the gene of interest in the LORE 1 lines, phenotyping assays of both symbioses were carried out. These assays were done on plants from two different seed bags for each LORE 1 lines, with an average of 14 plants per seed bags. All the mutant lines were presenting root system development similar to the wild type (Figure 37 a). All mutants were colonized with *R. irregularis* and presenting normal developed arbuscules (data not shown). After inoculation with DsRed rhizobia, all mutants were showing nodule number and development similar to the wild type as for their infection threads (Figure 37 b&c).

3. Discussion

Holmer et al. (2017) concluded that the combination of next generation sequencing and the emergence of new model species are important steps towards advancing the understanding of the evolution of the nitrogen-fixing root nodule symbiosis. With regard to the aim of engineering nitrogen-fixing root nodule symbiosis in non-legumes it is crucial to understand which genes distinguish nodulators and non-nodulators. Within the Rosaceae, the genus *Dryas* contains very closely related nodulating and non-nodulating species, allowing full genome comparisons. Due to its basal phylogenetic position within the Rosaceae, *Dryas* represents a key genus for evolutionary studies. Based on this, my thesis attempts to develop this plant genus in a laboratory context as a model, while focusing on root endosymbioses.

To establish *Dryas* as a new model genus in the Rosaceae family, the first step was to develop and adapt protocols to introduce *Dryas* spp. into the laboratory. Secondly, an investigation into arbuscular mycorrhizal (AM) symbiosis was needed. Indeed, to date, no published study has been conducted on this symbiosis within this genus. Then finally, a first effort at genomic investigation and comparison has been carried out.

3.1. *Dryas* a new genus model

3.1.1. Introduction of *Dryas* spp. in a laboratory context

Dryas spp. had never been used routinely in laboratories. Before the setting up of any molecular or genetic study protocols, the development of cultivation methods under controlled and sterile conditions was necessary. I have tested several seed surface sterilization procedures. The most efficient one (between high germination rates while eliminating contaminations) was obtained with the use of concentrate hydrogen peroxide. An interval of four days to reach maximum germination is observed between *D. drummondii* and *D. octopetala*. This interval seems due to plant species. Indeed, this is consistent for all cultivars examined, not only for seeds produced in the greenhouse or botanical garden during the same month, but also for commercial seeds. However, given that *D. octopetala* has a very wide distribution, it is well possible that the four lineages examined do not encompass the entire variability of this species.

Once optimal sterilization and germination rates had been established, I have carried out different growth protocols from conventional pots to plates, to hydroponic system and jars. These distinct growth systems combine diverse advantages for research such as sterile culture, conditions for root system observations and for inoculation with the microsymbiont. *Frankia* strains able to nodulate *D. drummondii* have not yet been successfully cultured (Pawlowski and Demchenko 2012), necessitating infection with crushed nodules. Because these nodules carry a rich fungal and

bacterial microbiome on the surface, inoculation of *Dryas* spp. with these nodules while maintaining a gnotobiotic system is challenging. On the other hand, plants grown in pots do not represent the most suitable system for root analyses. The process of cleaning the soil from the roots with water (deionized or not) could interfere with experiments due to putative osmotic shock; furthermore, either brushing the soil or harvesting root systems entails the risk of breaking thin and fragile lateral roots and root hairs. To circumvent this issue, plants can be grown in Petri dishes. For *Dryas* spp. this system was suited for early stages of development; for longer experiments exceeding five weeks, root and shoot growth required more space. Furthermore, shielding of plates never totally protected the roots from light, and a long exposure of roots to light tends to interfere with the analysis of root responses to any treatment. Exposure of roots to direct light modifies their transcriptome (Hemm et al. 2004) and often leads to stress responses, which can perturb the analyses and cause misleading effects. Hydroponic systems offer the possibility to observe the roots in a non-invasive way while also shielding them from light. They can be used with or without an inert substrate that mimics physical soil contact. The fact that *Dryas* spp. can grow in well-aerated soil but can also tolerate flood periods (West et al. 1993), suggested the use of hydroponics as a method of choice. Moreover, this system leads to healthy, lignin-poor plants that are suitable for a variety of experiments.

As mentioned in introduction, *Dryas* are woody shrubs showing predilection for calcareous soil. Among the different growth medium tested ¼ Hoagland was retained. Indeed, besides the fact that Hoagland was originally developed for woody plants, it is the medium that contains the most calcium compared to the other tested media. And, it gives the healthiest growing plants.

As soon as *Dryas* spp. could be grown under a controlled environment, vegetative and sexual propagation was investigated. In the wild, the clonal growth of *Dryas* spp. enables individuals to persist and grow in extreme environments where sexual proliferation is often unsuccessful (Wookey et al. 1995), and where individual clones of *D. octopetala* commonly live for more than 100 years (Kihlman 1890; Crawford 1989). The growth of cuttings was obtained in three weeks without the use of hormone cocktail and with a high success rate (over 65%). This easy protocol for the vegetative propagation of *Dryas* spp. by cuttings is an important tool for performing experiments on a high number of plants that have the same genotype, and it obviates the requirement for seeds.

Dryas is a perennial plant genus with bisexual flowers. *D. drummondii* has been described to flower in its fifth year (Lawrence et al. 1967), indicating a long generation time. Nodulation, plant growth and flowering processes in *Dryas* spp. seem to be extremely dependent on the environment and on light quality and intensity. Kohls et al. (1994) succeeded to obtain *D. drummondii* nodules after growing them at a light intensity of 700 $\mu mol.m^{-2}.s^{-1}$. Sexual

reproduction of *Dryas* spp. was feasible in a laboratory context when sufficient light intensity of a suitable spectrum was provided (high-pressure sodium lamps provide light with a richer emission in yellow-orange and a red/far red ratio shifted to the far red compared to standard high-pressure mercury vapour lamps and fluorescent lamps). Compared to the fifth year previously suggested by Lawrence et al. (1967), under high-pressure sodium lamps flowers and seeds of *Dryas* spp. were obtained in less than a year after seed sterilization or vegetative propagation. In the field, *Dryas* flower primordia are formed during the summer, *i.e.*, far in advance of flowering, which occurs in the next year shortly after snowmelt, with most individuals flowering within a month (Lawrence et al. 1967). This behaviour suggests that the development of floral primordia and blooming depends on photoperiod or vernalization (or both). Therefore, changes of light period and temperature might further speed up the induction of flowering and shorten the generation time.

In their natural habitat, *Dryas* depend mainly on insects for pollination (Kevan 1975; Roslin et al. 2013; Tiusanen et al. 2016). While the large and open flowers of *D. octopetala* (Figure 20 f) made manual pollination possible, *D. drummondii* flowers were never completely open during full bloom (Figure 20 e) which made manual pollination difficult. Nevertheless, pollen could be easily collected. Hybrids are often used in genomic studies for genome comparison, but the structure of *Dryas* flowers with more than 50 stamens makes the emasculation of these flowers difficult. However, hybridization between *Dryas* spp. occurs naturally in the wild, and is the main reasons why the taxonomy of the *Dryas* genus is so controversial. In areas where different *Dryas* spp. cohabit, natural hybrids were described between *D. integrifolia* and *D. octopetala* (Philipp and Siegismund 2003), or between *D. drummondii* and *D. integrifolia* (known as *D.* x *lewinii*). The German botanist Franz Sündermann also created *D.* x *suendermannii* by crossing *D. drummondii* with *D. octopetala* (Packer 1994), now part of the collections of the botanical gardens of Vienna and Lindau where the hybrids are maintained by clonal propagation. Seeds from hybrids growing in these botanical gardens occur, but it is not possible to determine whether they are backcrossed with other *Dryas* spp. or with *D.* x *suendermannii* itself. Although the level of hybridization is unknown, these plants might still represent an interesting tool for genomic and phenotypic analyses.

3.1.2. Standard protocols adapted to *Dryas* spp.

Once, I have adapted in the laboratory growth of *Dryas* spp. under controlled conditions, several conventional protocols were used and optimized for this plant genus. Such essential protocols are ranging from transient root transformation to the extraction of nucleic acids (for genome sequencing and PCR analyses).

For a model plant, a protocol for genetic modification is important in order to analyse the expression of marker gene promoter-reporter gene fusions, or to perform reverse genetics. Hairy root transformation mediated by *Agrobacterium rhizogenes* is the most commonly used technique to introduce chimeric constructs into plant roots. The fact that this method does not transform the shoot is no hindrance to the study of root symbioses; *A. rhizogenes*-mediated hairy root transformation is routinely used not only in the model legumes *Lotus corniculatus* var. *japonicus* (Díaz et al. 2005) and *Medicago truncatula* (Boisson-Dernier et al. 2001) but also for actinorhizal plants like *Datisca glomerata* (Markmann et al. 2008), *Casuarina glauca* and *Discaria trinervis* (Svistoonoff et al. 2011) and non-FaFaCuRo plants like tomato (Ron et al. 2014). After a first optimization of the transformation protocol (depending of the bacterial optical density), a transformation of 55 to 70% for *D. drummondii* and a maximum of 30% for *D. octopetala* was obtained on a routine basis (Figure 27). Previous studies have shown that hairy roots induced by different bacterial strains can vary in morphology and production of secondary metabolites (Thwe et al. 2016); it was also shown that plant defence reactions, phytohormone signalling and secondary metabolism could be affected by high expression levels of the agrobacterial *rolB* gene (Bulgakov et al. 2018). Thus, the difference in the reactions of two closely related species to the same *A. rhizogenes* strain is interesting. At any rate, since only one *A. rhizogenes* strain was used in this study, the use of other strains might leave room for further optimization of hairy root transformation of *D. octopetala*.

To establish an organism as a model, it is essential to have access to its nucleic acids routinely and easily. The woody nature of *Dryas* and the composition of their leaves made the DNA and RNA extractions challenging. A first goal was to obtain enough DNA of high quality and yield, in order to perform *de novo* whole genome sequencing. The best method found to carry this aim out was resorted to caesium chloride (CsCl) gradient technic. It is a procedure taking several days and requiring several hazardous chemicals (such as CsCl, ethidium bromide, trichloromethane...). However, the DNA extractions obtained have allowed the genomes of both *Dryas* spp. to be sequenced in depth and *D. drummondii* one is already publicly available. In a second hand, I have tested several methods in order to obtain easily and quickly DNA extractions allowing robust PCRs. A one day protocol, based on Khanuja et al. (1999), was retained. The 230/260 and 280/260 ratio attesting of the DNA quality were the less variable and the closest to the expected theoretical values (Figure 28). Reproducible and robust PCRs were performed on extracted DNA with this method and this on several different *Dryas* spp., ecotypes and genotypes (Figure 29&30).

Through the *Dryas* spp. genome access and the reproducible PCRs, the ability to clone *Dryas* spp. genes is henceforth achievable (Figure 36 d). This combined with the capacity to

introduce chimeric constructs into root systems (Figure 27) opens the possibility to study *Dryas* genetics in depth, allowing cross-species complementation, as well as transient expression, protein localization, and reverse genetics using CRISPR/Cas or RNAi methods.

3.1.3. First *Dryas* spp. genomic data

Dryas are diploid with an estimated genome size of 250 Mbp (Griesmann et al. 2018) distributed over 9 chromosomes (Potter et al. 2007), less than *Malus* x *domestica* (apple) which is diploid or triploid with 750 Mbp, or *Rosa* spp. which are tetraploid or triploid with 600 Mbp (Jung et al. 2013). The first *D. drummondii* genome version is publicly available. All data related to this genome have been deposited in GigaDB (Griesmann et al. 2018). It is estimated to be assembled at 92.11% and 86.64% of the predicted gene models are annotated (Table 5 and Supplemental S2). The *D. octopetala* genome is also sequenced. Its genome quality has not been analysed, because *D. octopetala* was not included in Griesmann et al. (2018) and the sequencing raw data were not available during the writing of this thesis (they were hold back at the BGI centre). The analysis of *D. octopetala* genome quality is ongoing at the Helmhotz Zentrum München and after optimization will be a part of a future publication. So it will be also publicly available. The first genome size estimation of *D. octopetala* genome is 253 Mbp. These small genomes, combined with a generation time of less than a year, makes *Dryas* suitable as model genus for the Rosaceae family. Moreover, the first *Dryas* spp. genome versions show low heterozygosity and relatively good assembly that allow reliable genomic investigations.

I have performed a first genetic comparison on housekeeping genes, internal transcribed spacer (ITS) regions and some genes involved in symbiotic pathways by looking on the genomes in combination with PCR amplification and subsequencing. The high sequence conservation in those regions highlighted the similarities between *D. drummondii* and *D. octopetala* (Figure 29&30). However, some genes (such as *RAM1* and *CASTOR*) show the existence of deletion/insertion polymorphism (DIPs; Figure 30 and Table 7-9). When found in coding regions, these DIPs usually consist of a multiple of three, preserving the reading frame of the encoded protein. When PCRs were performed on several genotypes and ecotypes of both *Dryas* spp., all investigated plants were homozygotes for these regions in *RAM1* and *CASTOR* genes (Figure 30). Moreover, these DIPs are species-specific. These differences can therefore be used as markers to distinguish *D. drummondii* from *D. octopetala* but also due to their homozygotic status in the parental plants, they could be used on hybrids.

On the other hand, despite a relatively correct assembly quality for a first genome version, in genetic comparisons (presented in part 2.3.1) several sequencing and gene prediction errors have been highlighted. Indeed, when looking for orthologues, several genes did not present

annotation in one or both *Dryas* spp. The gene prediction and annotation were performed using a computational tool and supplemented by two transcriptomes. However, the latter were obtained from seedlings or leaves resulting in a lack of root genes representativity. RNA extraction from more tissues will improve annotation quality and coverage. Moreover, additional genomic DNA extraction allowing the construction of larger and more libraries would allow in the future a finer genome assembly to a chromosomes level.

Together with their small sequenced genomes, the basic but indispensable procedures and protocols for cultivation, vegetative and sexual propagation, hairy root transformation and nucleic acid isolation described above, *Dryas* emerges as a new model genus to study important traits associated with survival in arctic conditions, including the formation of root symbioses with bacteria (*Frankia* with *D. drummondii*) and ectomycorrhizal (ECT) fungi (*D. octopetala* and *D. integrifolia*).

3.2. Endosymbiosis polymorphic trait

3.2.1. Lack of *Frankia* inoculum for *Dryas drummondii*

Dryadoideae subfamily is gathering all described nodulating genera of the Rosaceae (*Cercocarpus*, *Chamaebatia*, *Dryas* and *Purshia*). All these four genera enter into symbiosis with Cluster II Frankia bacteria. This cluster of Frankia have not yet been successfully cultured (Pawlowski and Demchenko 2012). Until now in published studies, only Kohls et al. (1994) have succeeded to obtain *D. drummondii* nodules in laboratory. They started from *D. drummondii* crushed nodules and soil collected close to nodulated *Dryas* sp. These inoculums were able to induce nodulation not only with *D. drummondii* plants but also with other Dryadoideae and non-rosaceous actinorhizal plants (such as *Alnus glutinosa*, *Elaeagnus angustifolia* and *Myrica cerifera*). However, all attempts to nodulate *Dryas* spp. with crushed nodules or isolated Frankia from other Rosaceae failed to produce effective symbiosis. This might suggest that *Dryas* sp. is more selective than other rosaceous plants in terms of its bacterial partner. In their publication, Kohls et al. (1994) obtained only few nodules on *D. drummondii*, after 12 weeks. As suggested by Dalton and Zobel (1977) rosaceous plants are slow to nodulate. Kohls et al. (1994) concluded that this was maybe due to the slow growing character of these plants. In addition, their assays were conducted under high light intensity (700 $\mu mol.m^{-2}.s^{-1}$ which is significantly over the 125 $\mu mol.m^{-2}.s^{-1}$ being the maximum light intensity used for different professional plant productions). During this thesis, I conducted two *D. drummondii* inoculation assays in our greenhouse: a first one with some soil from Alaska and a second one with *D. drummondii* crushed nodule. However, for both assays, the starting material for inoculation was in quantity enough for only one pot. Apart from the fact that only two attempts were possible, the failure to obtain

nodulated *D. drummondii* is most probably due to few points: the starting material and the growth conditions. Indeed, the provided soil sample was too much dry (almost dehydrated) and the *D. drummondii* nodule, obtained in the botanical garden of Munich in early spring, was old and most probably not yet in developmental process. Either inside this nodule or the dry soil, the living bacteria might have not been enough vigorous and numerous to be able to inoculate new plants. Another limiting point for these tests is the fact that the university facilities have lamps providing only a light intensity of 150 µmol.m^{-2}.s^{-1} (far below the 700 µmol.m^{-2}.s^{-1} used by Kohls et al. (1994). For future *Frankia* inoculations, we should first be able to have better growth conditions (especially regarding light conditions) and obtain freshly collected *D. drummondii* nodules during their developmental process.

3.2.2. Arbuscular mycorrhiza within *Dryas* genus

Dryas spp. ECT and actinorhizal symbioses have been the object of several observations and analyses in the scientific literature (Lawrence et al. 1967; Debaud et al. 1981; Newcomb 1981a; Fitter and Parsons 1987; Melville et al. 1987; Melville et al. 1988; Harrington and Mitchell 2002; Markham 2009; Ryberg et al. 2009; Bjorbækmo et al. 2010). Nevertheless, there is an obvious lack of data on AM symbiosis within this genus.

To fill this gap, investigations were carried out on this symbiosis with both *Dryas* spp. One of them was an *in-situ* analysis of fungus phyla associated with *D. octopetala* roots. Indeed, *D. octopetala* were collected in the Switzerland Alps (as well as neighbouring plants) by Prof. Didier Reinhardt. Ascomycota, Basidiomycota and Gloromycota fungi associations with roots were looked for by PCRs. *D. octopetala* roots were found to be associated with the two first phyla but never with Glomeromycota. However, the detection of this last fungal phylum in gDNA extracted from the neighbouring plant roots ensure its presence into the soil (Figure 34). In a second hand, I have inoculated *D. drummondii* and *D. octopetala* plantlets with two different species of AM fungi. During root system survey after inoculation, *D. drummondii* harboured AM fungal hyphae, vesicles, and well-developed arbuscules. However, it was never the case of *D. octopetala* (Figure 32). Taken together this constitutes strong evidence of an AM symbiosis loss within *Dryas* genus. It is one of the first observation of endosymbiosis polymorphic trait within the same plant genus and moreover inside the FaFaCuRo clade.

As mentioned by Cosme et al. (2018) non-host plant are defined by the evidence of colonization absence, which can lead to misclassification because of insufficient investigation. However, *D. octopetala* roots were inoculated with two different AM fungi species, with spores and/or with chive nurse pot system. Some assays were also done by combining in the same pot both *Dryas* spp. Roots were analysed at several different time point post inoculation and all

developmental sections of the root system were observed (from lignified part to root meristem of primary and lateral roots). In all of these conditions AM fungal hyphae were never observed penetrating *D. octopetala* roots. These different conditions and observations allow to minimize the risk of misclassification of *D. octopetala* as non-host plant. However, in order to dispel any doubts on the fact that *D. octopetala* is a real non-host plant, in the future, it could be investigated roots with more varieties of AM fungi and during several stages of plant development (such as during flowering time or after vernalization period and on older plants).

Since we have at our disposal seeds of a hybrid between *D. drummondii* and *D. octopetala*, root screening was carried out after inoculation in nurse pot with *Rhizophagus irregularis*. The preliminary root screening of these hybrids has given a ratio of 83% uncolonized plants versus 17% of AM competent plants (Figure 35). If these hybrids were F1 generation, their use could have allowed to test the dominant or recessive trait of the AM symbiosis. However, the level of hybridization of the *D.* x *suendermannii* seeds is unknown. Indeed, these seeds were collected from hybrid plants growing outdoors in the botanic garden of Vienna. Due also to the presence of the parental species *D. drummondii* and *D. octopetala* in the botanic garden, it is not possible to determine whether they are backcrossed with other *Dryas* spp. or with *D.* x *suendermannii* itself. The variation level of hybridization was confirmed by the difference in the allelic composition of some tested genes (as presented in Figure 30 for *RAM1* and *CASTOR* in 3 different *D.* x *suendermannii* plants). So, from this analysis, it is not possible to determine if the AM symbiosis is a dominant or recessive trait and/or if this polymorphic trait is depending of a single important affected gene or several ones. Although the level of hybridization is unknown, these plants might still represent an interesting tool for genomic and phenotypic analyses. Moreover, it is still possible to find back the mother plants in the botanical garden in order to check by PCR or sequencing their hybridization level. The hybrids already screened are currently the object of vegetative propagation for a second AM screening and DNA extractions. The screening of more plants with genetic analysis on them should help in the near future to narrow down and/or to determine which loci are important for the endosymbiosis polymorphic trait between *Dryas* spp.

3.3. Genomic investigation on symbiotic genes

Mechanisms of AM incompatibility had been mostly discussed at the plant physiological level. Several phylogenomic studies (Delaux et al. 2014; Favre et al. 2014; Bravo et al. 2016) provided steps towards an evolutionary genetics explanation by using non-host plants as tool to shed light on symbiotic processes. These genome-wide comparison studies between AM host and non-host plant species identified numerous candidate genes with potential roles in AM symbiosis. Moreover, they have confirmed the current hypothesis that independent non-host plant lineages

lost their symbiotic capacity due to convergent deletions of the orthologs of important symbiotic genes. In order to identify the causative factors of the *Dryas* spp. endosymbiotic polymorphic trait, I have carried out a targeted genomic comparison. To do this, *M. truncatula* was used as genome of reference. This choice was led by the fact it was at the time of the analysis probably the best annotated model legume genome available and served as the common used in the phylogenomic comparisons of Delaux et al. (2014), Favre et al. (2014) and Bravo et al. (2016).

The first step was to determine the orthologous genes in *Dryas* spp. In collaboration with Maximilian Griesmann the first *Dryas* spp. genome versions were included with 37 other genomes (Supplemental S5 part 1) in OrthoFinder. The fact that 5 other Rosaceae genomes were included has allowed to confirm the difference of orthologous genes number between *M. truncatula* and *Dryas* spp. Indeed for example one *Dryas* spp. gene could correspond up to 8 genes in *M. truncatula* (Table 8 a lines 18-25; Supplemental S5 part 3 tree n°1) or in the other direction one *M. truncatula* gene has two *Dryas* spp. orthologs (*e.g.* Table 8 a line 9,10; Supplemental S5 part 3 tree n°3). In all of these cases, there is the same number of orthologous genes in the other Rosaceae. That highlights some specific duplication in Fabales or in Rosaceae. *Dryas* spp. genomes are in their first version, so they are not yet at their optimal quality level regarding the gene annotations and sequence. Indeed during the genomic comparison, side by side between both *Dryas* spp., it has been highlighted few regions containing series of ambiguous nucleotides (*e.g.* in scaffold3035_cov254_60194_55004, Table 8 a lines 15,16) or with few sequencing errors (*e.g. Dryoc1228S17759,* Table 8 b line 36, where PCR sequencing has invalidated the gap in the sequence between *D. drummondii* and *D. octopetala*). Moreover, several genes were not annotated (see part 2.3.1 and 3.1.3). Nevertheless, a gene prediction was found for all non-annotated genes (corresponding simultaneously to a synthetic region and to the best blast hit) via FGENESH+ tool.

First, a list of 37 genes was established based on the symbiotic "tool kit" described by Delaux et al. (2013a) and on important characterized symbiotic genes. Secondly, efforts were made on receptor like kinase (RLK) families, containing important known genes (LYSM: *e.g. NFP*, *EPR3*; LRRI-1: *e.g. SYMRK*; LRRIII: *e.g. ALB1*). This was led by the fact that fungi never enter into *D. octopetala* roots so it was hypothesized that the symbiosis should be blocked at the upstream part of both symbiosis signalling pathways. From these lists pairwise comparisons were carried out between *Dryas* spp. orthologous genes. Some genes are presenting DIPs between both *Dryas* spp., however all of them in CDS are characterized by a multiple of three and do not lead to frame shifts or premature STOP codons. This is the case of the *RAM1* gene which present a specific insertion of 42 bp in *D. octopetala* compared to *D. drummondii* and other Rosaceae. In part 2.3.2 investigations were carried out through cross complementation of the *L. japonicus ram1-3* mutant with both *Dryas* spp. *RAM1* gene. The restoration of the arbuscular wild type phenotype

with both *Dryas* spp. gene versions has shown that this kind of DIP does not bring a loss of function in the protein (at least in this particular case). Obviously more careful investigations should be done on all DIPs in order to be sure that the addition of few amino acids in the protein does not affect the symbiosis phenotypes (such as *RPG*, *NIN* in RNS genes and in *CASTOR, CYCLOPS* in the common symbiosis genes).

The pairwise comparison of the LRRIII-RLK and LYSM-RLK orthologous genes has highlighted particular polymorphisms between both *Dryas* spp. in three genes (*e.g.* a premature STOP codon in *Dryoc3475S24779* ortholog of *MtLYR2*, a gap in the kinase domain of *Dryoc124516S20838* and the *D. octopetala* orthologue of *Drydr265S09332* which is fragmented). Because of the lack of an available *Dryas* spp. mutant library, we decided to investigate the phenotype of *L. japonicus* insertion mutant lines available for these candidates. However, one of them does not have an ortholog in *L. japonicus*. For the two remaining candidates, one of them has two orthologs in *L. japonicus*, one on the chromosome 1 and the second on the virtual chromosome 0. But among the *L. japonicus* LORE 1 insertion mutant lines only the gene on the chromosome 1 is affected by a transposon insertion. Thus, two and three insertion lines were subject to rhizobia and AM inoculations in order to check if the mutation in the remaining LYSM and LRRIII orthologous candidates (respectively) lead to a loss of symbiosis. The use of two separate seed bags per mutant lines and at least two different insertion lines per gene allow to reduce to risk of background mutations repercussions. The phenotyping of these *L. japonicus* mutants for both endosymbioses did not show any difference compared to the wild type. So, the mutation in the candidate genes could not explain the loss of symbiosis. However, because *Dryas* and *Lotus* belong to two distinct plant families with different redundancy gene level, it is not possible to fully exclude that the mutation in the *D. octopetala* or *L. japonicus* gene would result in a different phenotype. In future, creation of *Dryas* spp. mutants (*e.g.* on specific genes by RNAi or CRISPR) and "cross complementation" (by introducing *D. drummondii* genes in *D. octopetala*) will present attractive tools. Indeed, this will avoid working in a different plant system and so redundancy effect which could be Fabaceae specific.

Upon the 106 *M. truncatula* genes from the "tool kit" list and the two RLK families investigated, no gene absence was found neither in *D. drummondii* nor in *D. octopetala* orthologs. In addition, for all LRRI-1 genes in *D. drummondii* an orthologous one was found in *D. octopetala*. This does not follow the current hypothesis above-mentioned of the evolutionary pattern of symbiotic genes between host and non-host plants. Indeed, Delaux et al. (2014), Favre et al. (2014) and Bravo et al. (2016) phylogenomic studies have highlighted an erosion of a core set of symbiotic genes in non-host plant lineages. In Xiang et al. (2016), it is shown that *D. octopetala* separated from other Dryadoideae 38 Mya. Therefore, the loss of endosymbiosis in

D. octopetala could be too recent to allow time for the expected gene erosion to occur. A more detailed and broad analysis of *Dryas* spp. genomes regarding this endosymbiotic polymorphic trait at both gene presence/absence and genetic regulation level could be a major tool for studying plant evolution and more specifically for the evolution of symbiotic genes.

4. Conclusion

One aim of the host team is to study the evolution of root symbioses and to investigate and compare the connections between signalling pathways and developmental modules present in non-host and host relative plants. Among plant families with an important agronomic and economic interest, Rosaceae represent a particularly attractive family to test evolutionary hypotheses related to nodulation. However, no nodulating Rosaceae were established and developed as model. So, this thesis has the goal to develop *Dryas* genus as a new model for root symbioses studies among the Rosaceae. Here I provide the pioneer step towards the development of *Dryas* spp. in a laboratory context for genetic and molecular studies. Indeed, we have shown through the setting up of various methods (from seed sterilization to vegetative propagation via several growth systems) that this plant genus can be adapted in a laboratory environment. Although some optimizations are still possible, with a generation time of less than a year, easy DNA extraction procedure allowing robust PCRs, established *A. rhizogenes* transformation and a small diploid genome (*ca.* 250 Mbp), *Dryas* emerges as a new model genus.

This genus was selected because it contains closely related non nodulating and nodulating species. Even if until now the right *Frankia* strain which can nodulate *D. drummondii* is not available in our laboratory as well as most probably the ideal growth conditions for root nodule symbiosis (RNS) establishment, the investigations done on the arbuscular mycorrhiza (AM) symbiosis have highlighted a loss of symbiosis in *D. octopetala*. This constitutes a rare and unexpected case of endosymbiotic polymorphic trait within the same plant genus, as well as an indication of the non-nodulating status of *D. octopetala*.

The *de novo* whole genome sequencing was carried out on both *Dryas* spp. *D. drummondii* genome is already publicly available and the *D. octopetala* one will be also released after optimization. The close relation between these two species allows convenient genetic comparisons. During the first targeted gene comparison done in order to discover the causative genetic of the loss of AM symbiosis, no symbiotic gene erosion in *D. octopetala* has emerged. This could highlight a loss of endosymbiosis too recent within *Dryas* spp. to allow time to weaken symbiotic genes.

More broad genomic comparison between both *Dryas* spp. should determine important genes or genetic regulatory mechanisms which could be the initial step of symbiosis loss. In addition, genetic analyses with other Rosaceae would be also an important tool for evolutionary studies (regarding floral and fruit evolution and agronomical adaptation to diverse biotopes, within this plant family). Due to the natural distribution of *Dryas* spp., its development as a model genus will be an asset for the study of important traits associated with survival in arctic and alpine

conditions (related to climate change, as well as ecological studies of plant-microorganism and plant-pollinator relationships). By combining the *A. rhizogenes* transformation and genetic comparison finding, complementation of *D. octopetala* with *D. drummondii* symbiotic gene(s) in order to restore endosymbioses to this plant would provide decisive step to further understand and determine symbiotic evolution. Moreover, it will contribute to the development of biotechnological solutions to implement RNS in major crop plants.

5. Material and Methods

5.1. Material

5.1.1. Plants

- ***Dryas* spp. seeds**

Seeds of *D. drummondii* "DA462" and *D. octopetala* "DA460" were purchased from the seed producer Jelitto (Schwarmstedt, Germany). Samples of seeds and plants from the same seed bags used for the *de novo* whole genome sequencing are registered in the collection of the herbarium of the Botanische Staatssammlung München under the number M-0290345 and M-0290346 respectively.

The Nymphenburg Botanical Garden of Munich supplied seeds for *D. drummondii* "BGM". *D. drummondii* ecotypes "Albe." (origin Clearwater County, Alberta, Canada, collected in 2000); and "Alas." (origin Alaska, USA, collected in 2002) were found in and supplied by the Kew Millennium Seed Bank (Royal Botanic Gardens, Kew, London, UK). Respectively they are identified in their database under the serial number 147165 and 178408.

D. octopetala ecotypes "E548" and "Scha." were harvested in the wild in the Italian Alps (approximate GPS coordinates: 46°24'36.7"N 11°37'48.2"E) and German Alps (approximate GPS coordinates: 47°25'44.5"N 11°07'45.4"E), respectively.

D. x *suendermannii* seeds were supplied by the botanical garden of the university of Vienna. They correspond to the IPEN number XX-0-WU-0008729.

- ***Lotus japonicus* seeds**

L. japonicus LORE1 insertion lines were obtained from the mutant bank of Aarhus University (Małolepszy et al. 2016). They were propagated in our greenhouse and based on the instruction of the *Lotus* LORE1 database homozygote plants for the mutation of interest were selected. *L. japonicus* seed bag numbers used in this thesis are listed in Supplemental S6 c.

5.1.2. Bacteria

For rhizobia inoculation of *L. japonicus* roots, it was used *Mesorhizobium loti* MAFF303099 carrying DsRed as marker (Maekawa et al. 2008). For *Frankia* inoculation of *Dryas* sp. two different inoculum sources were used: a nodule from *D. drummondii* (collected at the botanical garden of Munich in March 2015) and Alaskan dried soil (collected by a Prof. Katharina Pawlowski's collaborator, stored in a plastic bag, and sent by post under normal conditions).

Transgenic hairy roots were induced by *Agrobacterium rhizogenes* strain AR1193 (Offringa et al. 1986) as described in Díaz et al. (2005).

M. loti and *A. rhizogenes* strains were stored in 20% glycerol at -80°C.

5.1.3. Fungi

For arbuscular mycorrhiza (AM) symbiosis phenotyping, *Rhizophagus irregularis* DAOM 197198 (Agronutrition, Toulouse France) and *Gigaspora rosea* DAOM 194757 (provided by Prof. Christophe Roux from the LRSV-Toulouse) were used.

5.1.4. Primer

ITS (internal transcribed spacer) primers were designed based on Cheng et al. (2016); All other primers were designed based on the first annotated version of *Dryas* spp. genomes and listed in the Supplemental S7.

5.2. Methods

5.2.1. Seed sterilization

- ***Dryas* spp.**

Based on advice from seed producers and on results from Nichols (1934) who could show that without refrigeration, germination of several alpine species was considerably reduced, seeds of *Dryas* spp. might require cold stratification prior to germination. They were therefore stored at 4°C. Several solutions for surface seeds sterilization were tested. *Dryas* spp. seeds were surface sterilized by immersion in sterilization solutions (10 min. for *D. octopetala*; 15 min. for *D. drummondii* and from 10 to 15 min. for *D. x suendermannii* depending of the seed set) and washed three times with sterile H_2O. As a routine 30% of H_2O_2 is used as sterilization solution; the other solutions tested were 0.05% of Tween80 in 70% Ethanol (used in the lab for surface sterilization of *Allium schoenoprasum*), 1.2% of NaClO in 0.1 % of SDS (used in the lab as routine for *L. japonicus* seeds).

- ***Lotus japonicus***

L. japonicus seeds were scarified either with sandpaper or immersed in liquid nitrogen (by four sessions of 15 sec. of submersion, followed by 15 sec. at room temperature). Then they were surface sterilized with 1.2% of NaClO in 0.1% of SDS for 10 min. and rinsed with sterile H_2O followed by incubation in a spinning wheel in water for two hours.

5.2.2. Plant growth

- ***Dryas* spp.**

Sterilized seeds were transferred on 1% agar-water plates and incubated in the dark at 22°C for 12 and 8 days for *D. octopetala* and *D. drummondii*, respectively. Several sources of agar were tested such as Bacto™ agar (Becton Dickinson and company) and agar Kalys HP 696 (Kalys SA, Bernin, France) and Gelrite agar (Duchefa, Haarlem, The Netherlands). Based on Bliss (1958), in order to ensure optimal germination rates, the germination assays were set up in the dark. After germination, seedlings of *Dryas* spp. transferred on plates prior to be grown on plates, in a hydroponic system or in pots.

As routine, growth on plates was performed on ¼ Hoagland's (using the protocol for N-free medium (Hoagland and Arnon 1950) and adding 1 mM KNO_3, pH 5.8) with 0.4% of Gelrite agar (Duchefa, Haarlem, The Netherlands), at 22°C, 55% of humidity with 16h-light/8h-dark cycles. After 1 week, plantlets were transferred either into pots (containing soil or a 2:1 mix of sand:vermiculite) or into a hydroponic system.

The hydroponic system consisted of a box in two parts: the first one contained 250 ml of growth solution (¼ Hoagland, 1 mM KNO_3, pH 5.8) and the second one was a floating part with 24 holes in which the plantlets were inserted (Figure 25 a). In order to avoid that seedlings or young plantlets fell down in the solution compartment, plantlets were introduced through thin slits on adhesive tape, which plugged the holes. The solution was changed every 3.5 days. The hydroponic system was kept at 22°C, 55% humidity with 16 h-light/8 h-dark cycles for a maximum of 4 months.

Plants in pots were transferred to the greenhouse (day temperature 21-24°C, night temperature 18-21°C, with additional lighting from 6:00 h to 10:00 h and from 15:00 h to 22:00 h).

- ***Lotus japonicus***

After sterilization treatment the swollen seeds were put on 0.8% Bacto Agar (Difco) water plate and incubated for about 60 h in a growth chamber at 24°C in the dark for germination. Then the seedlings were removed from the dark and grown for a total of 17 days (24°C, 60% air humidity, 16 h-light / 8 h-dark). Afterwards, plantlets were used for hairy root transformation or transferred in growth pots or Weck-jars. They were transferred in growth chamber (24°C, 55% humidity with 16 h-light/8 h-dark cycles) or in the greenhouse.

5.2.3. *Dryas* spp. vegetative propagation

Young and soft shoots of *Dryas* spp. were cut after the third internode (2-5 cm) above the woody part of the shoot. These explants were directly transferred into moist soil, then kept under plastic cover in the greenhouse. High humidity was maintained under the cover by spraying with water every two days for two weeks, after which spraying was stopped and cuttings were kept with moist soil under the cover until new leaves had developed.

5.2.4. Arbuscular mycorrhizal inoculation and observation

AM assays were performed in 2:1 mix of sand:vermiculite with 4 to 6 seedlings per pot either with chive (*Allium schoenoprasum*) nurse pots containing *R. irregularis* or *G. rosea* as described in Kistner et al. (2005) or with *R. irregularis* spores. For AM quantification 300 spores per plant were applied and at least 500 spores per plant for AM screening on *D. x suendermannii* and for *L japonicus* transformed via *A. rhizogenes*.

The plants were grown in a climate chamber at 24°C constant temperature, 60% air humidity and 16h light / 8h dark cycles. Pots were fertilized once per week with 20 ml of

¼ Hoagland (1.5 mM KNO_3 and 100 µM KH_2PO_4 pH 5.8) and twice a week with 20 ml of sterile dH_2O.

AM fungi in colonized roots were stained with acid ink (Vierheilig et al. 1998). Root length colonization was quantified using a modified gridline intersect method (McGonigle et al. 1990) with 20X magnification at a light microscope (Leica, type 020-518500 DM/LS).

For confocal laser scanning microscopy using a Leica SP5, fungal structures were stained with 1 $\mu g.\mu l^{-1}$ WGA Alexa Fluor 488 (Molecular Probes, www.lifetechnologies.com) (Panchuk-Voloshina et al. 1999).

5.2.5. Root nodule symbiosis inoculations

- ***Frankia* inoculations**

Full *D. drummondii* nodule was surface sterilized with 30% H_2O_2 for 5 min., rinsed 5 times in sterile dH_2O and mechanically homogenized with a mortar and pestle in a 1% NaCl in sterile dH_2O. The crushed nodule suspension was applied to the surface of the substrate adjacent to the plants.

Alaskan soil sample was mixed with some propagating substrate and used to fill horticultural pot.

Two plants of *D. drummondii* were inoculated with either *Dryas* sp. nodule suspension or Alaskan soil. Plants were grown for 10-12 weeks prior inoculation, in the greenhouse. Pots were irrigated once per week with ¼ Hoagland nitrogen-free nutrient solution, pH 5.8-6.0, supplemented with 0.1 mM KNO_3. Twice weekly, the pots were flushed with dH_2O. 24 weeks after inoculation, root systems were excavated and observed with binocular microscope for presence of nodules.

- **Rhizobial inoculation**

To evaluate root nodule symbiosis status of *L. japonicus* mutants, germinated seedlings were grown in closed Weck jars in sand:vermiculite for 2 weeks. Then they were inoculated with a suspension of *M. loti* set to a final OD_{600} of 0.05. Inoculated plants were incubated for 5 weeks prior root observations. Nodule number was reported then microscopic observation of red fluorescent was performed with a fluorescence stereomicroscope (Leica MZt6 FA).

5.2.6. gDNA extractions and PCR reactions

The caesium chloride (CsCl) gradient DNA extraction method was performed according to Ribeiro et al. (1995) starting with 6 to 10 g of leaves (mix of young and old) ground by hand using pistil and mortar with 4 g of PolyclarAT in liquid nitrogen. For the others extraction methods, 2 young leaves with apical meristem were used as starting material. They were ground with a tissue lyser (2 times at 30 Hz during 30 sec) in 2 ml Eppendorf tubes containing 2 metal beads each, after being shock frozen in liquid nitrogen. The “classical CTAB" extraction method

is based on the procedure described in Doyle and Doyle (1987), whereas the "PVP/NaCl" extraction method is based on Khanuja et al. (1999). The latter consists of an adapted version of the CTAB protocol with modified extraction buffer (compared to the classical CTAB method it contains +25% of EDTA, +5.5% of NaCl, +25% of CTAB, -68% of β-mercaptoethanol, and with addition of 4% (w/v) of PVP) and followed by an isopropanol precipitation step in high salt solution before the RNAse A treatment.

Invitrogen™ Quant-iT™ Qubit™ dsDNA Kit (Carlsbad, CA, USA) and Nanodrop™ (ThermoFisher, Germany) were used according to manufacturer's instructions in order to respectively determined DNA concentration and to show the presence of contaminants in the DNA extractions.

PCRs were performed on 1 µl of DNA (an average of 100 ng of DNA) using GoTaq® DNA polymerase (Promega, Madison, WI, USA), SYBR Green buffer and 0.2 µM of each primer. Amplifications were carried for 5 min at 95°C, followed by 35 cycles (30 sec at 95°C, 30 sec at 60°C, and 40 sec at 72°C), and a final extension for 40 sec at 72°C. Electrophoresis were performed with 4 µl of PCR reaction on 3% agarose gel for 100 min at 130 V. DNA was visualized with UVP UVsolo touch from Analytik Jena© (Jena, Germany) after 10 min in an Ethidium bromide bath at 2 $ng.ml^{-1}$.

Some PCR were done with MyTaq™ Plant-PCR Kit (Bioline Reagents, London) according to manufacturer's instructions, this Kit allows to perform PCR directly on tiny leave sample without DNA extraction step.

To evaluate if *L. japonicus* LORE1 mutants were homozygous for the mutation of interest, gDNA was extracted with classical CTAB method prior PCRs performed with the adapted primers. PCR programs and primers sequences provided on the Lotus database website have been used.

5.2.7. RNA isolation

Leaves and seedlings were shock frozen in liquid nitrogen. RNA of ground material (with same procedure previously describe for DNA extraction) was extracted using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, CA, USA) without adaptation in the protocol except for older and thick leaves where polyclarAT was added. The RNA was treated with Invitrogen™ TURBO DNA-free Kit (Carlsbad, CA, USA) and tested for purify and integrity with a Bioanalyzer RNA kit from Agilent (Agilent Technologies, Palo Alto, CA).

5.2.8. Hairy root transformation

A. rhizogenes AR1193 bacteria carrying a Golden Gate LIIIβ F A-B (Binder et al. 2014) plasmid containing the mCherry gene under control of the Ubiquitin promoter (*AtUbi10*pro) as transformation marker (Pimprikar et al. 2016), were grown in liquid culture (LB medium with

50 µg.ml^{-1} of rifampicin, carbenicillin and kanamycin) at 28°C overnight. Bacteria were collected via a centrifugation step (15 min at 4 369 x *g*) and resuspended in water to obtain the wanted OD_{600} (0.01; 0.1; 1; 7.2). As routine procedure for *Dryas* spp. and *L. japonicus* OD_{600} was set up at 1. Cut hypocotyls of 10-12 days old *Dryas* spp. seedlings or 17 days old *L. japonicus* seedlings were dipped in the bacteria solution and placed on ¼ Hoagland (1 mM KNO_3, pH 5.8) 0.4% Gelrite (Duchefa, Haarlem, The Netherlands) plates. The plates were kept for 4 days in the dark at 22°C, then under a 16h-light/8h-dark cycle with 55% of humidity. To prevent overgrowth of bacteria and dehydration, the plants were transferred onto new plates every 1.5 week. After 4 to 6 weeks post transformation, roots were screened using a Leica MZ16 FA stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) with the N3 filter from Leica (BP 546/12;600/40).

5.2.9. Bioinformatics

All genome parts (sequencing, assembly, assembly evaluation, annotation, comparative genomic analysis) and transcriptome sequencing are described in Griesmann et al. (2018). Determination and visualisation of syntenic regions were performed via tools available on CoGe website (www.genomevolution.org; (Lyons and Freeling 2008). Blasts, alignments and pairwise comparison were performed with CLC Genomics Workbench 7.0 (www.qiagenbioinformatics.com). All statistical analyses were performed in R-studio (version 0.99.484; www.rstudio.com). All microscopic images were processed with ImageJ (http://rsb.info.nih.gov/ij/) software in order to integrate scale bars; and all figures were done with Inkscape (www.inkscape.org).

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S4 **Workflow to find the orthologous *NSP1* gene in *Dryas octopetala* which was unannotated.**

S5 **Maximum-likelihood phylogenetic trees for the three families of receptor like kinase.**

S6 **Overview of the workflow for the choice of LORE 1 insertion mutant lines.**

S7 **List of primer used.**

Supplemental S1

Overview of important protocols and procedures tested in order to use *Dryas* spp. as a model genus in a laboratory context.

Aim	tested protocol specificity	remarks and conclusion
Seed sterilization	**33 % H_2O_2[#]**	* ca. 85 % and 40 % of germination for *D. drummondii* and *D. octopetala*, respectively * <3 % of contamination
	1.2 % NaClO	*ca. 80 % and 40 % of germination for *D. drummondii* and *D. octopetala*, respectively * ca. 20 % of contamination
	"EtOH"	* 0 % of germination * <1 % of contamination
Growth solution	MS or B5 or FP	* reddish seedlings (anthocyanin production)
	"FAB"	* reddish seedlings (anthocyanin production; but less than on MS or B5) * darker green cotyledons than on B5
	1/4 Hoagland[#]	* reduction of the anthocyanin production * darker green cotyledons and leaves * longer primary root than on "FAB" * more of lateral root than on "FAB"
Growth systems	Agar plates	* roots not protected from ligth * suitable for small plantlets (not more than 6 weeks old) * easy non-invasive root observations
	pot/solid substrate	* roots protected from light * suitable for long experiments and propagation * "invasive" root observation (digging out and washing of the roots could easily break lateral roots and root hairs) * strong root lignification
	hydroponic	* roots protected from light * suitable for long experiments * non-invasive root observations * delay of the root lignification process
DNA extraction	**"CsCl" Ribeiro et al. (1995)[#]**	* good yield and good quality for *de novo* whole genome sequencing * 260/280 ratio around 1.9-2.0 * 230/280 ratio >2.0 * long protocol (≈4 days)
	"classical CTAB" Doyle and Doyle (1987)	* low yield * 260/280 ration variable but around 1.8 * 230/280 ratio <1.8 * not usable for robust PCRs * 1/5 day protocol
	"PVP/NaCl" Khanuja et al. (1999)[#]	* medium yield *260/280 ratio less variable ≈1.8 *230/280 ration >1.8 * usable for robust PCRs * 1 day protocol

[#] most suitable protocols for *Dryas* spp.

Overview of different protocols tested.

Adapted from Billault-Penneteau et al. 2019

Supplemental S2

Transposable elements in *D. drummondii* assembly and summary of *D. drummondii* genes with function description.

	Length (bp)	% in genome
Type I: Retro-TEs		
LTR/GYPSY	4 838 912	2,08
LTR/Copia	1 992 692	0,86
LTR/other	23 666 120	10,16
LINE	492 460	0,21
SINE	35 331	0,02
Type II: DNA-TEs		
DNA/hAT	4 206 347	1,81
DNA/Helitron	118 467	0,05
DNA/Ginger	1 200	0,00
DNA/Mu-like	1 726 334	0,74
DNA/MITE	6 109 669	2,62
Other DNA-TEs	4 851 839	2,08
Simple_repeat	323 351	0,14
Unknown	20 364 235	8,74
Total TEs		27,96

Transposable elements in *D. drummondii* assembly.

From Griesmann et al. 2018

	Number	Percent (%)
Total	25 030	
InterPro	19 216	76,77
GO	12 188	48,69
KEGG	19 258	76,94
Swissprot	18 610	74,35
Annotated	21 687	86,64
Unanotated	3 343	13,36

Summary of *D. drummondii* genes with function description.

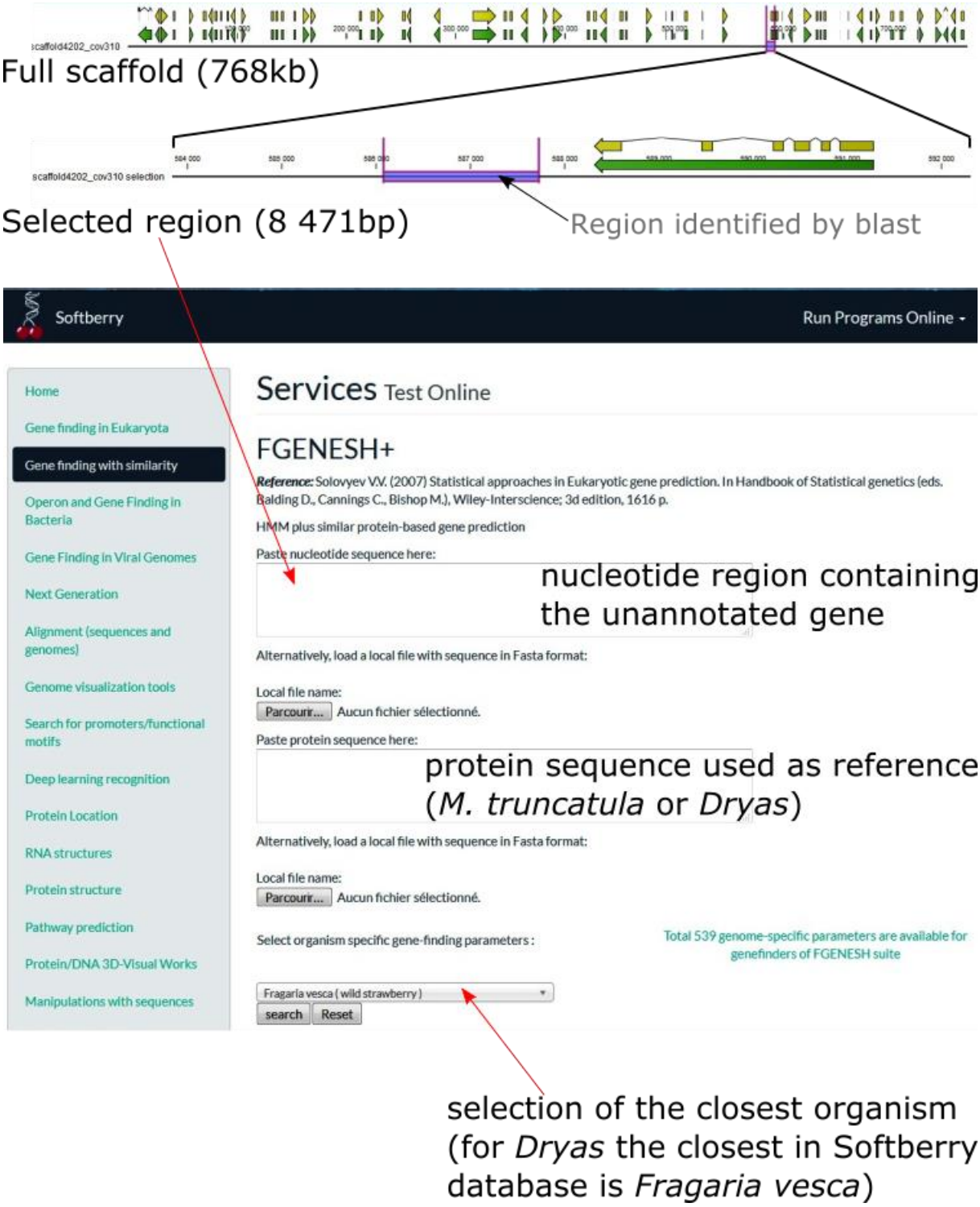
From Griesmann et al. 2018

Supplemental S3

Workflow of using FGENESH+ online tool in order to predict the annotation of an unannotated gene in *Dryas octopetala*: The example of *NSP1*.

Part. 1 Use of FGENESH+

Part. 2 FGENESH+ result



Part. 1 Use of FGENESH+.

By using MtNSP1 as query, tblastn and Syndfind (online tools from www.genomevolution.org) have highlited a region in *D. octopetala* genome which is not annotated. In a first step the full scaffold was extracted (scaffold4202_cov310 ; 768kb). Then a large nucleotide sequence was selected surounding the putative DoNSP1 (8 471bp). This region was paste in the online tool FGENESH+ from www.softberry.com together with the protein sequence of the DdNSP1 protein. Using Fragaria vesca (the closest organism of *Dryas* in the sofberry database) for genome specific parameters, FGENESH+ determined the prediction of the mRNA if it is possible.

Part. 2 FGENESH+ result.

FGENESH+ 2.6 Prediction of potential genes in Fragaria_vesca genomic DNA

Seq name: test sequence

Length of sequence: 8471

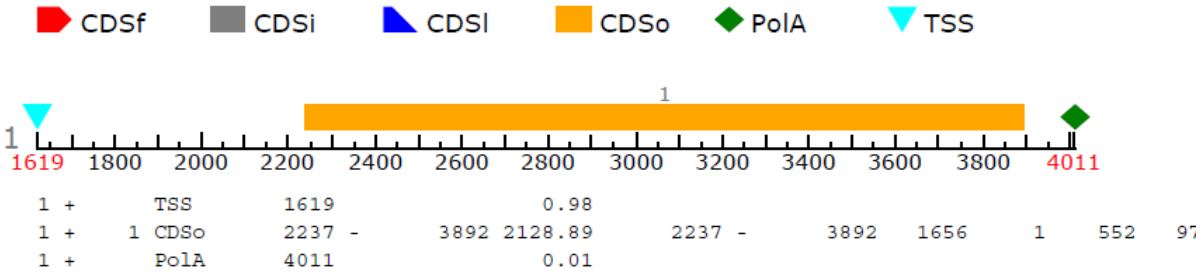
Homology: test sequence

Length of homolog: 552

Number of predicted genes 1: in +chain 1, in -chain 0.

Number of predicted exons 1: in +chain 1, in -chain 0.

Positions of predicted genes and exons: Variant 1 from 1, Score:2166.105078



1	+		TSS	1619		0.98						
1	+	1	CDSo	2237	-	3892 2128.89	2237	-	3892	1656	1	552 97
1	+		PolA	4011		0.01						

Predicted protein(s):

>FGENESH:[mRNA] 1 1 exon (s) 2237 - 3892 1656 bp, chain +

ATGACCACCGGAGAACCAGAGCCAAACCCCAACTCAGATCACATCTTGGACTGGCTTGAAGATTCAGTCACCTTCTT
TCCATCTTTCTTGGATGATCCTTACAATTCAAGTGATATCAACGGCTATCAATGGTGGGATGAGAGTCAGGATCTGA
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GCAATTGGGAGATGAGAGTGGATGAGAAAGATGGGTGTGCAGGGTTATGGTGGAAGGGGCAGCCTGTTTCTTTTTGT
TCATTGTGGAAGATGGATATGAAAGCAAGTGCCAAGTAA

>FGENESH: 1 1 exon (s) 2237 - 3892 551 aa, chain +

MTTGEPEPNPNSDHILDWLEDSVTFFPSFLDDPYNSSDINGYQWWDESQDLIHANTTSLNSPTSIAIATNTRPINPT
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LHHLNHNLPDERTEFLKLLRNMEAKGVILSENNMECSCSNCGDFATGFSRQVEYLWRFLDSTSSAFKGRESDERRVM
EGEAAKALTNRGEMNEGKEKWCERMKGAGFVGETFGEDAIDGGRALLRKYDSNWEMRVDEKDGCAGLWWKGQPVSFC
SLWKMDMKASAK

Supplemental S4

Workflow to find the orthologous *NSP1* gene in *Dryas octopetala* which was unannotated.

a – Best hits of tblastn using *Mt*NSP1 as query.

Org	Chr	Position	Coverage	E-value	Closest Genomic Feature	Distance to Genomic Feature (bp)
Medicago truncatula	8	7352592	100.0%	0.0	Medtr8g020840.1	overlapping
Dryas drummondii	Scaffold490	1417812	99.3%	0.0	Drydr490S04636	overlapping
Dryas octopetala	scaffold4202_cov310	586090	99.3%	0.0	Dryoc4202S14123	2896.5

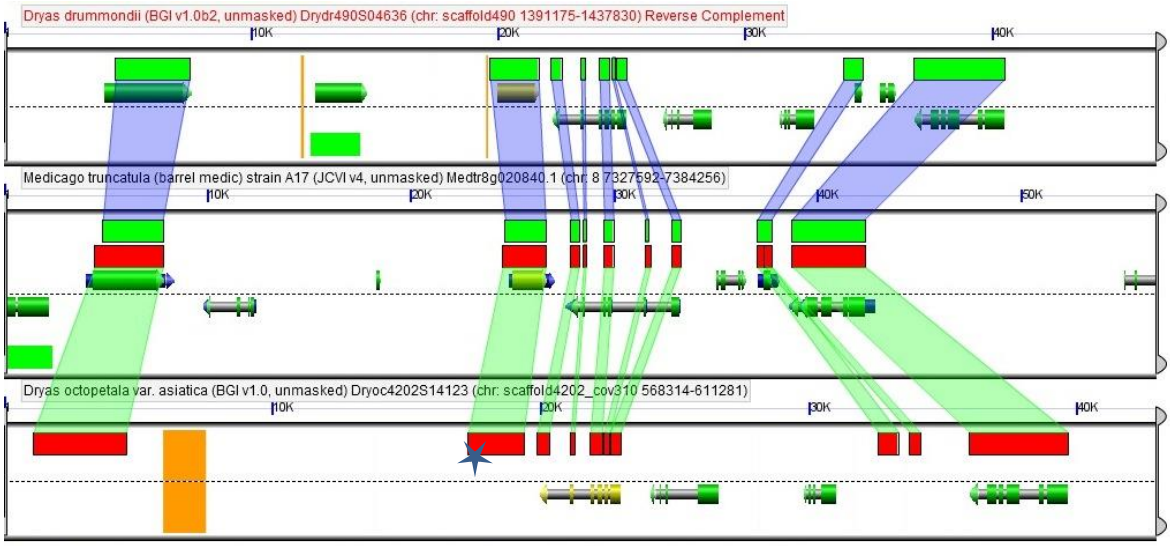
b – SynFind using *MtNSP1* as query.

Organism	Type	Name	Chr	Synteny Score
Medicago truncatula	query	Medtr8g020840.1	8	/
Dryas drummondii	Syntelog	Drydr490S04636	scaffold490	11
Dryas octopetala	Proxy region	pos 588314	scaffold4202_cov310	4

c – SynFind using *DdNSP1* as query.

Organism	Type	Name	Chr	Synteny Score
Dryas drummondii	query	Drydr490S04636	scaffold490	/
Dryas drummondii	Proxy region	pos 2175997	Scaffold45	10
Dryas octopetala	Proxy region	pos 588314	scaffold4202_cov310	13
Dryas octopetala	Proxy region	pos 486558	Scaffold2953_cov328	9
Dryas octopetala	Proxy region	pos 118530	Scaffold2378_cov267	6
Medicago truncatula	Syntelog	Medtr8g020840.1	8	25
Medicago truncatula	Proxy region	pos 18325935	2	5

d – Visualization of the Synteny regions.



Workflow to find the orthologous *NSP1* gene in *Dryas octopetala* which was unannotated.

(a) Using *Mt*NSP1 protein as query tblastn was run on *M. truncatula*, *D. drummondii* and *D. octopetala* genomes. The best hit in *D. drummondii* was corresponding to the orthologous gene found by orthofinder. (b-d) Syntelogous regions were investigated in both *Dryas* spp. and in *M. truncatula*, using either *MtNSP1* (b&d) or *DdNSP1* (c) as query. *D. octopetala* sequence identified by blast was corresponding to the region having the best synteny scores. It was an annotated region (red rectangle with a star) where FGENESH+ tool predicted a gene annotation. Tables and graphic adapted and coming from the online tools on the website CoGE, Lyons and Freeling 2008.

(data unpublished)

Supplemental S5

Maximum-likelihood phylogenetic trees for the three families of receptor like kinase.

Part. 1 -> List of species used in OrthoFinder with “theoretical” trees

Part. 2 -> Example of a “full” tree vs. its “simplified” version

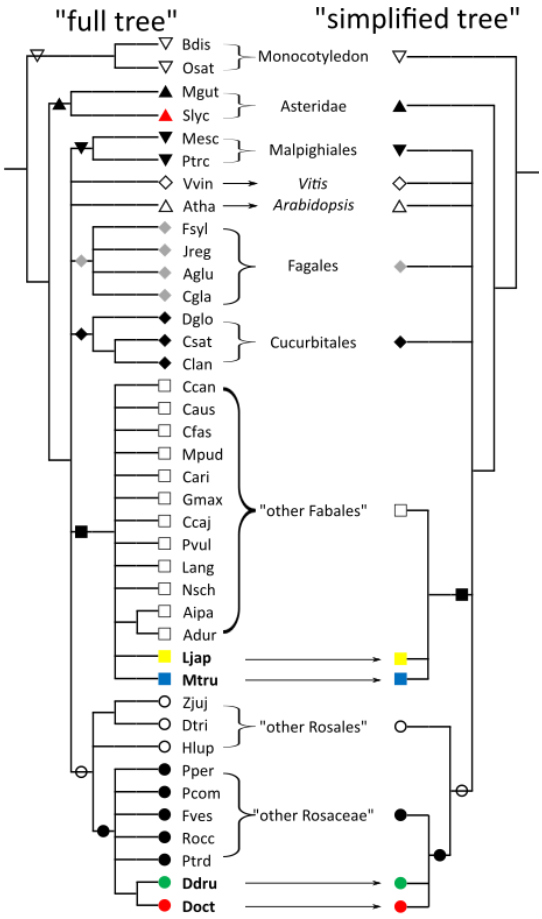
Part. 3 -> LYSM “simplified” maximum-likelihood phylogenetic trees

Part. 4 -> LRRIII “simplified” maximum-likelihood phylogenetic trees

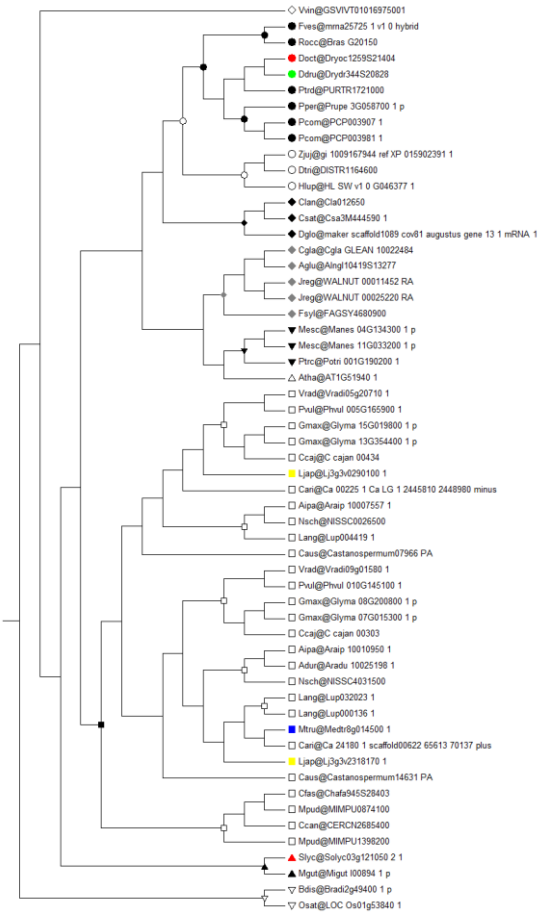
Part. 5 -> LRRI-1 “simplified” maximum-likelihood phylogenetic trees

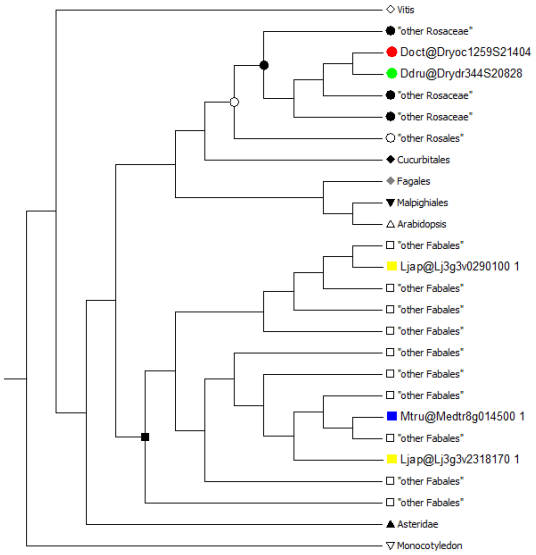
Part. 1 -> List of species used in orthofinder with "theoretical" trees.

Clade	Subclass	Order	Family	genus	species
Monocot		Poales	Poaceae	*Brachypodium*	*distachyon*
				Oryza	*sativa*
Eudicot	Asteridae	Lamiales	Phrymaceae	*Mimulus*	*guttatus*
		Solanales	Solanaceae	*Solanum*	*lycopersicum*
	Rosidae	Malpighiales	Euphorbiaceae	*Manihot*	*esculenta*
			Salicaceae	*Populus*	*trichocaprpa*
		Vitales	Vitaceae	*Vitis*	*Vinifera*
		Brassicales	Brassicaceae	*Arabidopsis*	*thaliana*
		Fagales	Fagaceae	*Fagus*	*sylvatica*
			Juglandaceae	*Juglans*	*regia*
			Betulaceae	*Alnus*	*glutinosa*
			Casuarinaceae	*Casuarina*	*glauca*
		Cucurbitales	Datiscaceae	*Datisca*	*glomerata*
			Cucurbitaceae	*Cucumis*	*sativus*
				Citrulus	*lanatus*
		Fabales	Fabaceae	*Cercis*	*canadensis*
				Castanospermum	*australe*
				Chamaecrista	*fasciculata*
				Mimosa	*pudica*
				Cicer	*arietinum*
				Glycine	*max*
				Cajanus	*cajan*
				Phaseolus	*vulgaris*
				Lupinus	*angustiflorus*
				Nissolia	*schottii*
				Arachis	*ipaensis*
					duranensis
				Lotus	***japonicus***
				Medicago	***truncatula***
		Rosales	Rhamnaceae	*Ziziphus*	*jujuba*
				Discaria	*trinervis*
			Cannabaceae	*Humulus*	*lupulus*
			rosaceae	*Prunus*	*persica*
				Pyrus	*communis*
				Fragaria	*vesca*
				Rosa	*occidentalis*
				Purshia	*tridentata*
				Dryas	***drummondii***
					octopetala

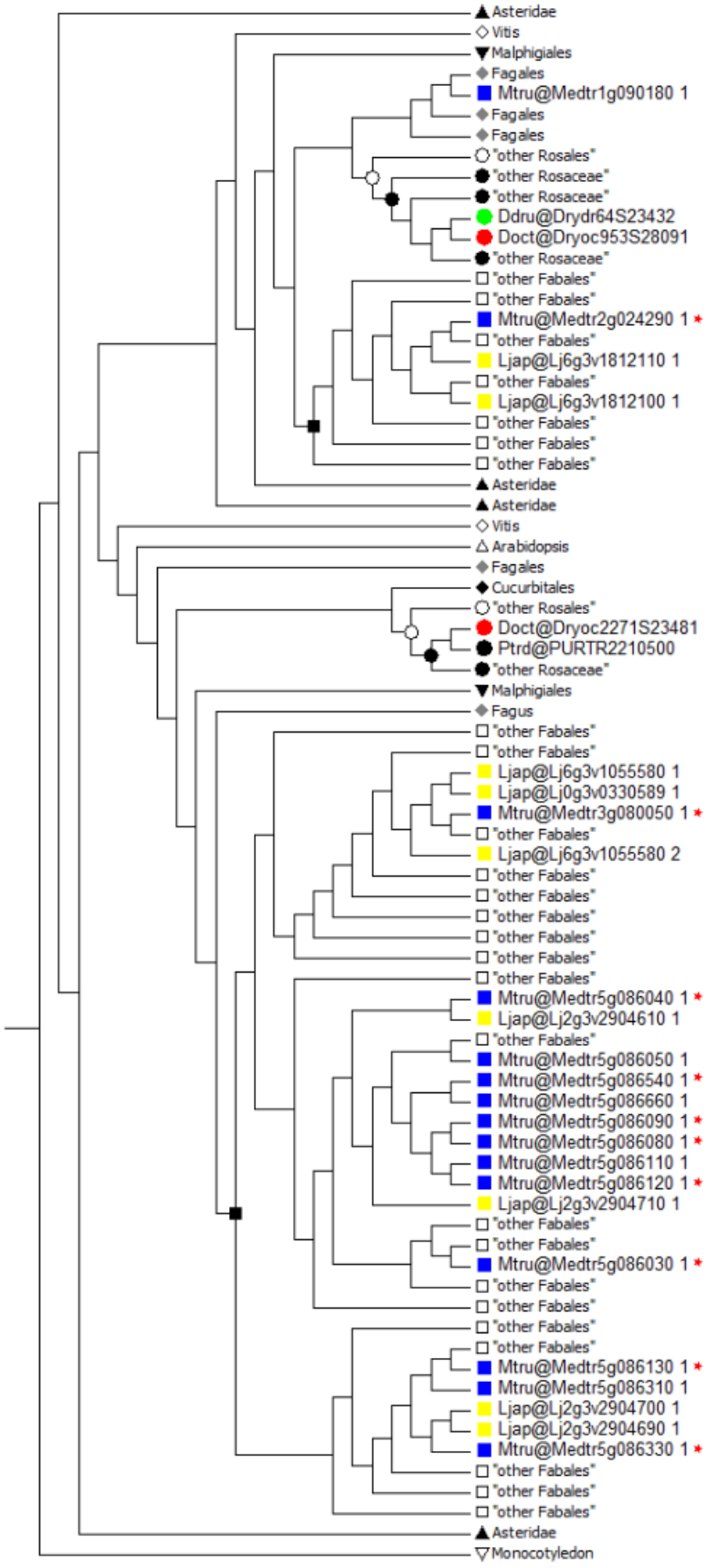


Part. 2 -> Example of a “full” tree vs. its “simplified” version.





Part. 3 -> LYSM "simplified" maximum-likelihood phylogenetic trees.



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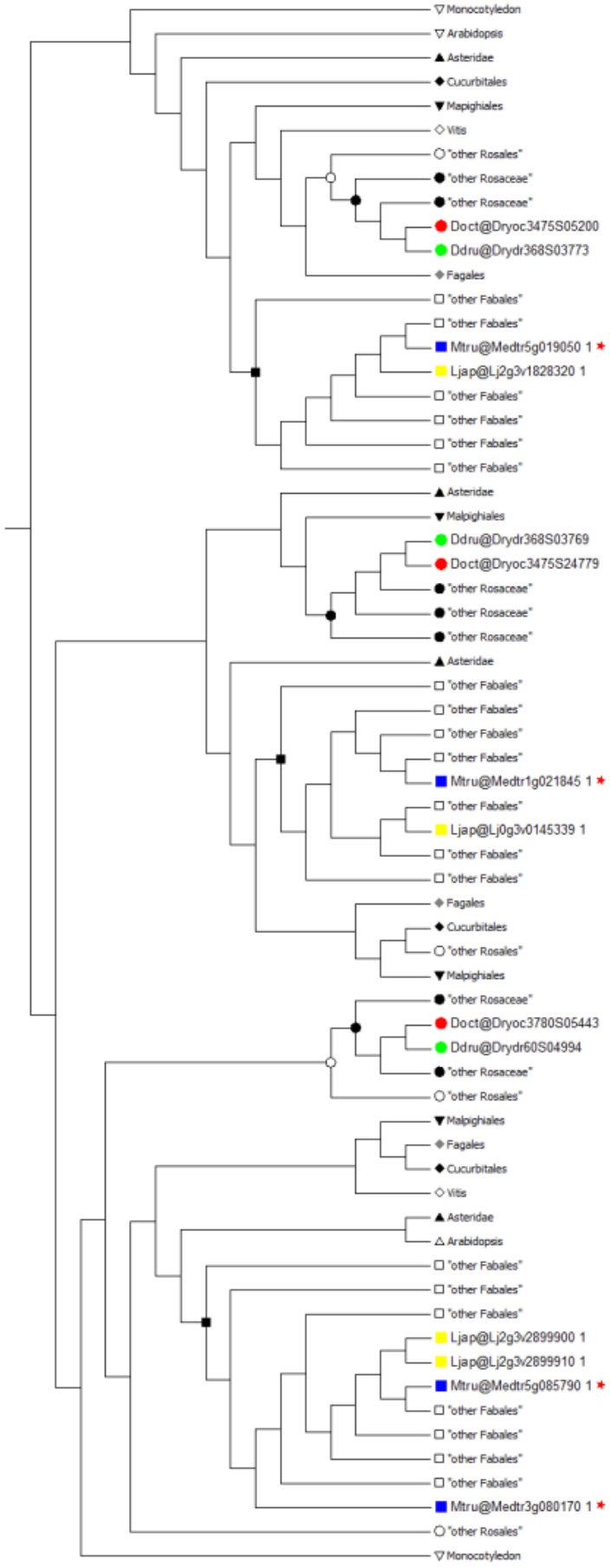
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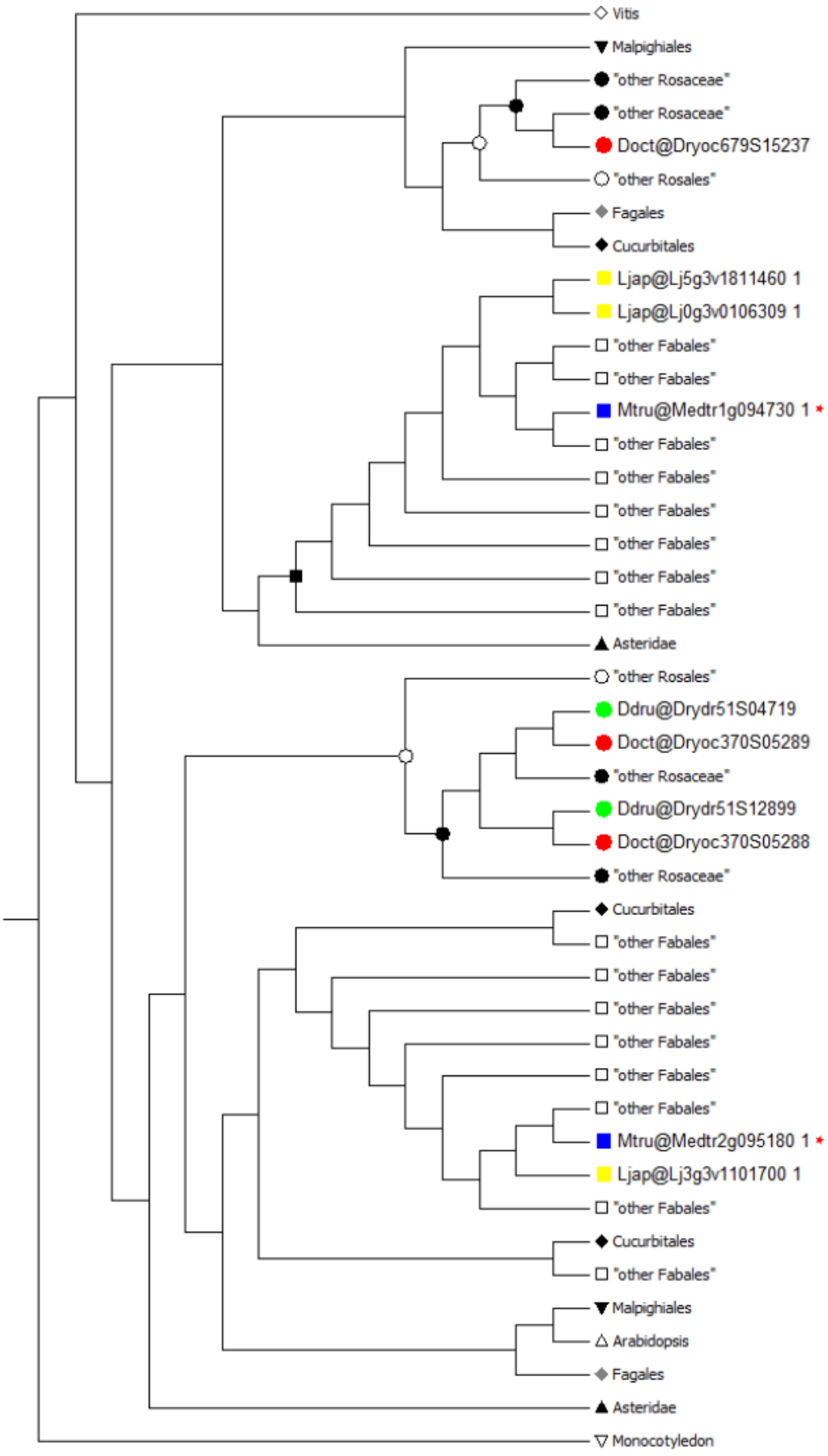
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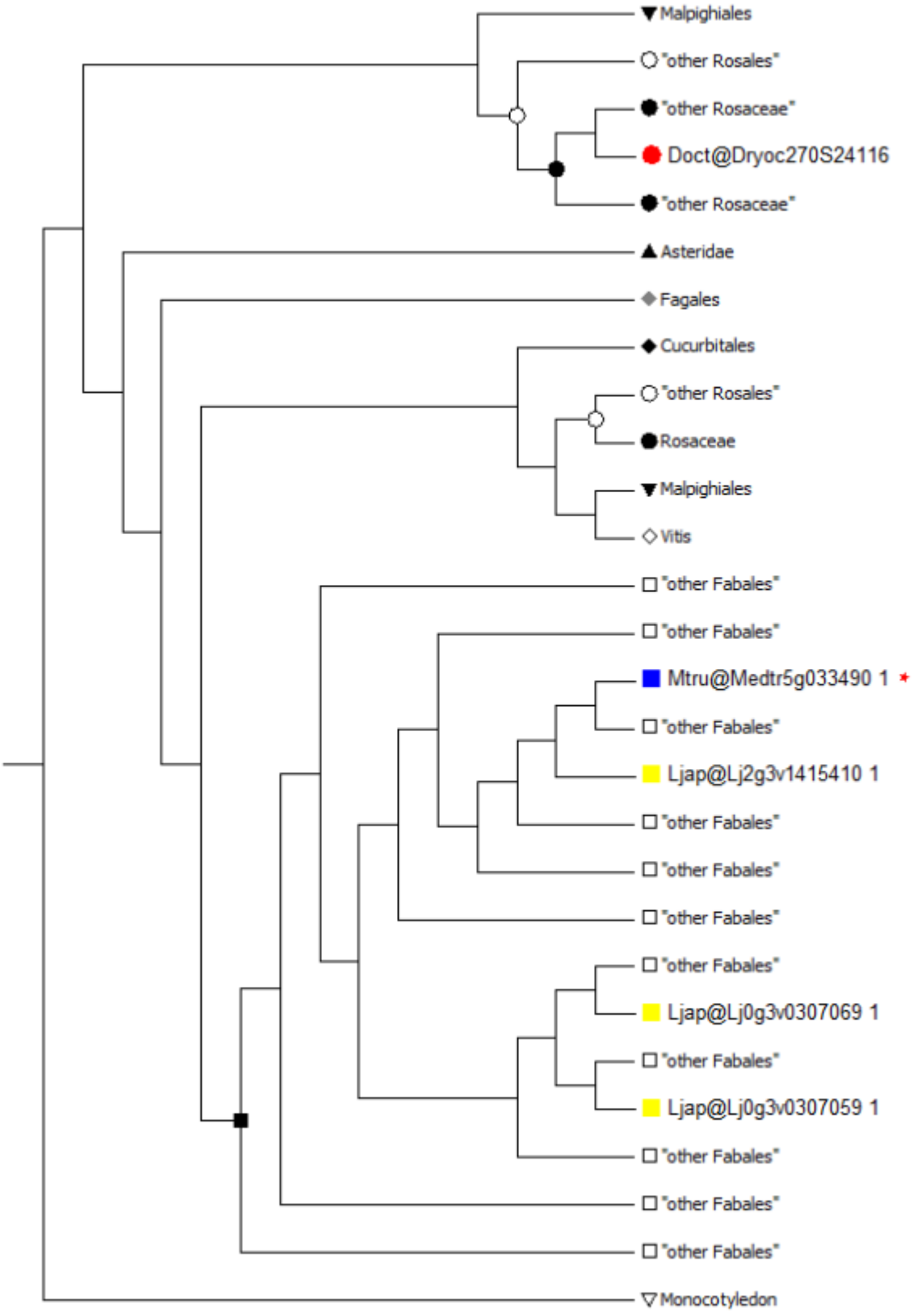
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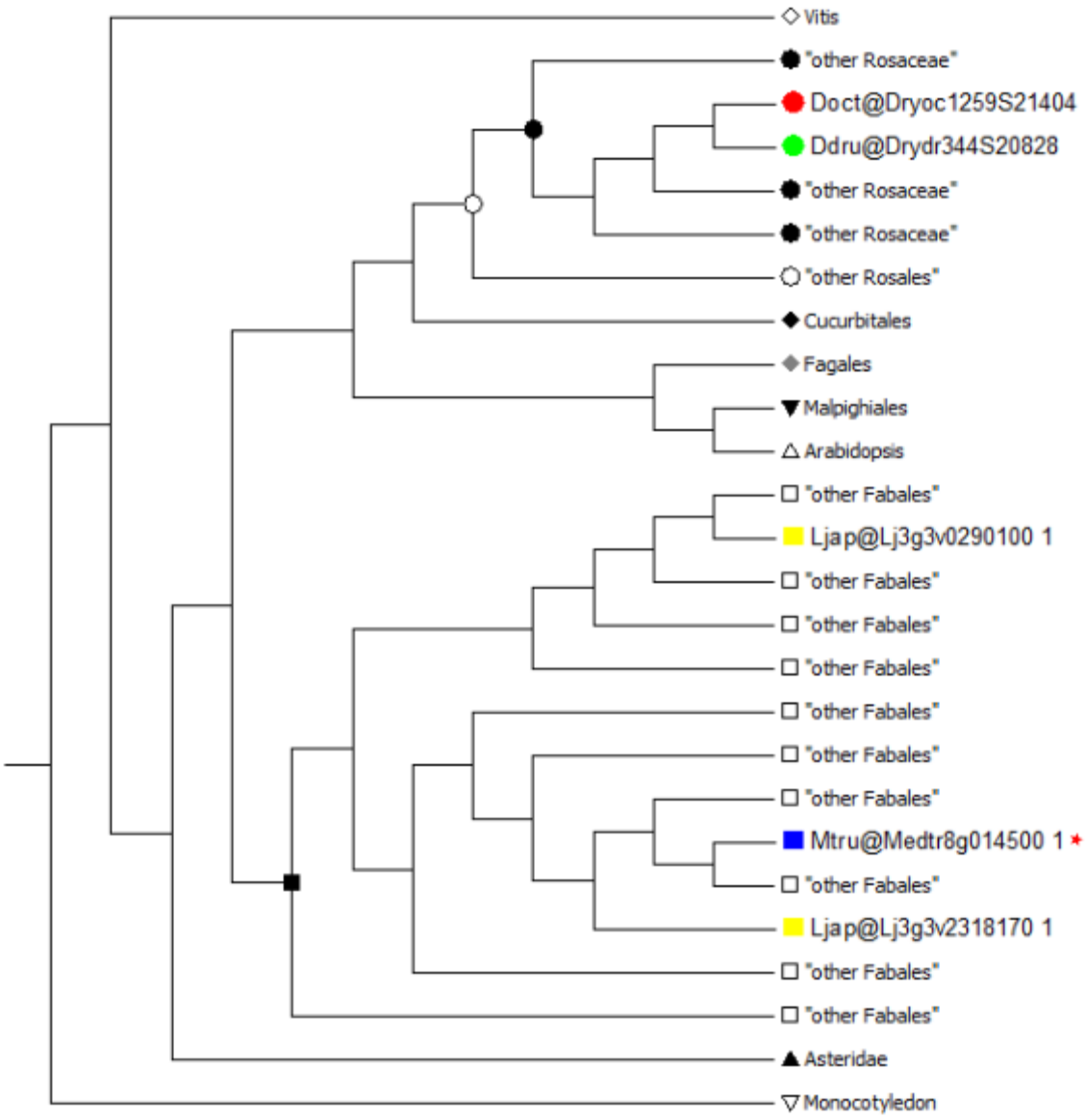
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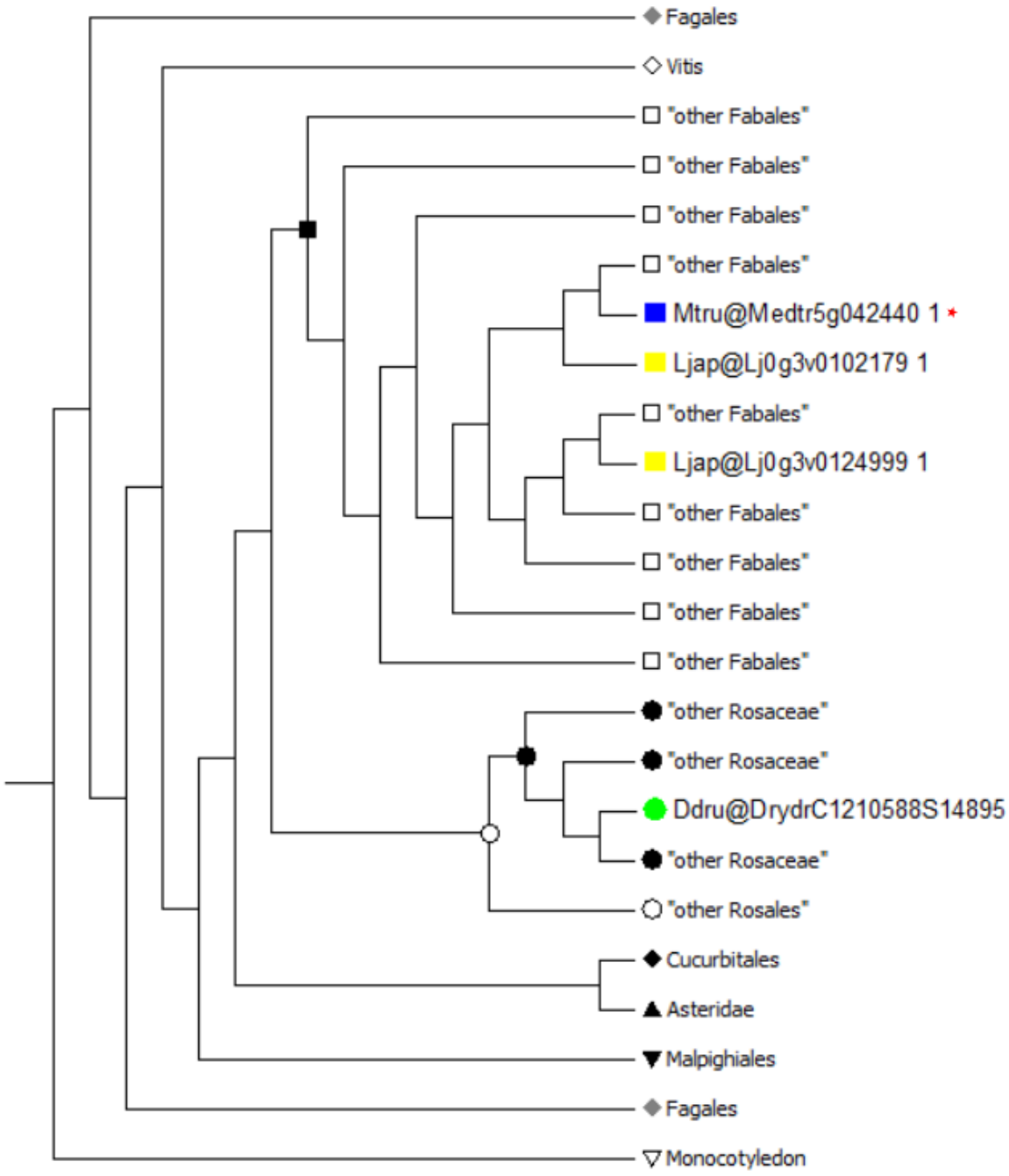
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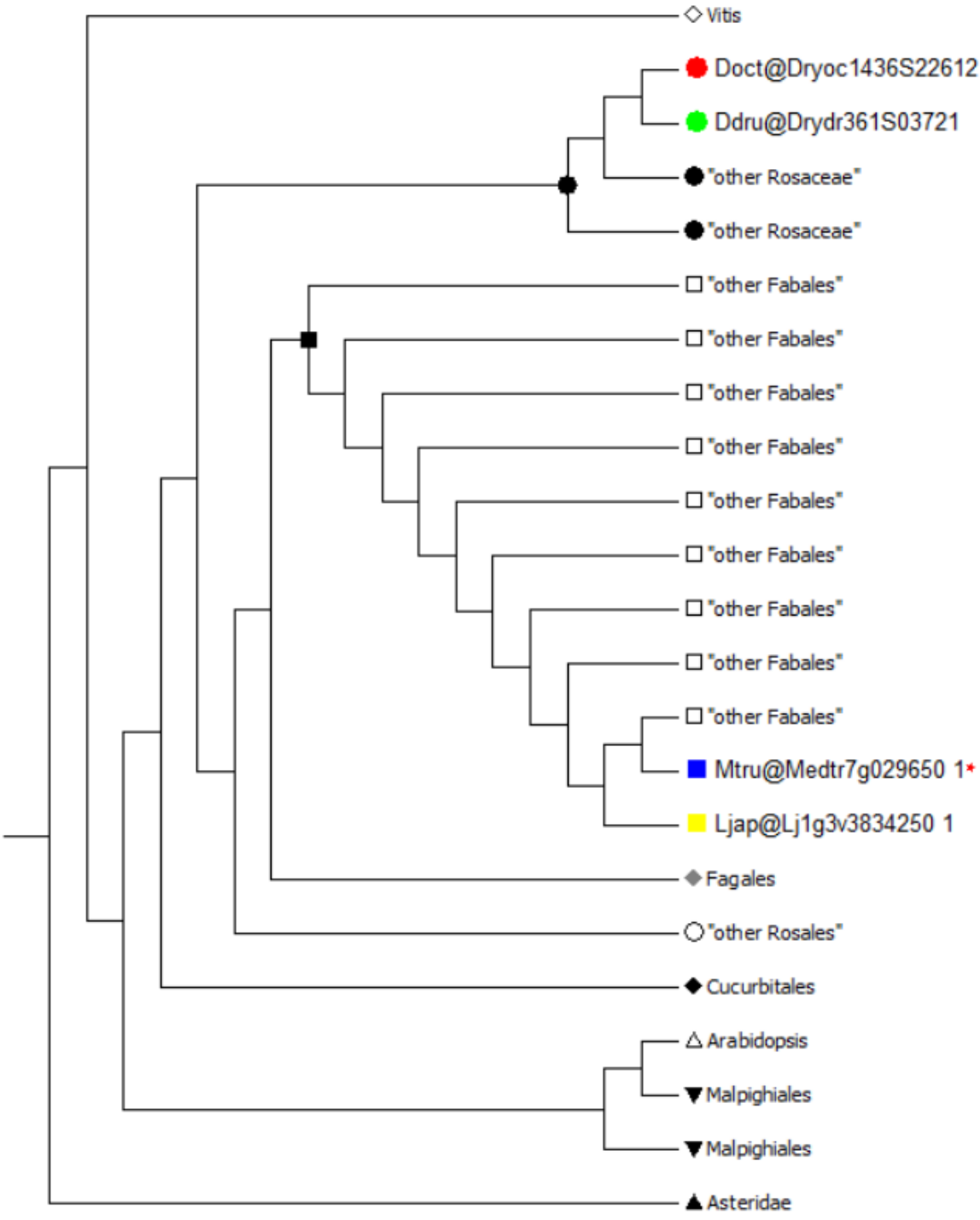
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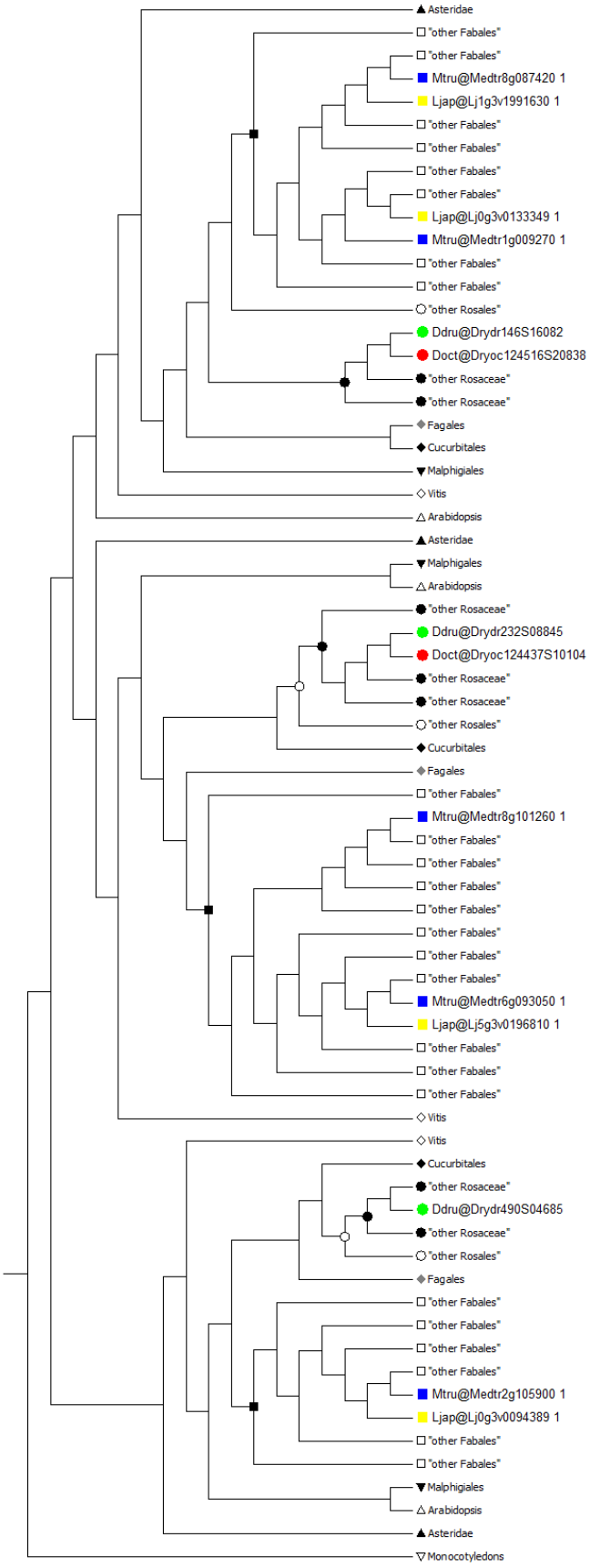
n°7

Part. 4 -> LRRIII “simplified” maximum-likelihood phylogenetic trees.



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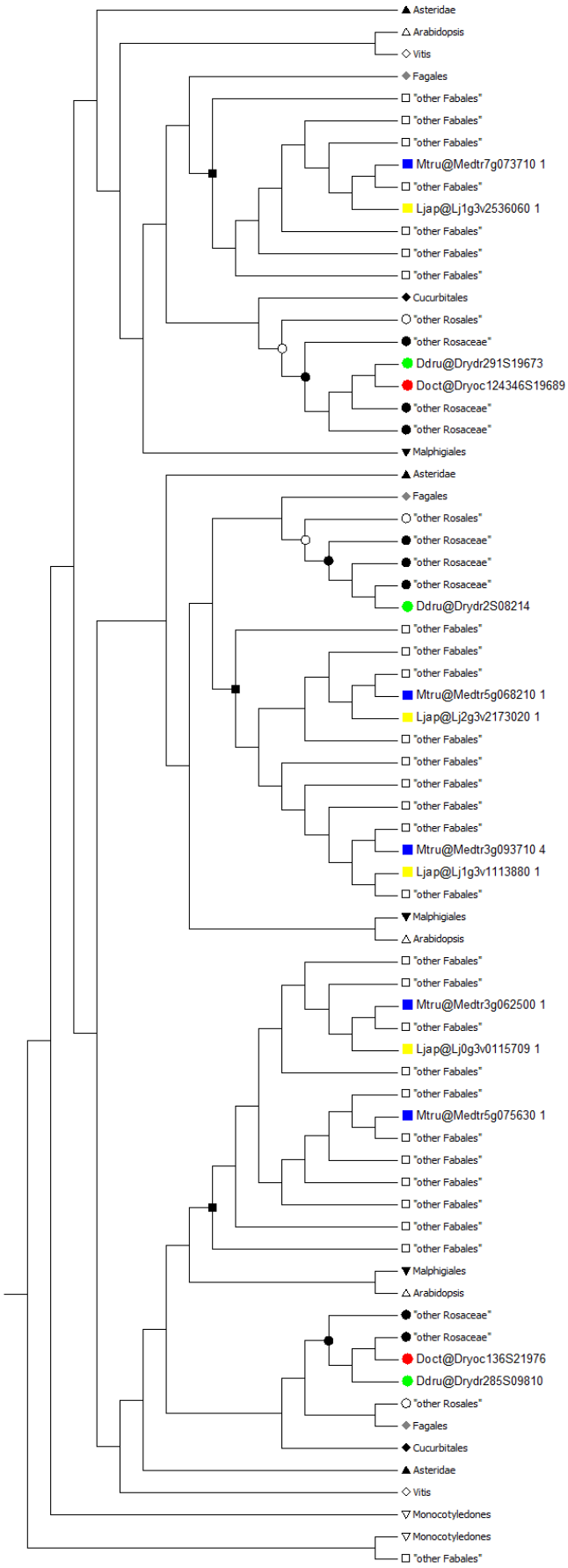
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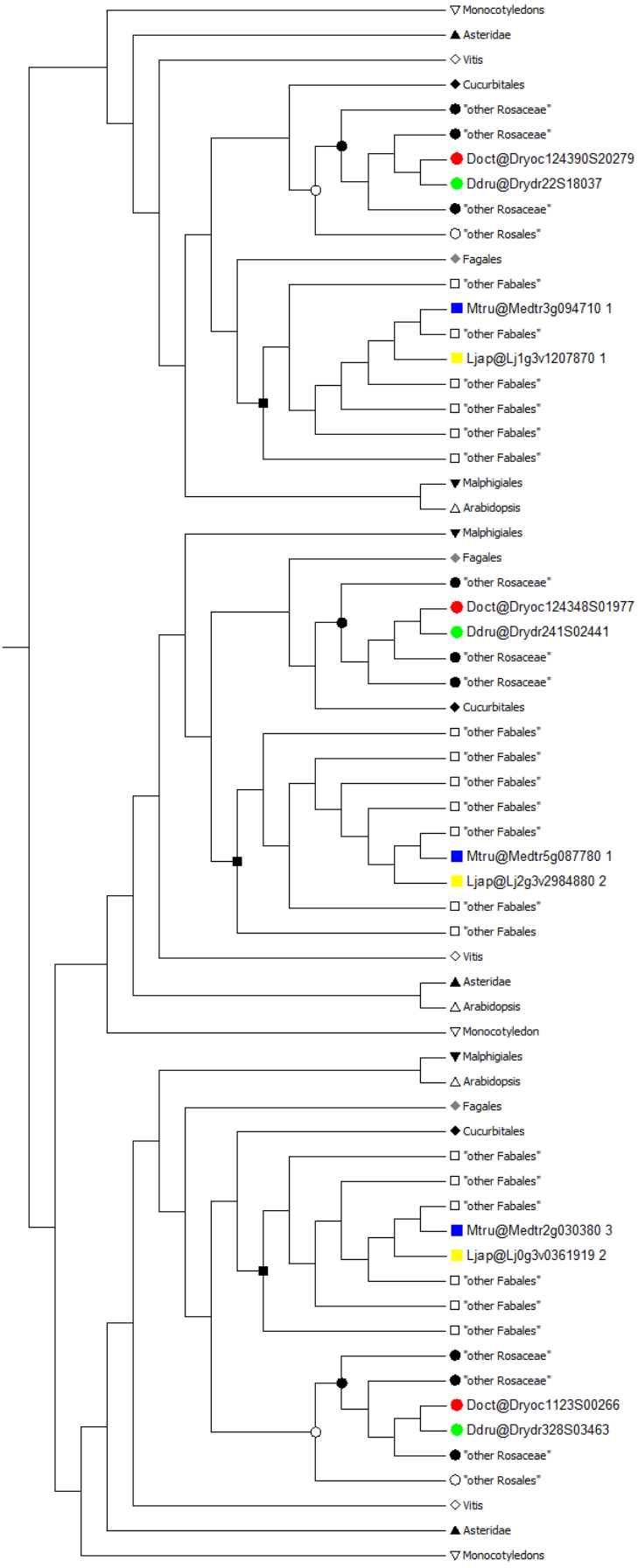
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Arabidopsis
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Monocotyledons

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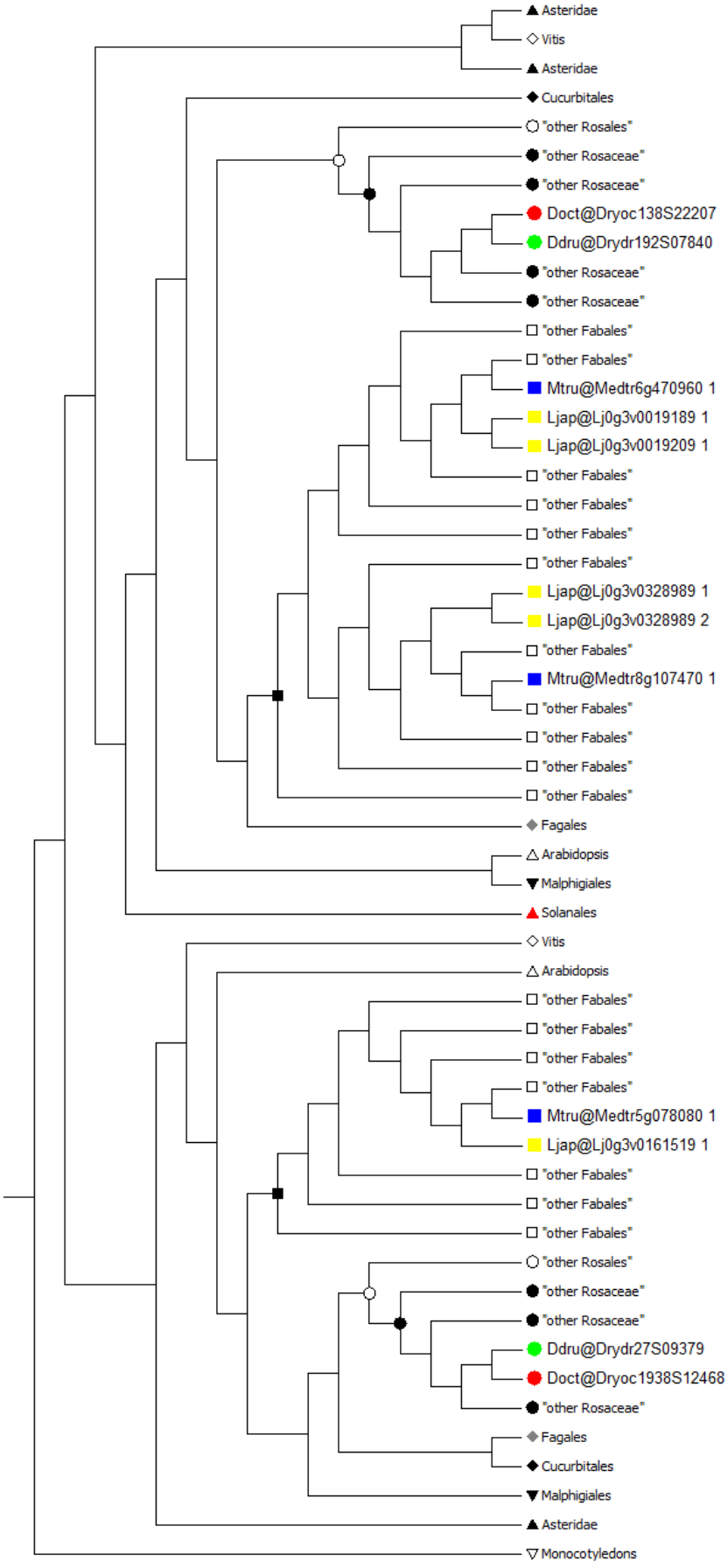
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Vitis
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Asteridae
Monocotyledons

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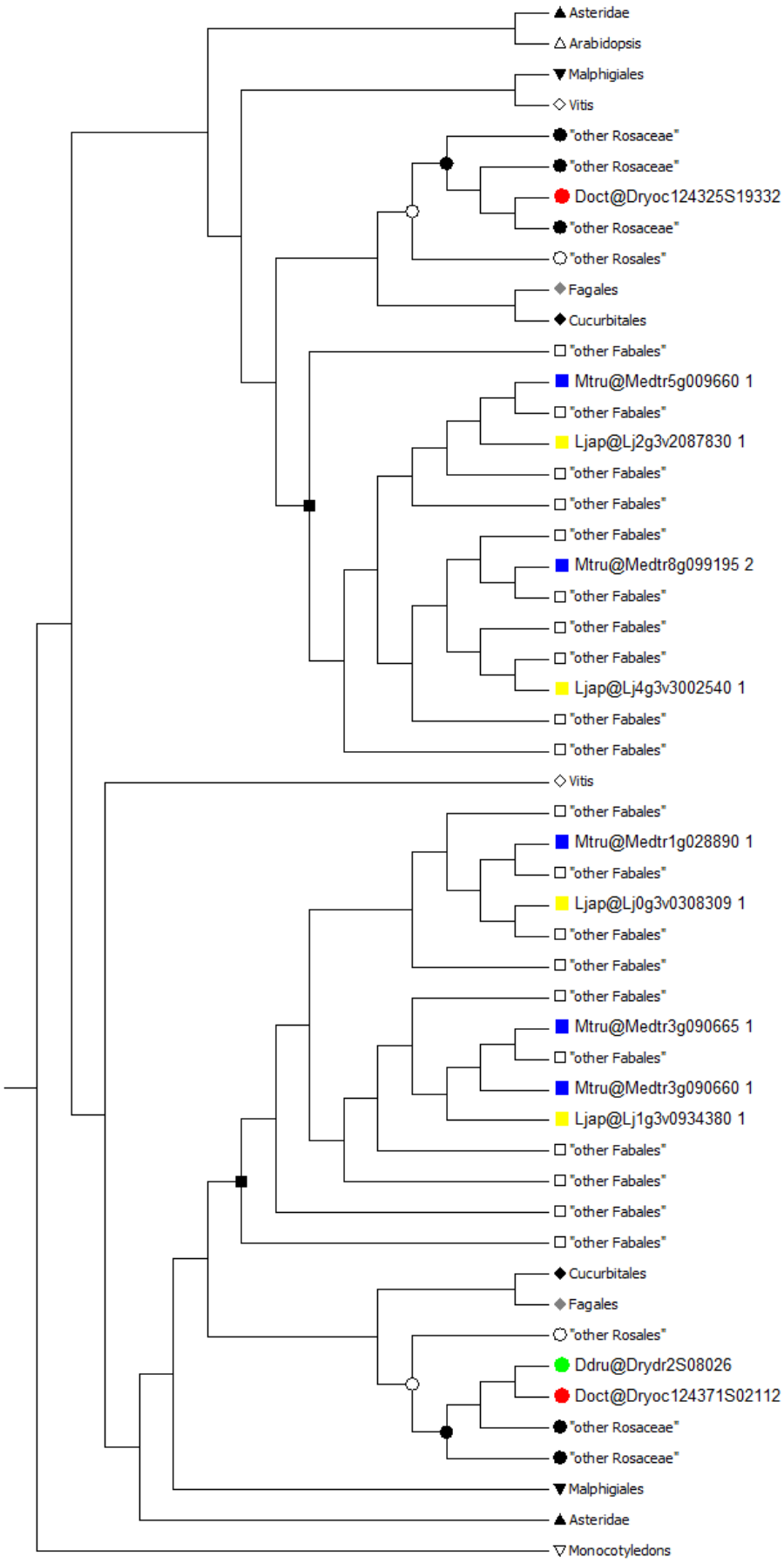
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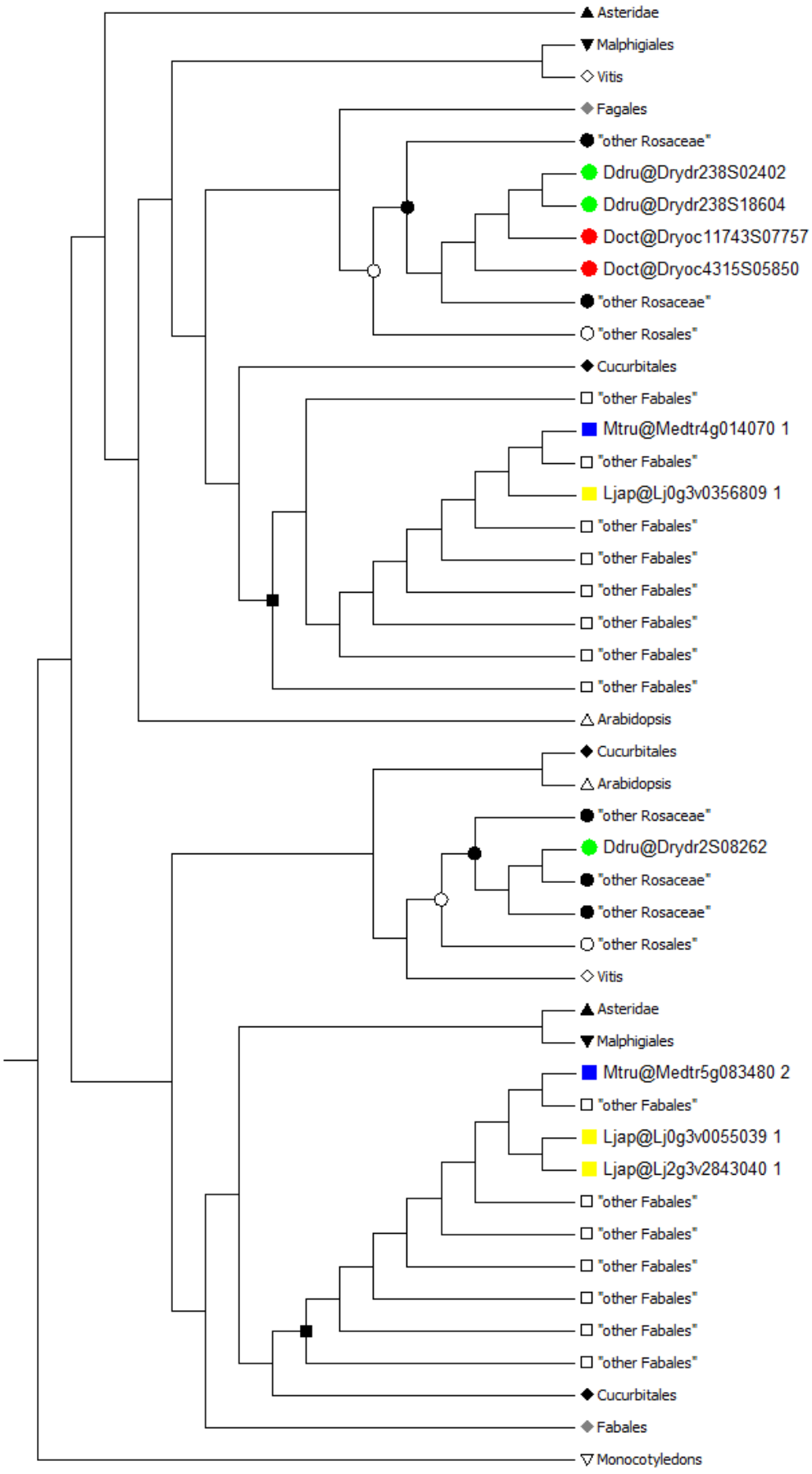
Monocotyledons

n°5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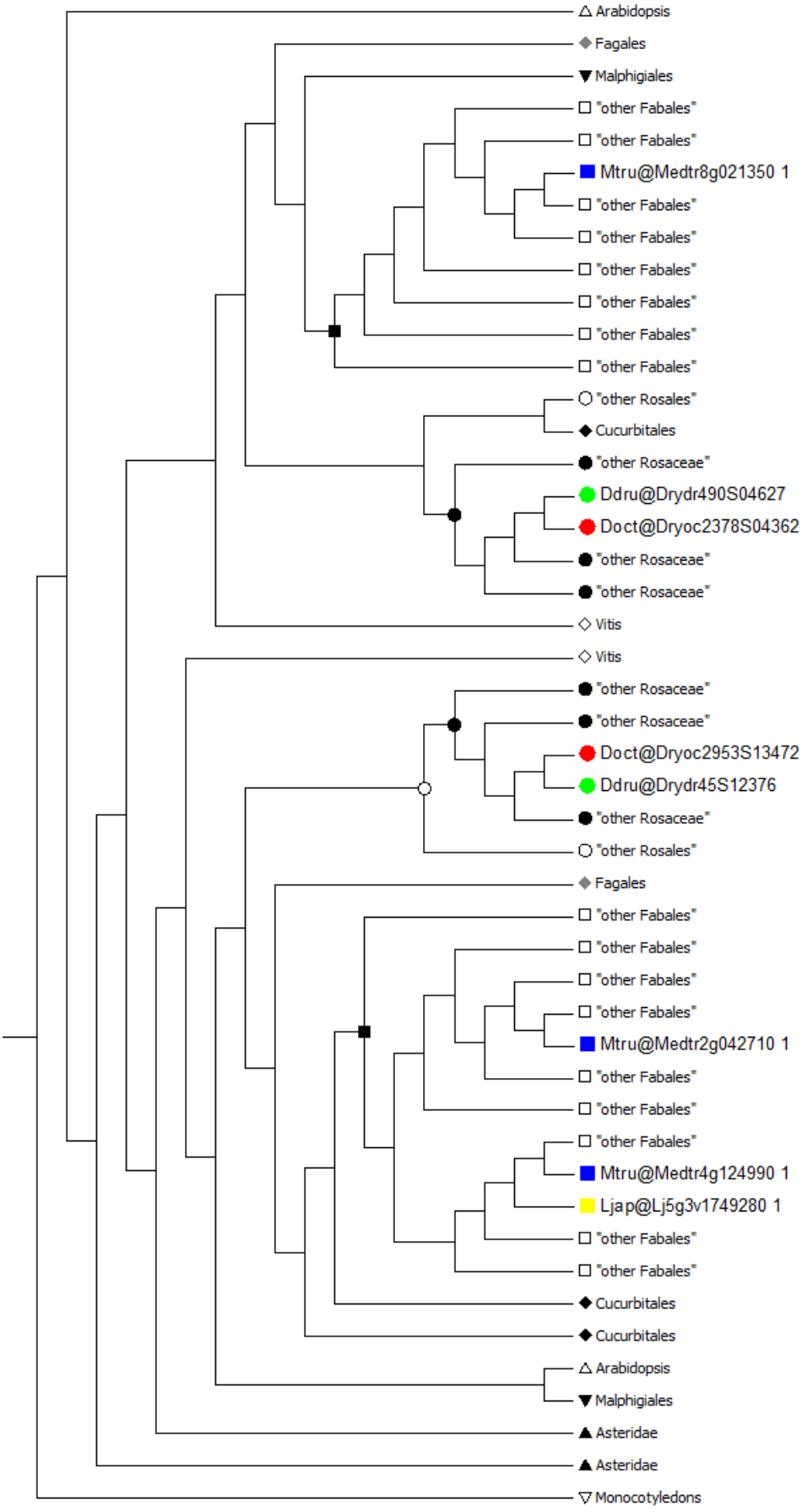
Monocotyledons

n°6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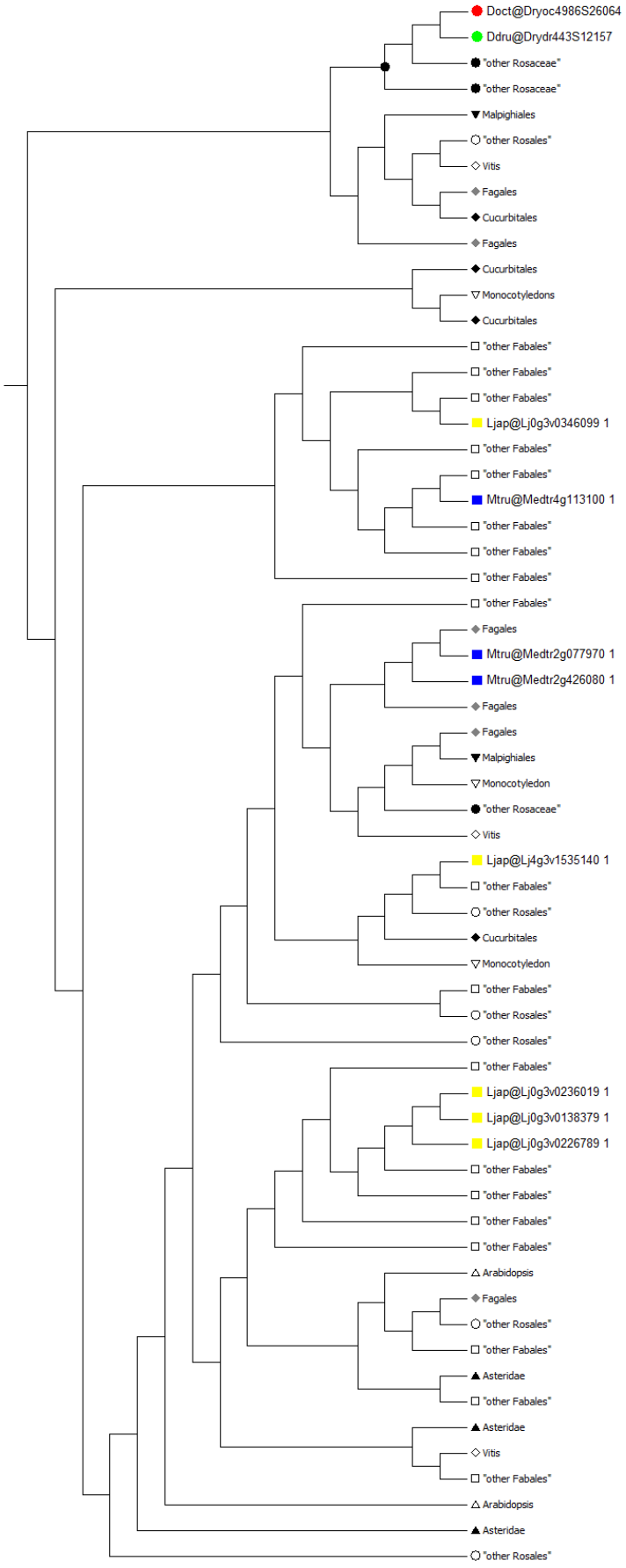
Malphigiales
Asteridae
Asteridae
Monocotyledons

n°7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Cucurbitales
Fagales
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Monocotyledons
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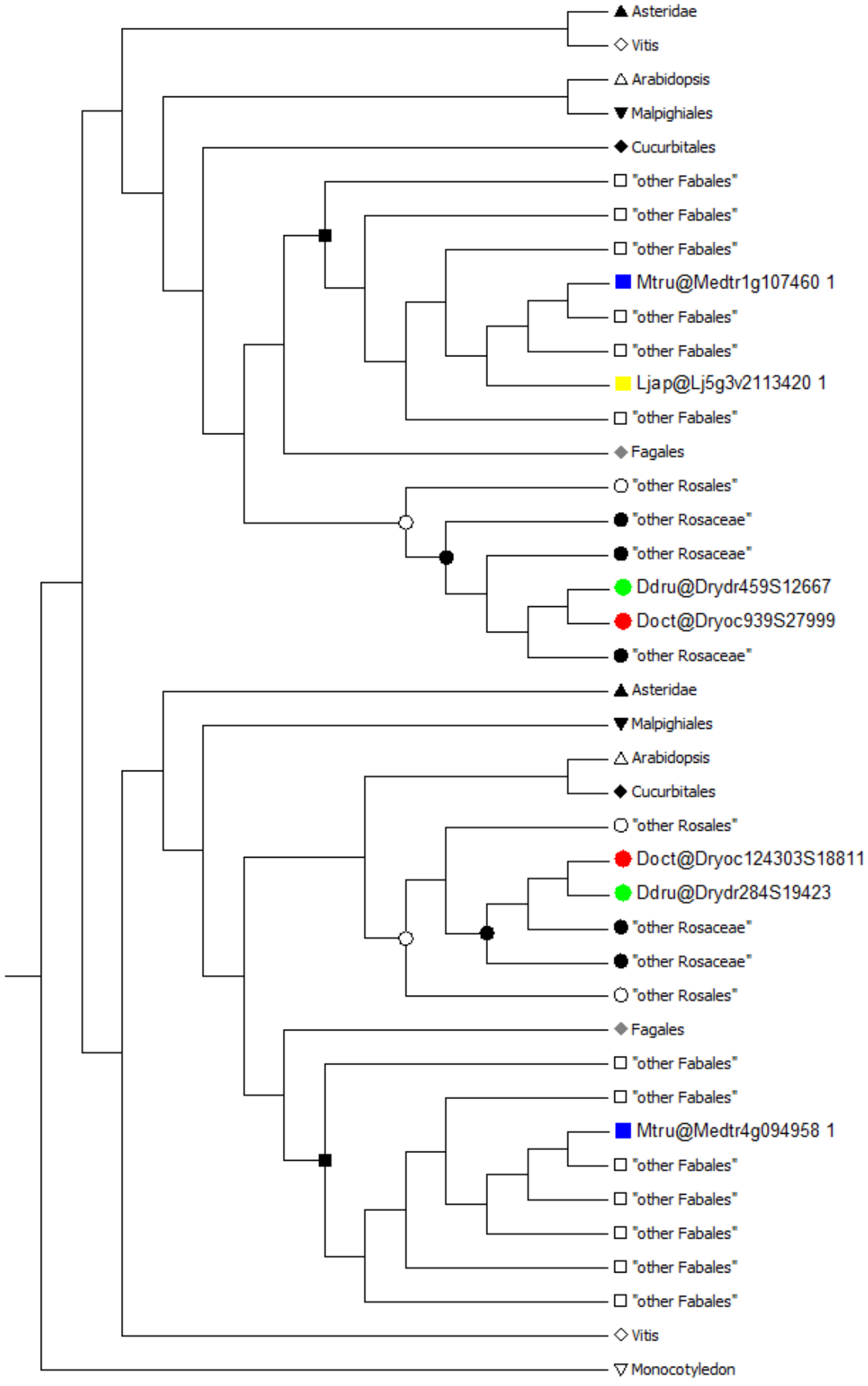
Asteridae
"other Rosales"

n°8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Doct@Dryoc939S27999
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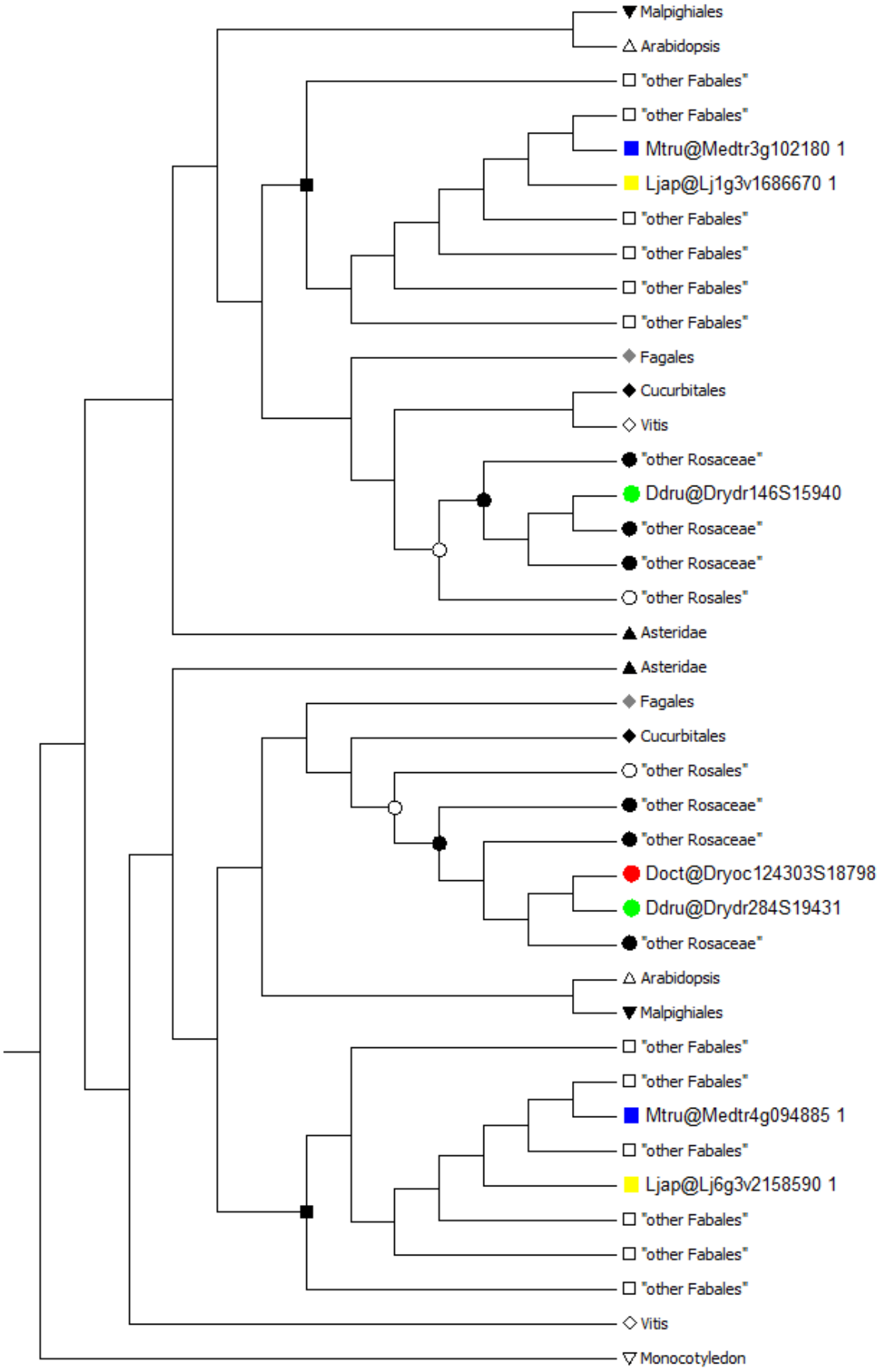
Monocotyledon

n°9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Cucurbitales
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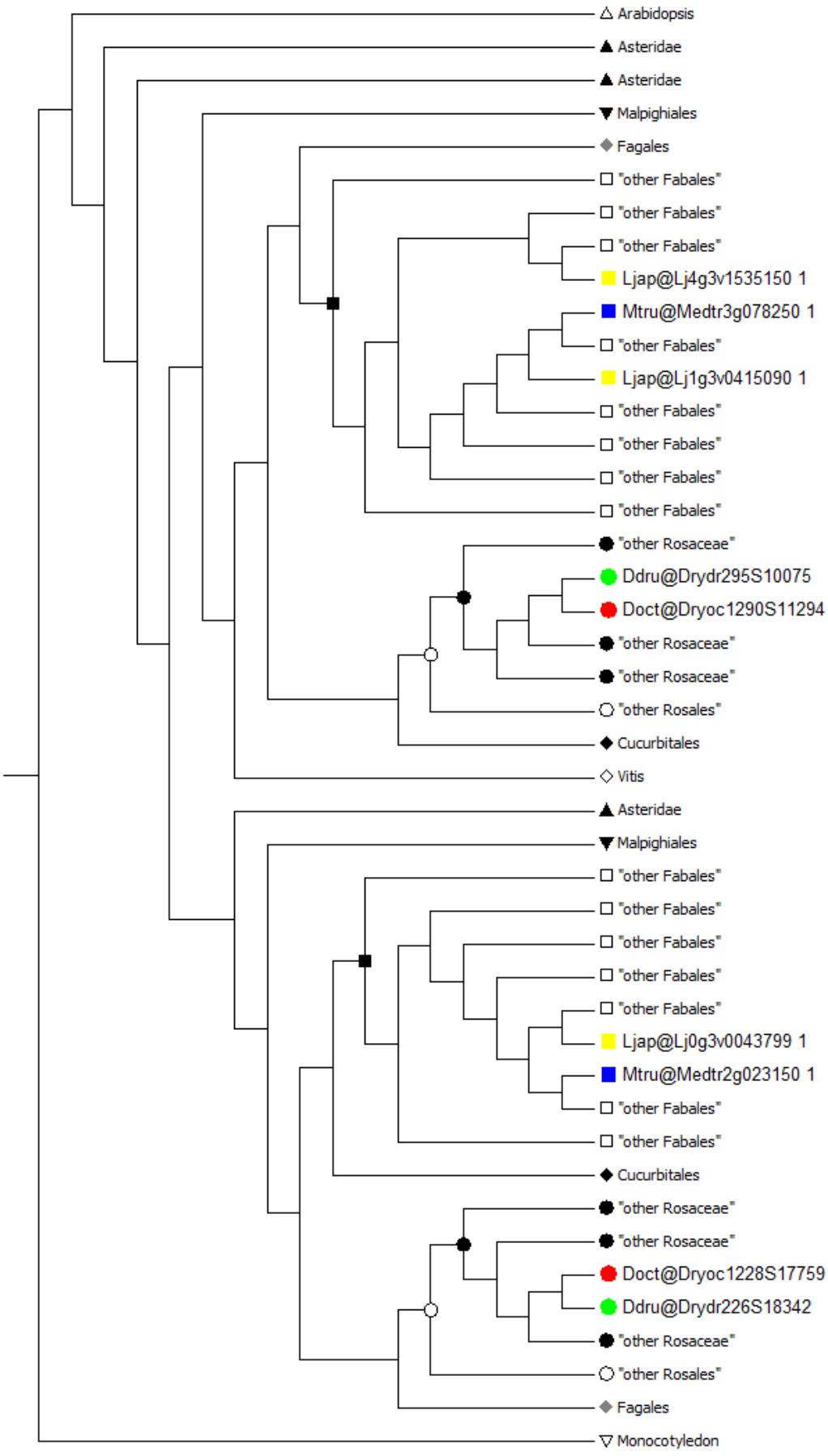
n°10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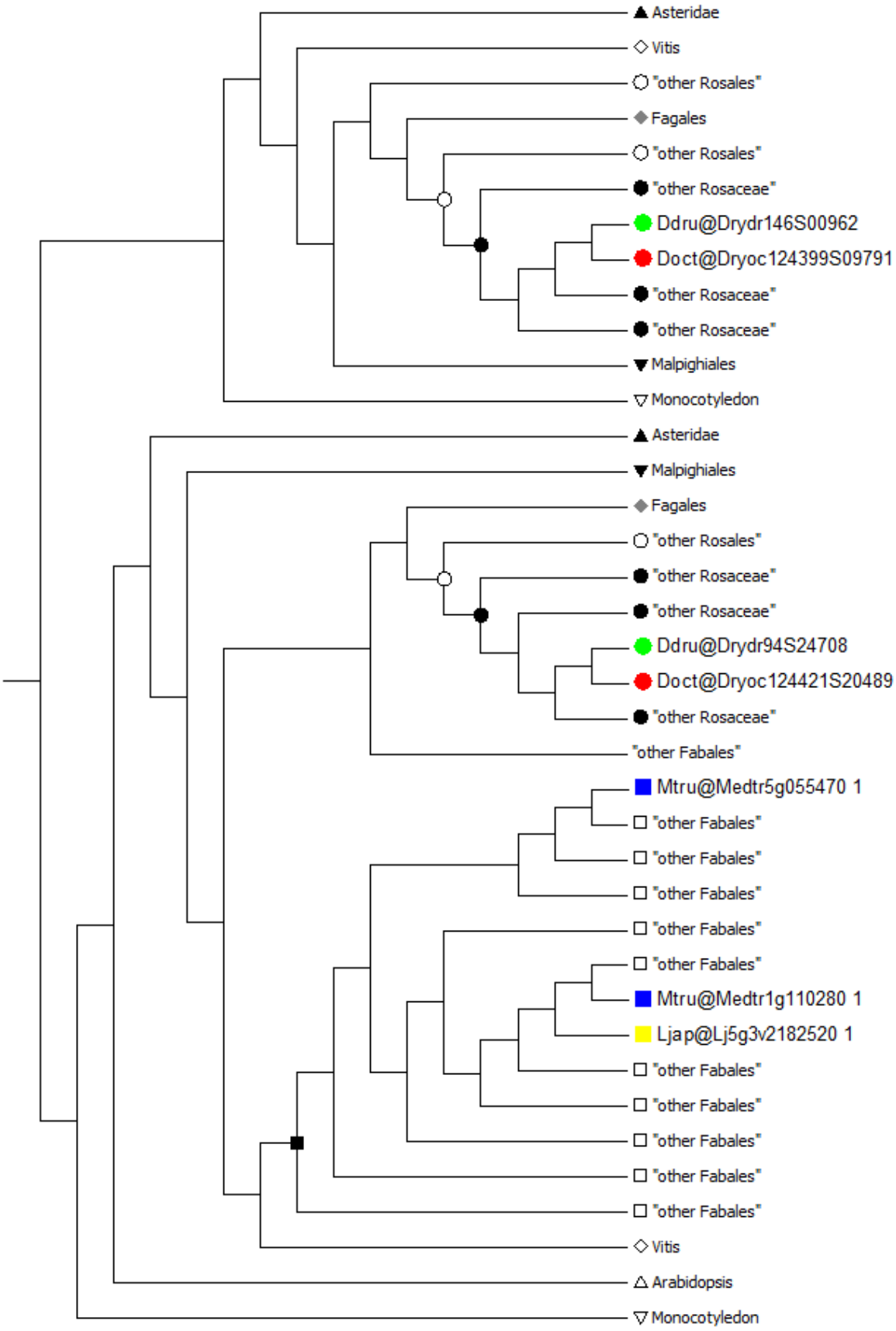
n°11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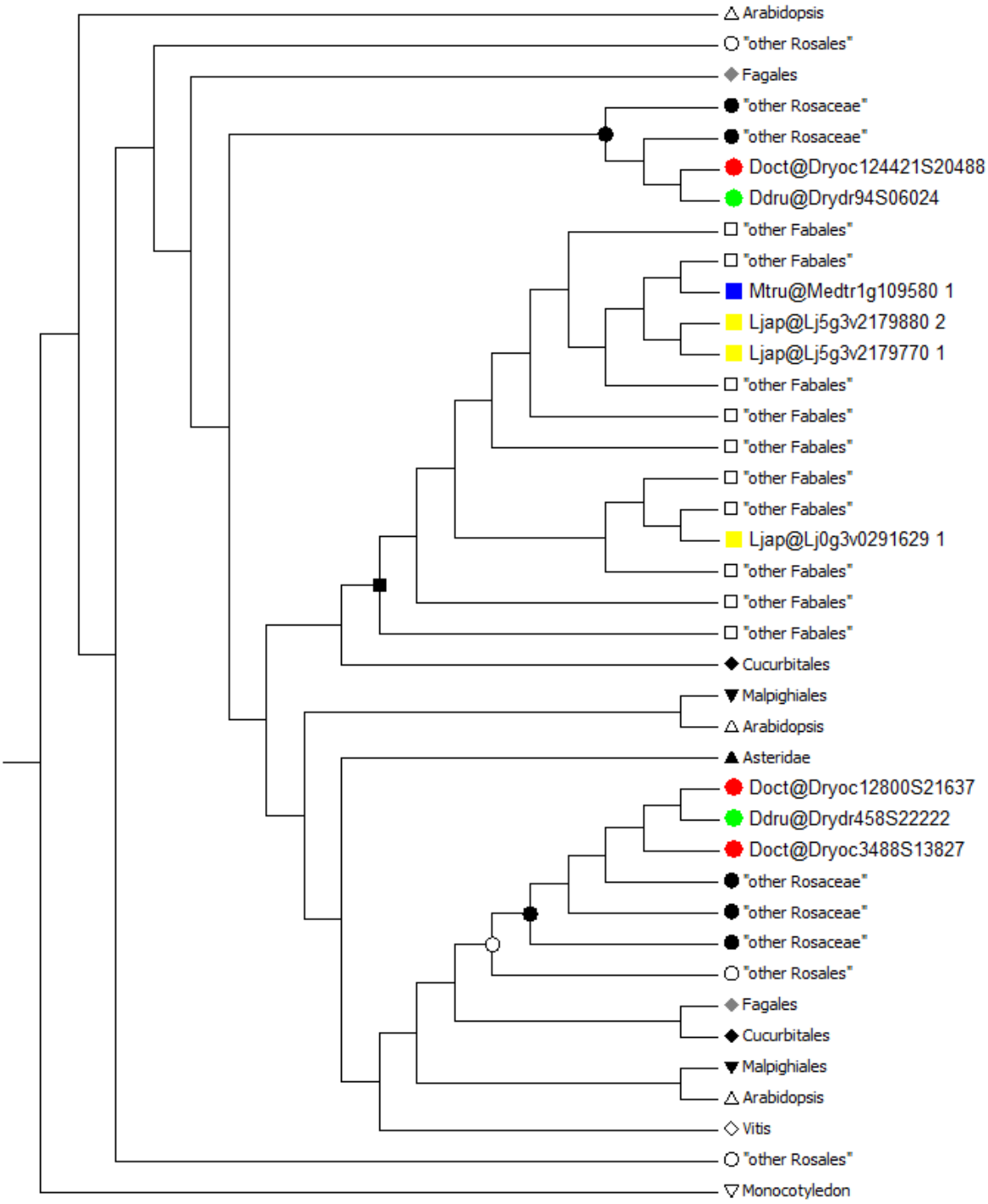
n°12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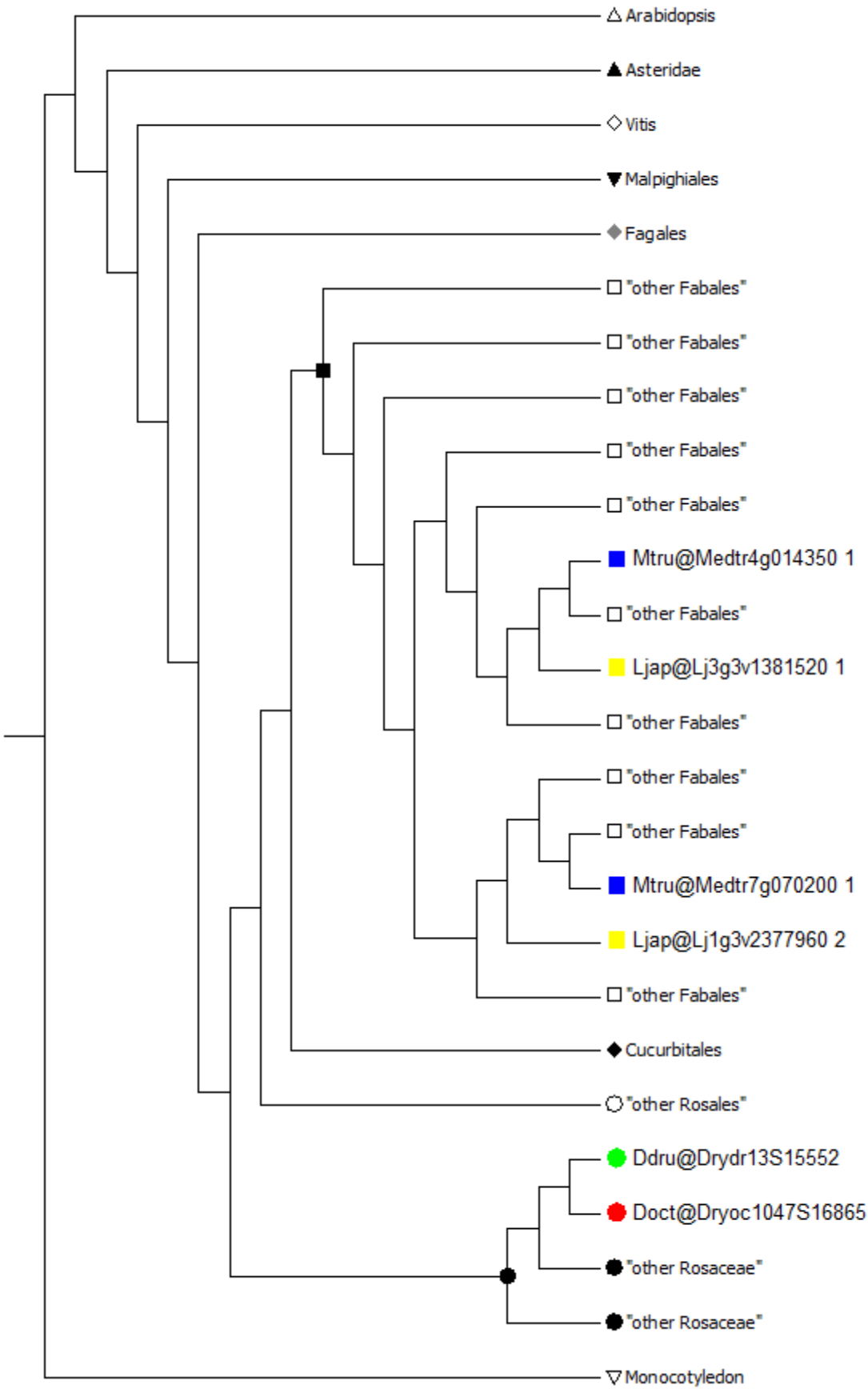
n°13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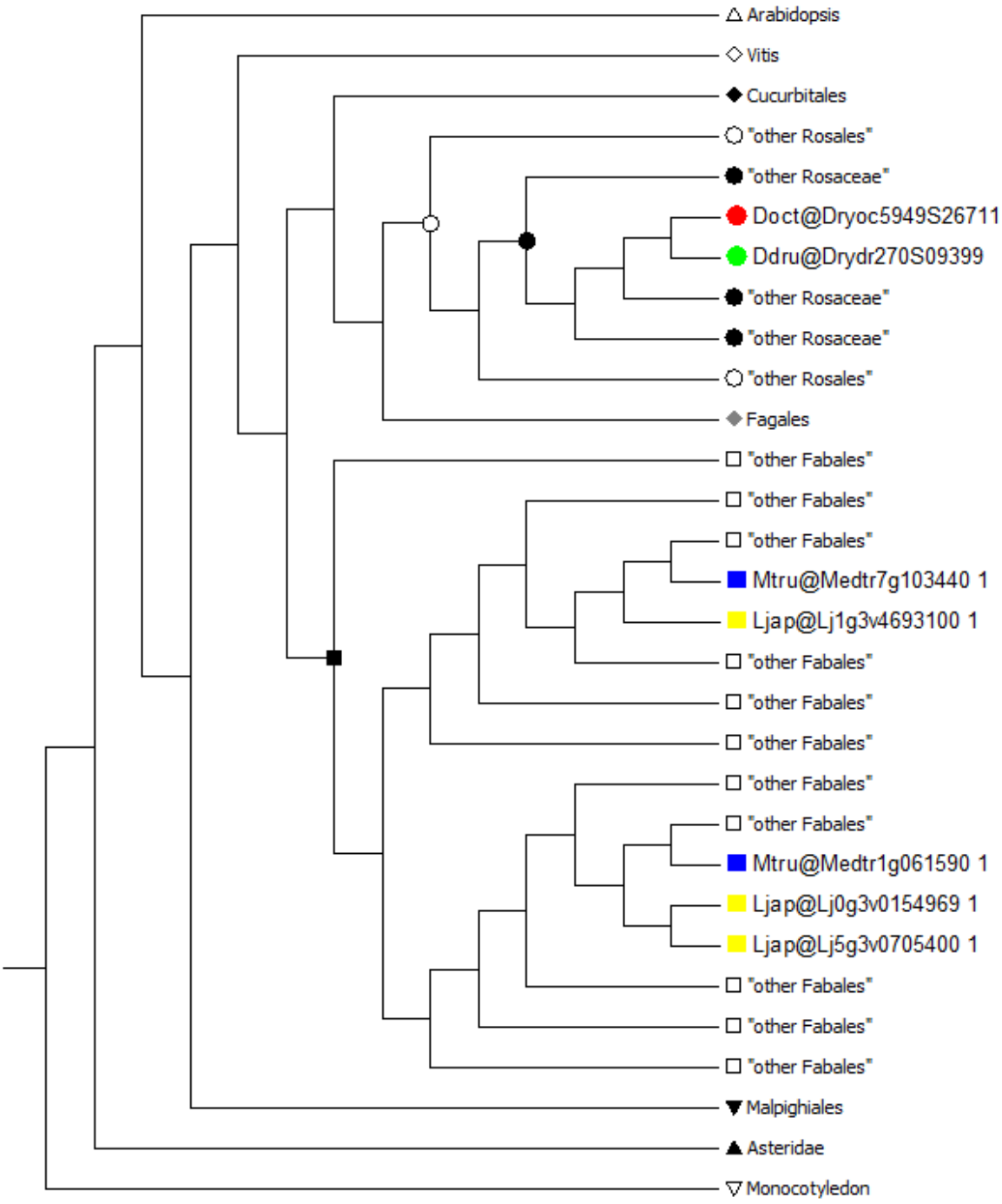
n°14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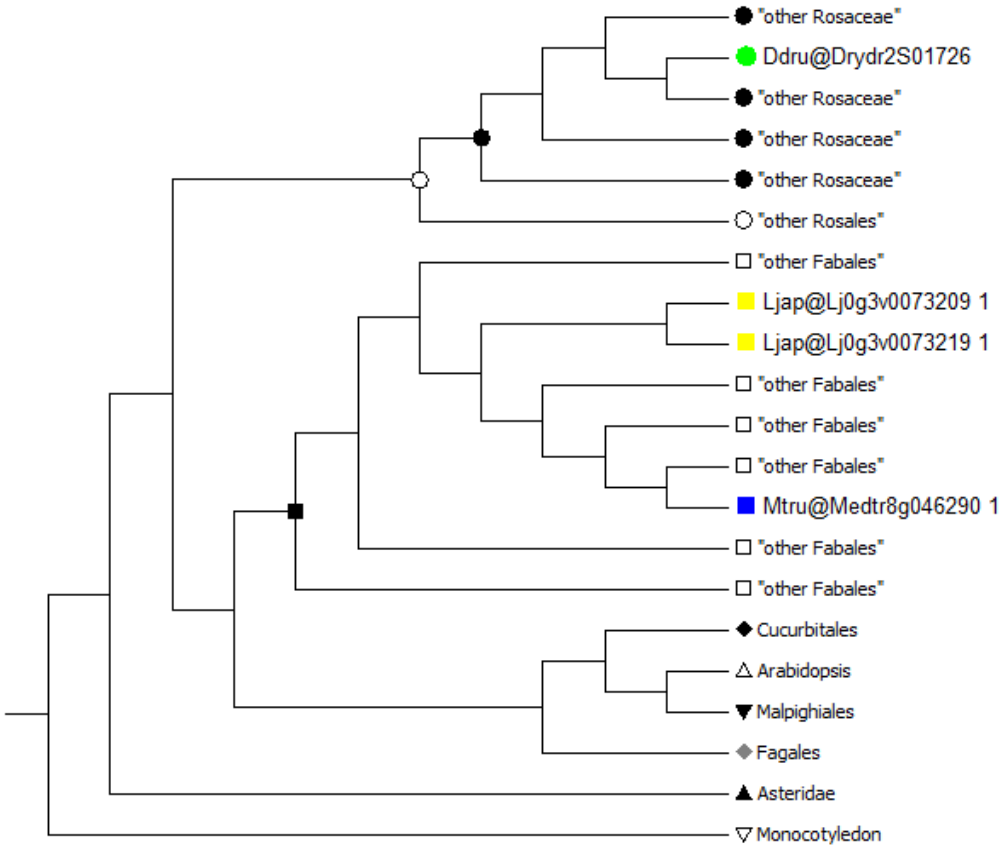
n°15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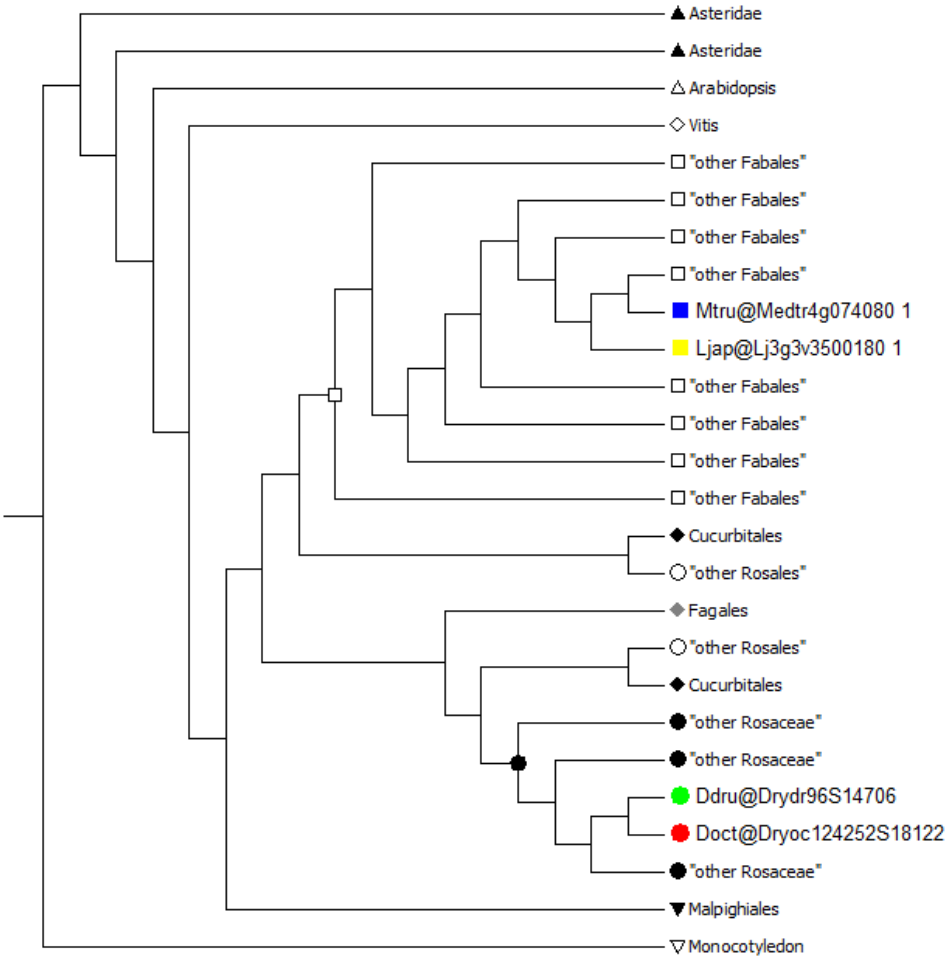
n°16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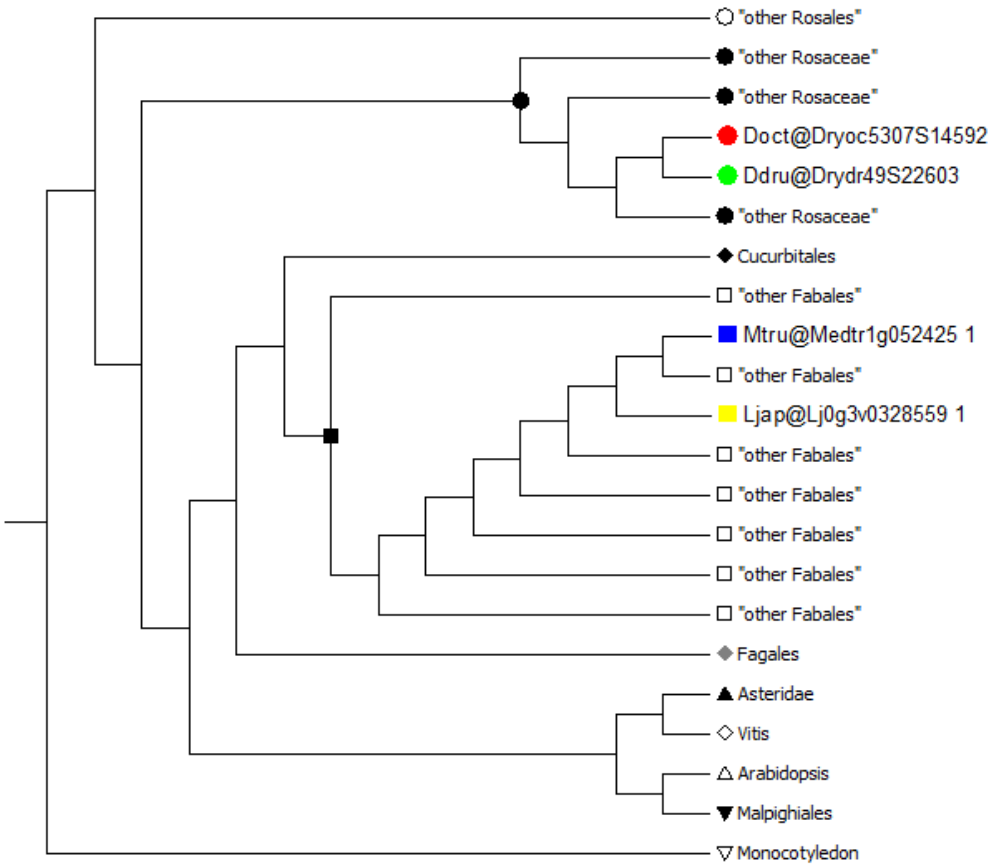
Monocotyledon

n°17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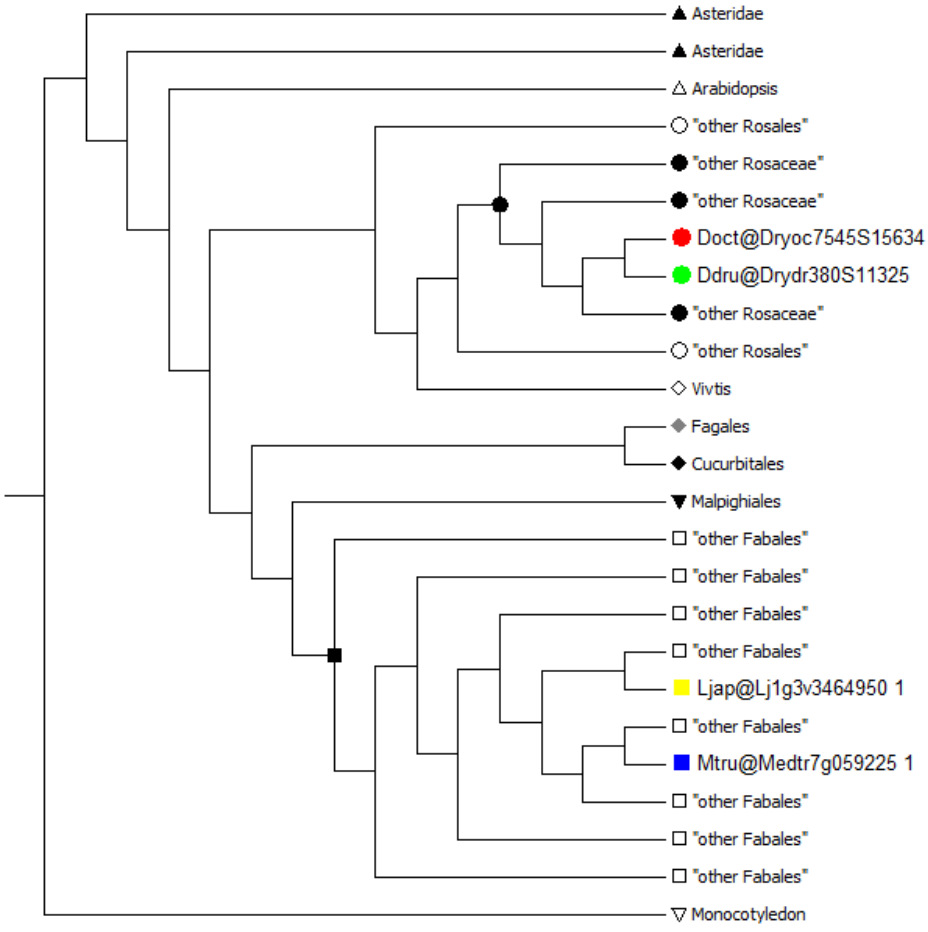
n°18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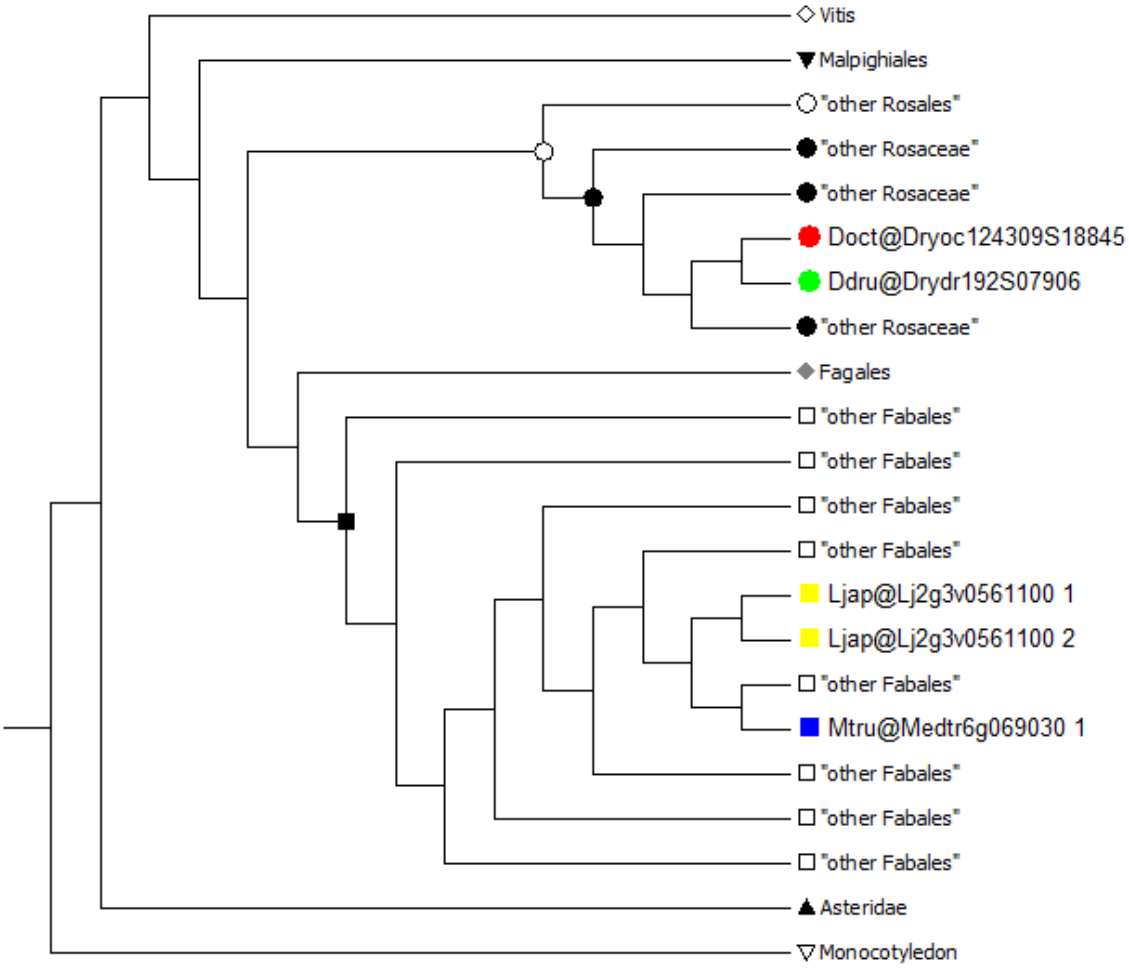
n°19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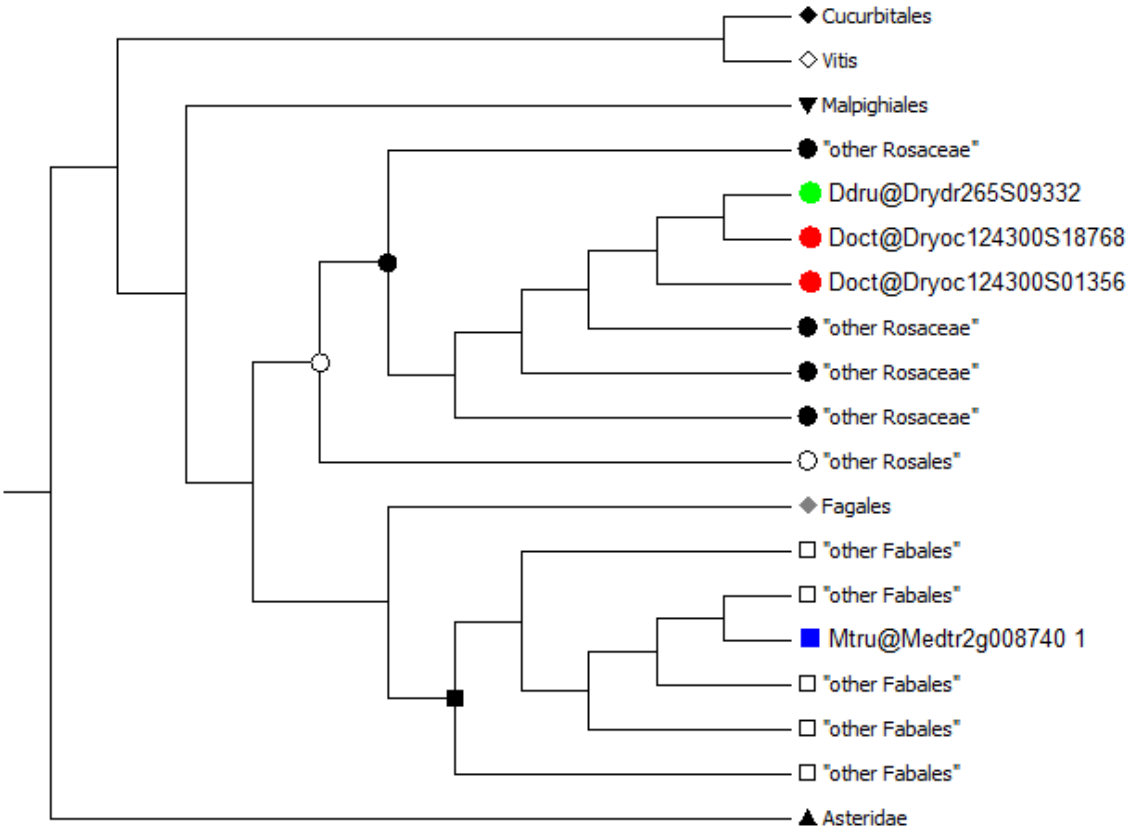
Monocotyledon

n°20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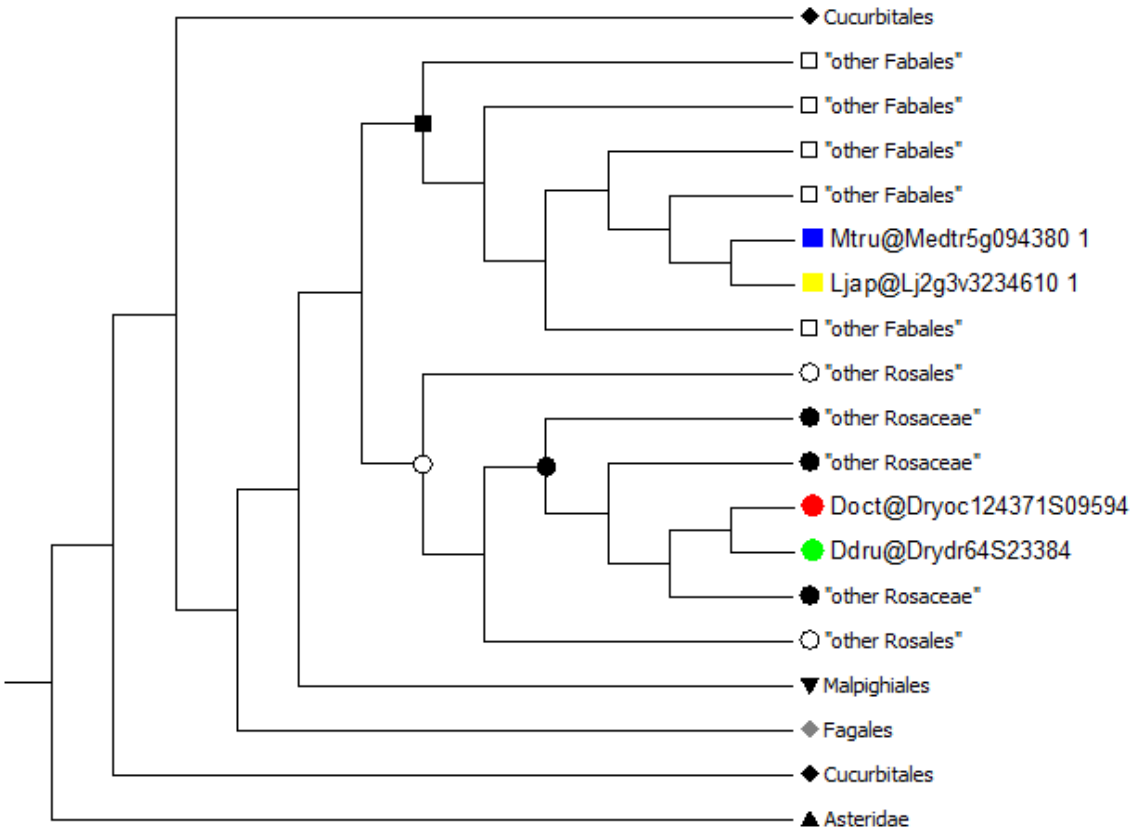
n°21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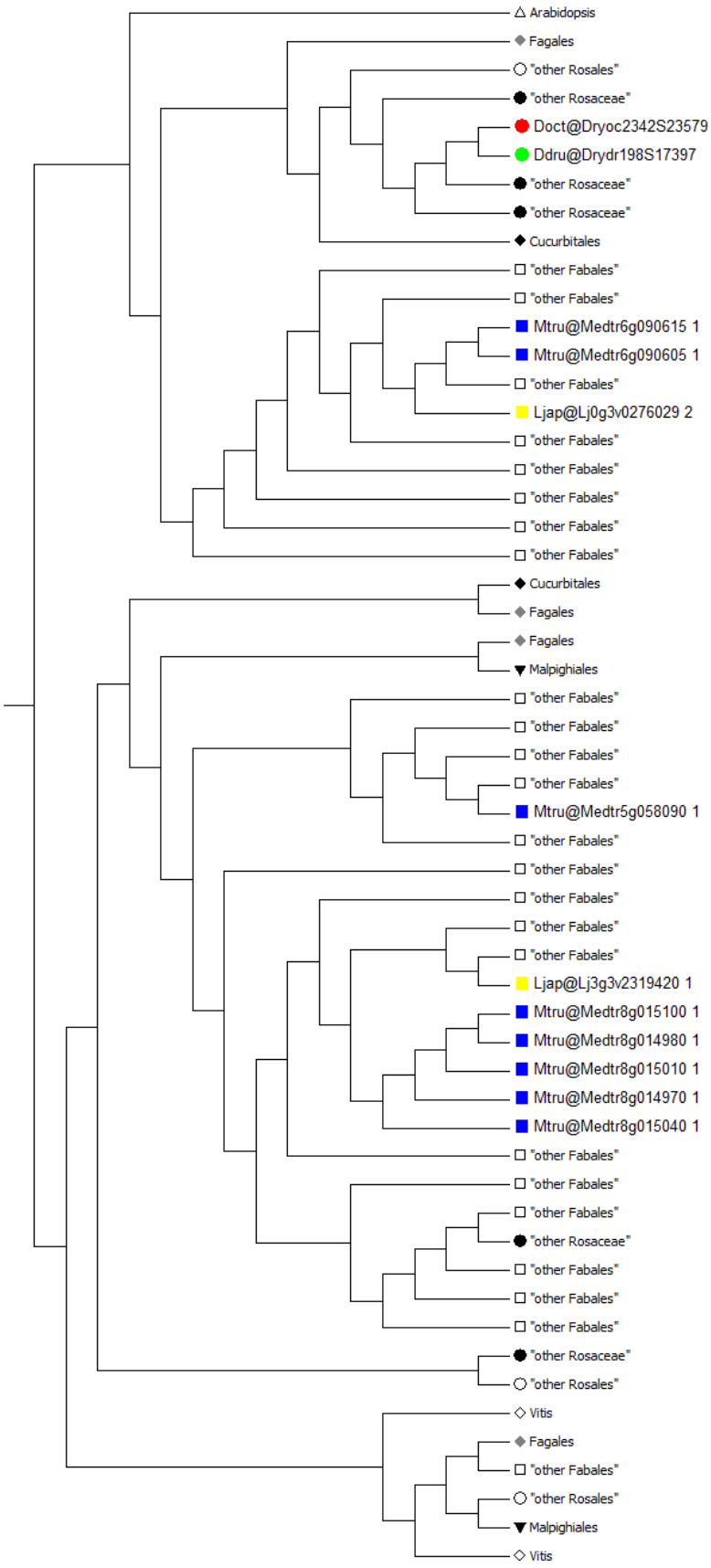
Malpighiales
Fagales
Cucurbitales
Asteridae

n°22

Part. 5 -> LRRI-1 “simplified” maximum-likelihood phylogenetic trees.



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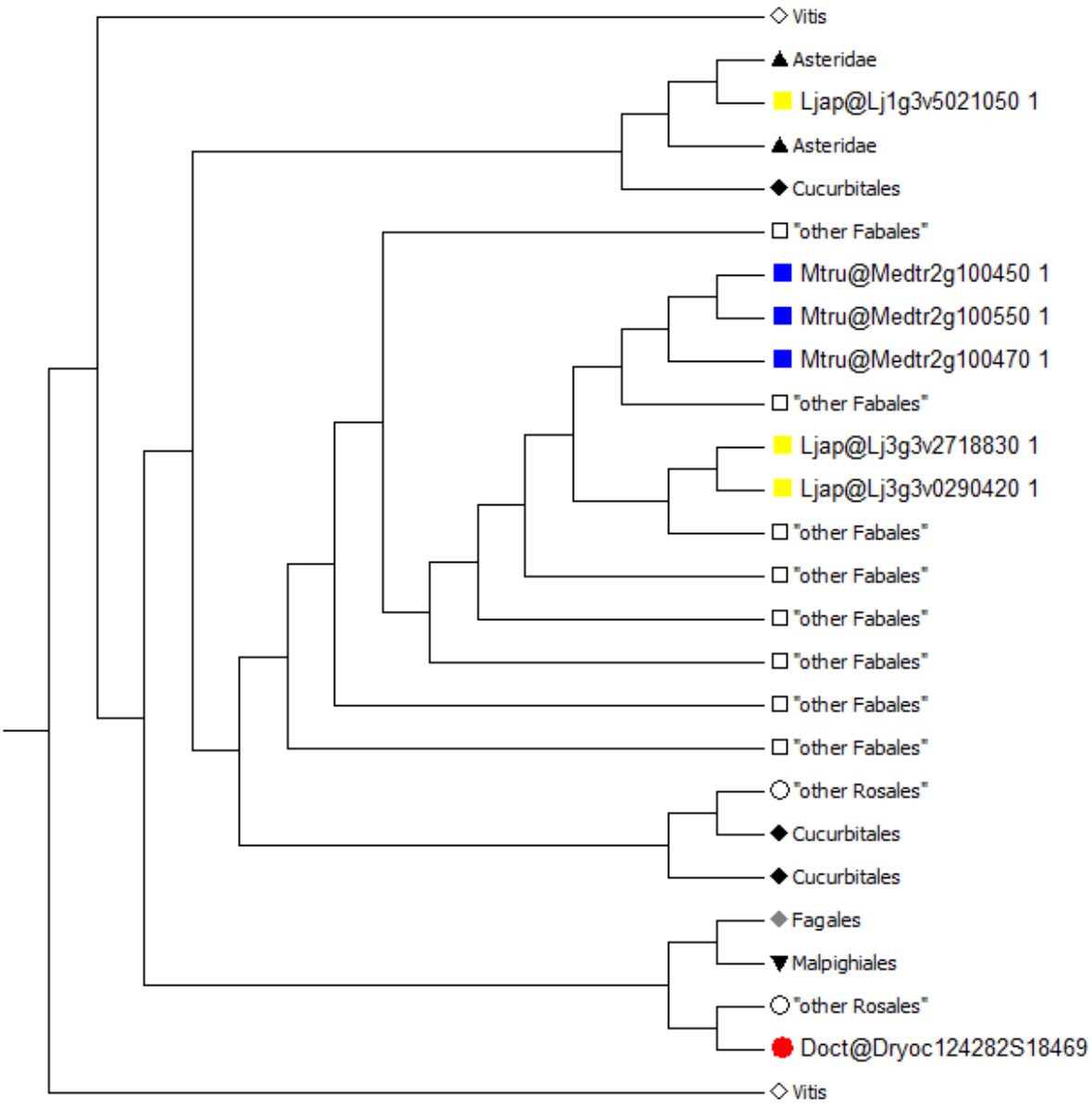
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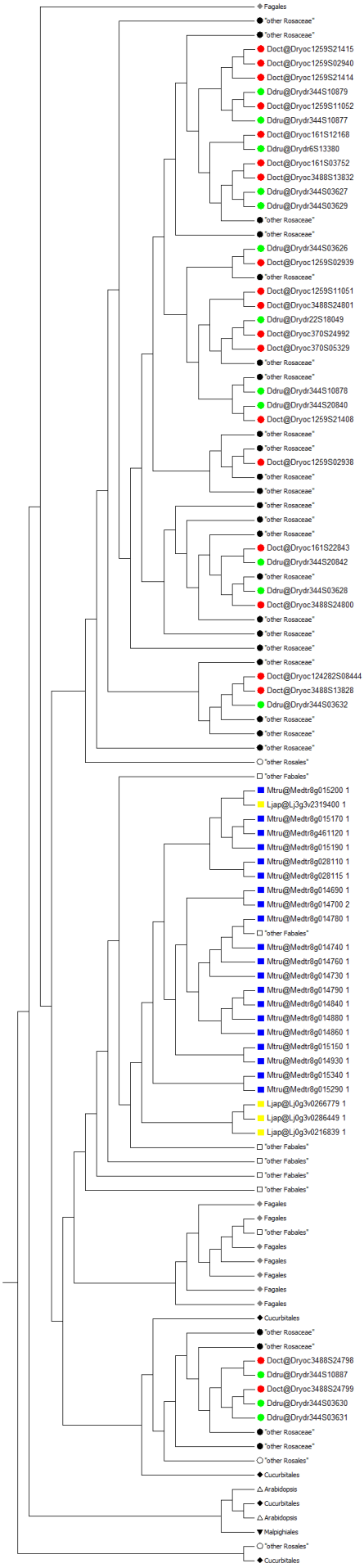
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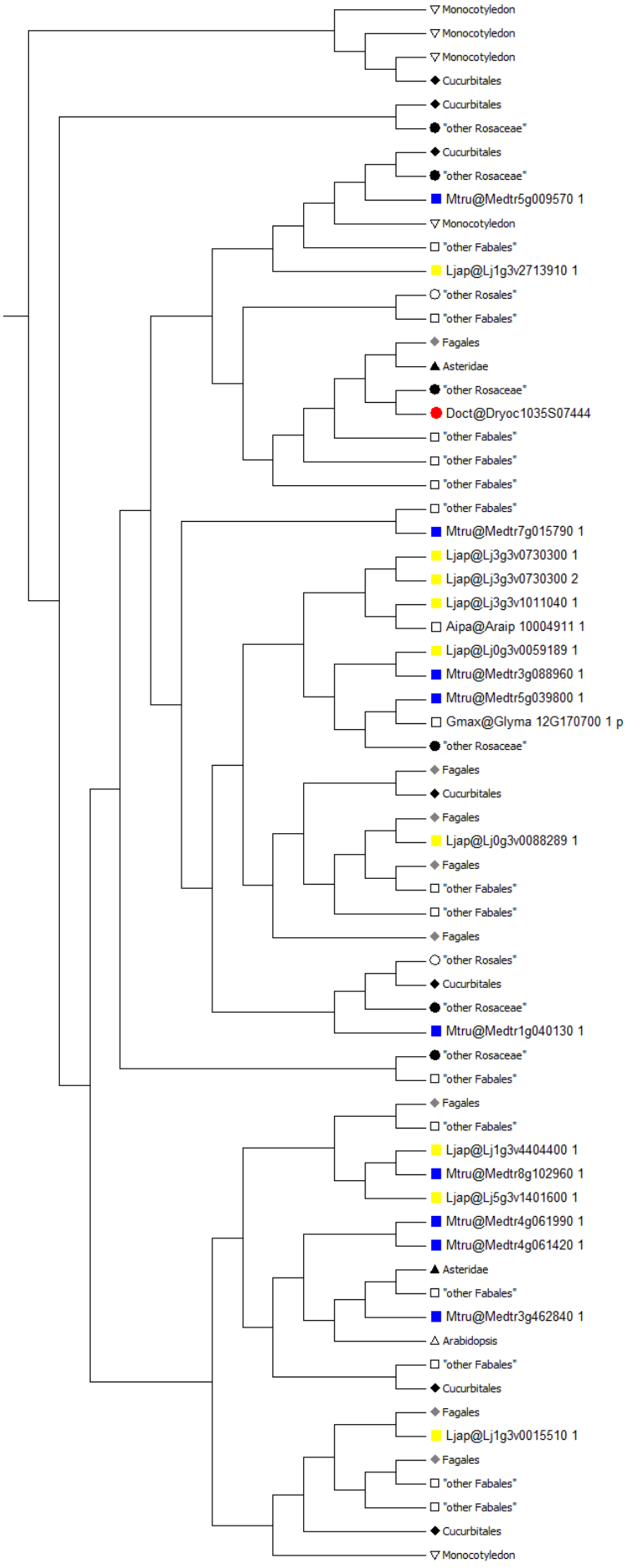
n°3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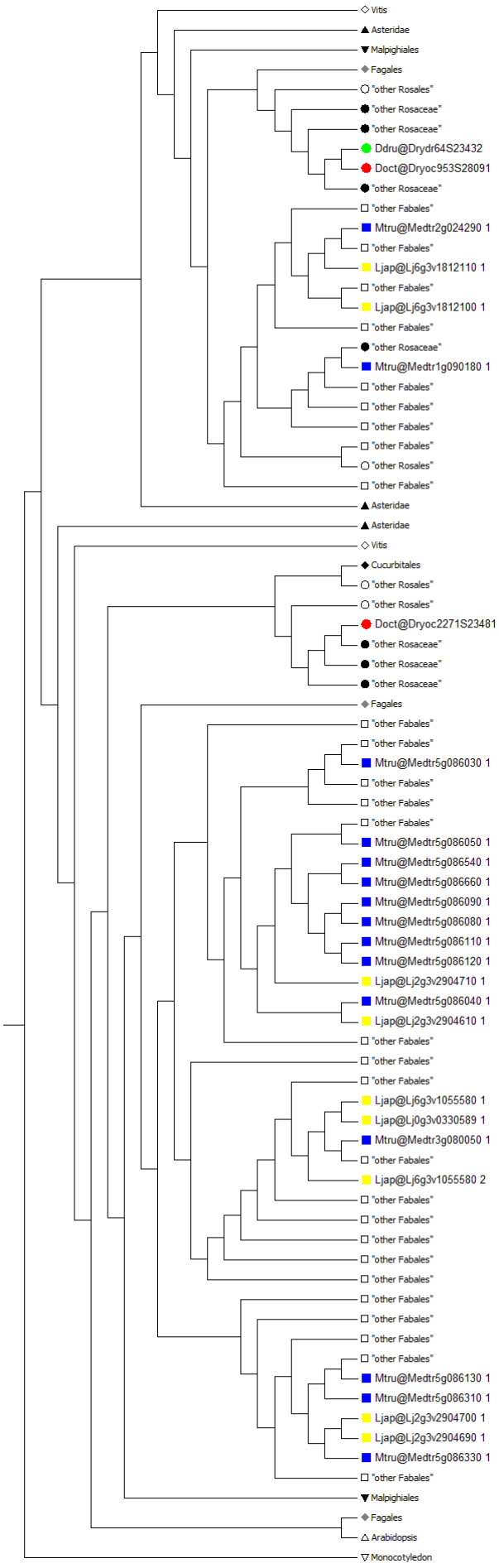
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n°5


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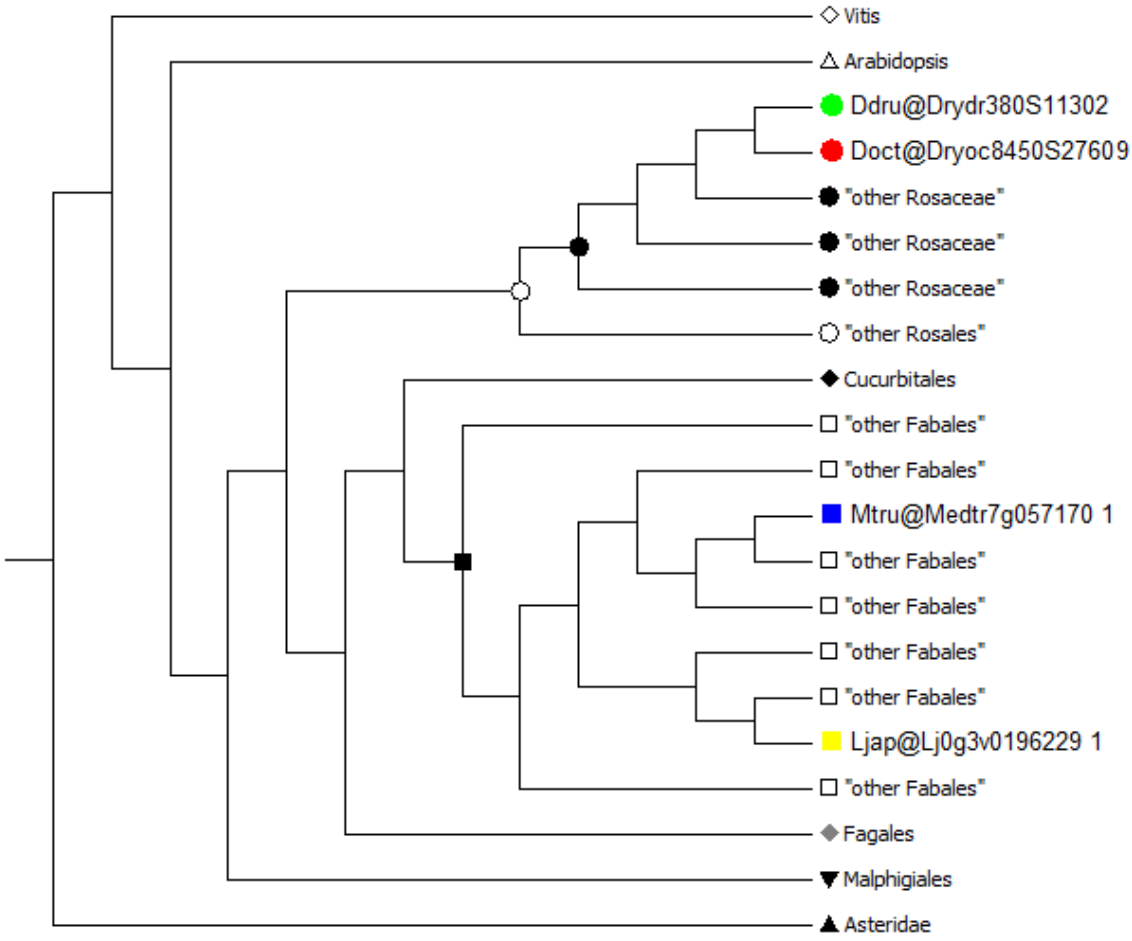
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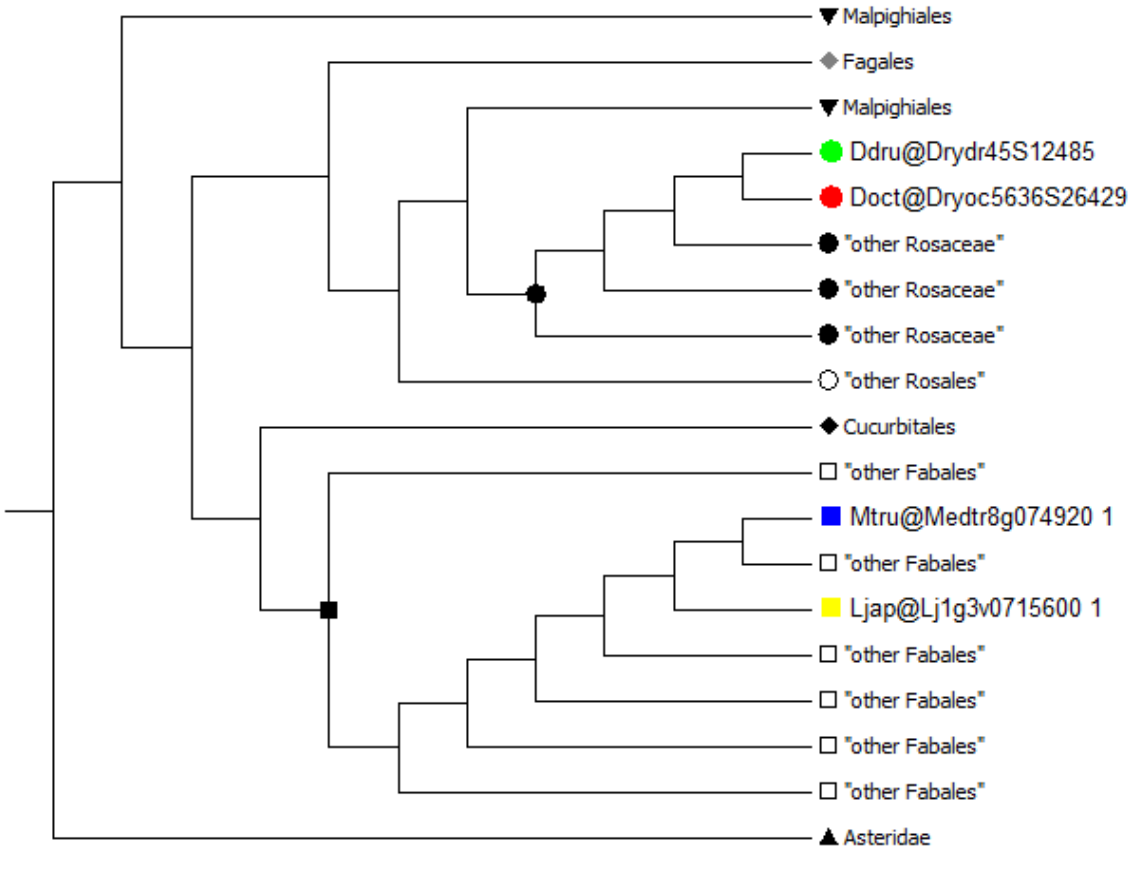
n°7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"other Fabales"
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"other Fabales"
Asteridae

n°8

Supplemental S6

Overview of the workflow for the choice of LORE 1 insertion mutant lines.

a

RLK family	*D. drummondii* ID	*D. octopetala* ID	Remarks	*L. japonicus* ID	# of LORE 1	
					in *Lotus* base	tested
LRRIII	Drydr146S16082	Dryoc124516S20838	gap in the kinase domain	Lj0g3v0133349	0	-
				Lj1g3v1991630	19	3
	Drydr265S09332	Dryoc124300S18768/Dryoc124300S01356**	fragmented gene	No *L. japonicus* gene		
LYSM	Drydr368S03769	Dryoc3475S24779	premature STOP codon	Lj0g3v0145339	3	2

b

RLK family	Targeted gene	*LORE1* insertional mutant line	Number of insertions			
			Exonic	Intronic	Intergenic	Total
LRRIII	Lj1g3v1991630	30035473	4	1	3	8
		30037601	7	3	0	10
		30039146	3	0	0	3
		30049695	3	2	0	5
		30052272	2	0	3	5
		30058354	5	1	3	9
		30073774	6	2	2	10
		30077439	3	0	0	3
		30092210	7	1	4	12
		30098812	5	2	3	10
		30104008	2	1	1	4
		30105750	7	4	4	15
		30122273	4	5	3	12
		30122957	6	1	6	13
		30126216	4	2	1	7
		30145017	4	0	2	6
		30150926	10	4	10	24
		30150926	10	4	10	24
		30003376	7	3	3	13
LYSM	Lj0g3v0145339	30000889	11	2	2	15
		30008011	1	2	0	3
		30136249	2	2	2	6

c

L. japonicus line	ZopRA seed bag number		Name
	seed bag n°1	seed bag n°2	In Fig. 39
30049695	111954	111955	lrrIII_1
30077439	111958	111957	lrrIII_2
30104008	111965	111964	lrrIII_3
30008011	111946	111947	lysm_1
30136249	111950	111951	lysm_2
WT GIFU	110895		GIFU

Overview of the workflow for the choice of LORE 1 insertion mutant lines.

(a) List of the three RLK presenting polymorphism between both *Dryas* spp. that could led distinct phenotype. The number of LORE 1 insertional mutant lines on the *L. japonicus* gene are reported. (b) List of all LORE 1 insertional lines with an insertion in the two "candidate" genes. In green is highlighted the lines used in part 2.3.3. (c) List of *L. japonicus* lines used in Figure 37 with the seed bag numbers reference in the ZoPRA database and the name given in the figure.

Supplemental S7

List of primer used.

"primer ID"	sequence	targeted gene	remarks
IST-p5_F	CCTTATCAYTTAGAGGAAGGAG	internal transcribed spacer (ITS) of nuclear ribosomal DNA	internal transcribed spacer (ITS) of nuclear ribosomal DNA
IST-u4_R	RGTTTCTTTTCCTCCGCTTA		
DK02_gi_F	ATGGCGTCCAAAAGTCCTGCATCG	Lj0g3v0145339	Check the 30008011 LORE1 insertion in Lj0g3v0145339 (GOI)
DK02_gi_R	AAGCATGAAGCTCCCTGTGGTGGC		
DK16_gi_F	TGGGAAATCCATGTCCACAGAGCA		Check the 30136249 LORE1 insertion in Lj0g3v0145339 (GOI)
DK16_gi_R	TGGATGTTCACGCCTCGAACCAGA		
DK12_gi_F	GAACCTCAGCAGAGGCCCTGAGCA	Lj1g3v1991630	Check the 30104008 LORE1 insertion in Lj1g3v1991630 (GOI)
DK12_gi_R	TGTGGAAAACCTGTAAGCAACCCCTGC		
DK10_gi_F	AGCTTCTTCACATAATTGATTTTGGGGCT		Check the 30077439 LORE1 insertion in Lj1g3v1991630 (GOI)
DK10_gi_R	GAACCTCAGCAGAGGCCCTGAGCA		
DK07_gi_F	AGCTTCTTCACATAATTGATTTTGGGGCT		Check the 30049695 LORE1 insertion in Lj1g3v1991630 (GOI)
DK07_gi_R	GAACCTCAGCAGAGGCCCTGAGCA		
GAPDH-F	CCCCAGTACGAATGCTCCCATGTTTG	Drydr226S18269 - Dryoc1436S22582	glyceraldehyde-3-phosphate dehydrogenase
GADPH-R	TTAGCCAAAGGAGCAAGACAGTTGGTGG		
EF1a_Mt_F	TGGGTTTGAGGGTGACAACATGA	Drydr51S22898+Drydr51S22899+Drydr51S12917 - Dryoc3749S25103+Dryoc3749S25104+Dryoc3749 S25112	elongation factor 1 alpha
EF1a_Rc_R	GTACACATCCTGAAGTGGAAGTCGGAGG		
26SrRNA_F	TACTGCAGGTCGGCAATCGG		26S ribosomal RNA
26SrRNA_R	TCATCGCGCTTGGTTGAAAA		
QRAM1_F1	CCAGCAAGAGAAGCAACACC	Drydr690S13573 - Dryoc1788S22993	Reduced Arbuscular Mycorrhization 1 (RAM1)
QRAM1_R1	GCACAAGCTAGGAGGAGGTG		
AS16_RAM1_f1	AAGTCCAATCGCCAGACCAT		
AS17_RAM1_r1	ACAGGAAGAGTAGGCACCATC		
AS20_ RAM1_f1	ATGATGATTAATTCTCTTTGTGG		Reduced Arbuscular Mycorrhization 1 (RAM1) for cloning all fragment
AS21_RAM1_r1	TCAGCATCTCCATGCAGAAGC		
AS22_ram1_atg_cF	ccGAAGACttTACGGTCTCaCACCATGATGATTAATTCTCTTTGTGG		for cloning fragment GG comp _ BpiI + BsaI sites
AS23_ram1_Bp1_cR	ccGAAGACatCcTCGATGGCAGGCAGTGTGTTC		for cloning fragment GG comp/ remove R sites _ BpiI site single nucleotide mutation
AS24_ram1_Bp1_cF	ccGAAGACatGAgGACTTCTTGGATGATTATC		
AS25_ram1_Es1_cR	ccGAAGACatCaTCTCTTTGATAATCATCC		
AS26_ram1_Es1_cF	ccGAAGACatGAtGGCTCATTCTTAGCGTACCC		
AS27_ram1_Bs1_cR	ccGAAGACatGTtTCTTCGGTCAATTGAGA		
AS28_ram1_Bs1_cF	ccGAAGACatAaACCACGAGGGGTGGTACTGC		
AS29_ram1_Bp2_cR	ccGAAGACatTCcTCTTTTGCAACGGCCTCAGC		
AS30_ram1_Bp2_cF	ccGAAGACatAgGACTACATGCTAGCAAGAAGG		
AS31_ram1_Es2_cR	ccGAAGACatTtTCTTTCACAGTCTCGATACACG		
AS32_ram1_Es2_cF	ccGAAGACatGAaACGGGCCTGTGTTTAACTG		
AS33_ram1_Bs2_cR	ccGAAGACatCaCGGATCATTGCTAACAAGTTCC		
AS34_ram1_Bs2_cF	ccGAAGACatCGtGACCAAGCACCCAATATTG		
AS35_ram1_cR5	ccGAAGACatCAGAGGTCTCaCCTTGCATCTCCATGCAGAAGCAGC		for cloning fragment GG comp _ BpiI + BsaI sites
QSTR_F1	CCACATTTCTTGATGCCTTGGC	Drydr87S24490 - Dryoc756S06838	stunted arbuscule (STR) / (1st intron-spanning)
QSTR_R1	AACATGGGGAAGAGCTGGTC		
QSTR_F2	CGTGCTCATGACCATTCATCAG		stunted arbuscule (STR) / (3rd intron-spanning)
QSTR_R2	CTGGCCTTCCAAATCCAGAAAG		
CASTOR_F	ATGTCCCTTGACTCCGACAC	Drydr411S21715* - Dryoc57S26677*	Ion channel (CASTOR)
CASTOR_R	GAAACGTCCGGTCGTCTTCTC		
NFP2_F	TGATCCCGGTGTCAAAGCTC	Drydr368S03767 - Dryoc124277S01099/Dryoc124277S01098***	homolog of NFR5 (Medtr5g019040) and LYS11 (Medtr8g078300)
NFP2_R	CCAAGAAGCGATTCCCGTCT		
L11Nf5_1F	CAAAAGAAAGCCATAGAGAAATGCC		
L11Nf5_1R	CATGTATGGGTAACCTTTGTATGAC		
RINRK101_F	TTCAGTTCAATCCCGGGCTC	Drydr284S19431 - Dryoc124303S18798*	homolog of Medtr4g094885
RINRK101_R	TTGCTCGAACACTTGCTTGC		
RINRK41_F	TGCAGGTCCCAAAACACACT	Drydr192S07840 - Dryoc138S22207	homolog of Medtr6g470960 - Medtr8g107470
RINRK41_R	CGAAGAGTAACGCAGGCAGA		
RINRK112_F	CAAGCATTTGACTGGGCCAC	Drydr226S18342 - Dryoc1228S17759	homolog of Medtr2g023150
RINRK112_R	AGACATGAGAAAGGGGTTGCA		
RINRK121_F	GGACTCTCAGCTCACCCAAC	Drydr146S00962 - Dryoc124399S09791	
RINRK121_R	CACTCACCTTACCGACACCC		
RINRK211_F	CACCCTCTCCCATTACCTGC	Drydr265S09332 - Dryoc124300S18768/Dryoc124300S01356**	homolog of Medtr2g008740
RINRK211_R	TCCTCACTCACCAATGGCTT		
RINRK221_F	ACCCTTTGAGGAGTTTGGCC	Drydr64S23384 - Dryoc124371S09594*	homolog of Medtr5g094380
RINRK221_R	GCCGAAGTCTCCATGGGAAA		
RINRK11_F	AGGCCTAGCGTACCTGTACA	Drydr146S16082 - Dryoc124516S20838	homolog of Medtr1g009270 - Medtr8g087420
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doRINRK11_s1R	GCAAAAGAAAATGGACAATAGGG	Dryoc124516S20838	
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doRINRK11_s3R	CCACTGAGATAACTATGATGCC		
doRINRK11_s4F	GTCATTCATGCAAACGTGAATG		
doRINRK11_s5R	CATATAACCCCCTATAGTGAGAC		
doRINRK11_s6F	GCTTGGAAGGTTGTCACACC		

List of primer used.

Curriculum Vitae

Profile

PhD in plant symbiosis genetics and holder of a technical diploma in horticultural productions

Name: Benjamin BILLAULT-PENNETEAU
Date of birth:
Mobil phone:
Business sector:
Postal address:
City/Country:
E-mail address:

Picture

Work experience and traineeships

- **PhD student under the supervision of Prof. M. PARNISKE**
Genetic analysis of plant root endosymbioses in the genus *Dryas* (Rosaceae)
Genetic department of the LMU; University of Munich
2015-2019
- **Master 2 trainee under the supervision of Dr. A. NIEBEL**
MtENR1-promoter is a direct target of the CCAAT-box transcription factor *Mt*NF-YA1
Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR2594 INRA-CNRS (Auzeville Tolosane, France)
January-May 2013
- **Master 1 trainee under the supervision of Dr. P. LAPORTE**
Localisation of the *Mt*NF-YA1 transcription factor expression during early nodulation stages
Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR2594 INRA-CNRS (Auzeville Tolosane, France)
November-December 2011
- **Bachelor project under the supervision of Dr. T. GUERINIER**
Production of recombinant proteins of the *At*SNRK1 complex in *E. coli*
Institut de Biologie des Plantes (I.B.P.) University of Paris Sud - CNRS - UMR 8618 (Orsay, France)
May-June 2011
- **"Discovery of the research"** 1 month at the INRA-Lusignan genetic resources department (Lusignan, France) - 2010
- **Horticultural apprentices in flower production**
Flutre's company (Eu, France)
September 2007 – July 2009
- **Farming traineeships:** Stud farm at Woking (UK) June-July 2005 ; Dairy farm at Cork (Ireland) June-July 2004 and at Druisy (France) July 2003.

Education

- From 01/2015: **PhD Thesis**
Ludwig-Maximilians-University of Munich
Faculty of Biology, Genetics, Research group Prof. Martin Parniske
- 09/2011 – 06/2013: **Microbiology Agrobiosciences Bioinformatics and systems biology (MABS) Master degree**
Specialization in plant sciences
Paul Sabatiers, University of Toulouse III
- 09/2009 – 06/2011: **General Biology Licence degree**
University of Poitiers
- 09/2007 – 06/2009: **Horticultural production technical postgraduate degree (BTS)**
Hortitheque, Mont Saint Aignan
- 09/2003 – 06/2007: **Integrated preparation**
ESITPA - School of Agricultural Engineering, Rouen
- 2003: **Science baccalaureate diploma**
Fenelon Sainte-Marie high school, Paris

International Conferences

- 3rd international Molecular Mycorrhiza Meeting (iMMM); Toulouse 2017
Talk: "*Dryas*, a new model for plant root symbioses – Discovery of a polymorphic AM symbiosis trait within the same plant genus"
- 12th European Nitrogen Fixation Conference (ENFC); Budapest 2016
Talk at the satellite symposium: 15th BNF-NL-Symposium – "*Dryas*: a model genus for root symbioses of the Rosaceae"

Publications

- Development of *Dryas* genus as model for genetic studies on root symbioses of the Rosaceae.
Billault-Penneteau B, Sandré A, Folgmann J, Parniske M, Pawlowski K. Frontiers in Plant Science. 2019
DOI:10.3389/fpls.2019.00661
- Phylogenomics reveals multiple losses of nitrogen-fixing root nodule symbiosis.
Griesmann M, Chang Y, Liu X, Song Y, Haberer G, Crook MB, ***Billault-Penneteau B***, Lauressergues D, Keller J, Imanishi L, Roswanjaya YP. *et al.* Science. 2018
DOI:10.1126/science.aat1743
- Two CCAAT-box-binding transcription factors redundantly regulate early steps of the legume-rhizobia endosymbiosis.
Laloum T, Baudin M, Frances L, Lepage A, ***Billault-Penneteau B***, Cerri MR, Ariel F, Jardinaud MF, Gamas P, de Carvalho-Niebel F, Niebel A. The Plant Journal. 2014
DOI:10.1111/tpj.12587

Skills

Horticultural: crop implementation and monitoring, phytosanitary and biological protection
Scientific: directed mutagenesis, DNA extraction (CTAB, CsCl gradient), Chromatin Imunoprecipitation, plant transformation (*A. rhizogenes* and *A. tumefaciens*), symbiotic inoculations.
Computer: Bioinformatic (CLC Genomics Workbench, R Statistic, Biopython), Image processing (Illustrator, ImageJ), Microsoft Office.
Language: French (native), English (fluent), Spanish (basic).